Universitat Autònoma de Barcelona

Molecular Basis of Ischemic Post-Conditioning: DJ-1 in Cardioprotection

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Molecular Basis of Ischemic Post-Conditioning: DJ-1 in Cardioprotection

– Ph.D. Thesis –

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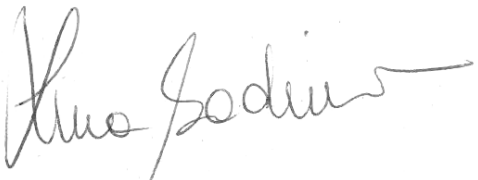

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Prof. Francisco Blanco Vaca

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"…mientras haya un misterio para el hombre,
¡Habrá Poesía!"

[Gustavo Adolfo Bécquer, *Rima IV*]

Abstract

Cardiovascular diseases are a leading cause of mortality and morbidity worldwide. Among them, acute myocardial infarction (AMI) is the most prevalent. Although revascularization is the definitive treatment for ischemia, it paradoxically entails the potential to exacerbate damage in a process known as ischemia/reperfusion (I/R) injury. The adult human heart retains minimal regenerative capacity. Therefore, after AMI, a non-contractile scar is formed decreasing cardiac output. Although the improvement of revascularization therapies has driven a drop in mortality within the past decades, AMI survivors with myocardial injury are at high risk of developing heart failure. The discovery of the conditioning phenomena, referring to the acquired cardioprotection derived from the application of repetitive brief cycles of ischemia and reperfusion, has evidenced the existence of endogenous cardioprotective programs. However, disappointing results have been obtained in clinical studies, highlighting gaps in the understanding of the molecular basis of cardioprotection.

We first explored the endogenous cardioprotective program triggered by ischemic post-conditioning (IPostC) in a swine model. To this end, we analyzed the mitochondrial proteomic response of the myocardium to ischemia, I/R, and IPostC, following a protein-protein interaction network approach. We identified both cardiac metabolism, and the up-regulation of DJ-1 as important drivers for cardioprotection. DJ-1 is a multifunctional protein playing intriguing roles in cell survival under stress. Although it has been a matter of intense research, the function and regulation of DJ-1 remain to be fully understood. We hypothesized that its regulation would provide a new therapeutic opportunity for myocardium salvage. Then, we characterized the dynamics of DJ-1 over ischemia and I/R. Using an *in vitro* model, we found that DJ-1 is cleaved and secreted by endothelial cells (ECs) during ischemia, and evidenced a role in regulating the ectopic ATP-synthase activity. Whilst ischemia led to a nearly 3-fold increase in the extracellular ATP generation, the knockdown of DJ-1 abrogated the effect. The ectopic ATP-synthase is located within the plasma membrane and it has been recognized to participate in the regulation of intracellular pH, and purinergic signaling, amongst others. Using a

recombinant DJ-1 model, we could further associate extracellular DJ-1 with the regulation of ECs function after reperfusion.

To test the therapeutic potential of recombinant DJ-1 administration, we employed a mouse model of AMI. As a result, the intraperitoneal administration of recombinant DJ-1 before AMI significantly reduced infarct size and modified the myocardium transcriptional response to reperfusion. Interestingly, gene sets associated with the immune response and G-protein coupled receptors (GPCRs) signaling encompassed the highest enrichments after AMI in the presence of the treatment. Whilst the immune response is a known player of I/R injury, GPCRs signaling has been extensively reported to participate in cardioprotection. Diminished leukocyte infiltration, oxidative stress, and apoptosis were also detected in the myocardium of the treated animals.

To further characterize the role of DJ-1 in I/R, we studied the functional implications of the previously reported RNA-binding activity of DJ-1. To this end, we analyzed a dataset resulting from the identification of DJ-1-binding transcripts and found that DJ-1 preferentially binds mitochondria-encoded transcripts and that RNA-binding to DJ-1 significantly increases during ischemia. The impact of DJ-1 depletion and I/R on mitochondrial protein expression and mitochondrial morphology was also investigated. While mitochondrial protein levels were found higher for the DJ-1 knockout cultures in normoxia, they significantly fell after I/R. Consistently, DJ-1 depletion resulted in unstable hyper-fused mitochondria, that were sensitive to I/R.

In conclusion, our findings highlight the implication of DJ-1 in the molecular basis of cardioprotection. We further provide the first lines of evidence regarding an extracellular activity for DJ-1 and characterize an additional aspect of DJ-1 maintaining mitochondrial stability.

Resumen

Las enfermedades cardiovasculares son la principal causa de morbimortalidad en todo el mundo, siendo el infarto agudo de miocardio (IAM) la más prevalente. Aunque la revascularización es el tratamiento definitivo para la isquemia, paradójicamente conlleva el potencial de exacerbar el daño en un proceso conocido como lesión por isquemia/reperfusión (I/R). El corazón humano adulto conserva una capacidad regenerativa mínima. Por tanto, tras un IAM se deposita una cicatriz no contráctil, disminuyendo el gasto cardíaco. Aunque la mejora de las terapias de revascularización ha permitido disminuir la mortalidad, los supervivientes de IAM con lesión miocárdica tienen un alto riesgo de desarrollar insuficiencia cardíaca. El descubrimiento del fenómeno de condicionamiento, referido a la cardioprotección adquirida tras la aplicación de ciclos alternos y breves de isquemia y reperfusión, ha revelado la existencia de programas cardioprotectores endógenos. Sin embargo, se han obtenido resultados decepcionantes en estudios clínicos, señalado lagunas en la comprensión de las bases molecular de la cardioprotección.

En primer lugar, exploramos el programa cardioprotector desencadenado por el poscondicionamiento isquémico (PostCo) en modelo porcino. Para ello analizamos el proteoma mitocondrial del miocardio en isquemia, I/R y PostCo, mediante redes de interacción proteína-proteína. Identificamos el metabolismo cardíaco y la inducción de DJ-1 como actores importantes en cardioprotección. DJ-1 es una proteína multifuncional implicada en la supervivencia celular bajo estrés, cuya función molecular y regulación aún no se han entendido por completo. Hipotetizamos que su regulación proporcionaría una nueva oportunidad terapéutica para proteger al miocardio. Posteriormente, caracterizamos la dinámica de DJ-1 en isquemia e I/R. Usando un modelo *in vitro*, encontramos que DJ-1 es escindido y secretado por células endoteliales (CEs) en isquemia, y evidenciamos un papel regulando la actividad de la ATP-sintasa ectópica. Mientras la isquemia condujo a un aumento de casi 3 veces en la generación de ATP extracelular, el silenciamiento de DJ-1 anuló el efecto. La ATP-sintasa ectópica se encuentra en la membrana plasmática de muchos tipos celulares y participa en la regulación del pH intracelular y señalización purinérgica, entre otros. Usando DJ-1 recombinante,

pudimos asociar su presencia extracelular con la regulación de la función de CEs en reperfusión.

Para explorar el potencial terapéutico de la administración de DJ-1 recombinante, empleamos un modelo murino de IAM. Como resultado, la administración intraperitoneal de DJ-1 recombinante antes de la inducción del IAM redujo significativamente el tamaño del infarto y modificó el transcriptoma. Varios conjuntos de genes asociados con la respuesta inmunitaria y señalización vía receptores acoplados a proteína G (RAPGs) abarcaron los mayores enriquecimientos después del IAM en presencia del tratamiento. Si bien la respuesta inmunitaria es un actor conocido de la lesión por I/R, la señalización por RAPGs participa en cardioprotección. Además, detectamos la disminución de la infiltración leucocitaria, estrés oxidativo y apoptosis en el miocardio de los animales tratados.

Finalmente, estudiamos las implicaciones funcionales de la capacidad de DJ-1 de unir ARN en I/R. Con ese fin, analizamos un conjunto de datos resultantes de la identificación de los transcritos que une DJ-1 y descubrimos la unión preferencial a transcritos codificadas por la mitocondria. Además, la unión de ARN a DJ-1 aumenta notablemente en isquemia, contribuyendo a la estabilidad mitocondrial. Si bien la depleción de DJ-1 resultó en un incremento de la expresión de proteínas mitocondriales en normoxia, esta cayó significativamente tras I/R. Del mismo modo, la depleción de DJ-1 resultó en un retículo mitocondrial hiperfusionado y sensible a I/R.

En conclusión, nuestros hallazgos destacan la implicación de DJ-1 en las bases moleculares de la cardioprotección. Proporcionamos las primeras líneas de evidencia con respecto a una actividad extracelular para DJ-1 y caracterizamos un aspecto adicional implicado en el mantenimiento de la estabilidad mitocondrial.

Resum

Les malalties cardiovasculars són la principal causa de morbimortalitat a tot el món, essent l'infart agut de miocardi (IAM) la més prevalent. Encara que la revascularització és el tractament definitiu per a la isquèmia, paradoxalment comporta el potencial d'exacerbar el dany en un procés conegut com a lesió per isquèmia/reperfusió (I/R). El cor humà adult conserva una capacitat regenerativa mínima. Per tant, després d'un IAM es diposita una cicatriu no contràctil, disminuint el rendiment cardíac. Encara que la millora de les teràpies de revascularització ha permès disminuir la mortalitat, els supervivents d'IAM amb lesió miocàrdica tenen un alt risc de desenvolupar insuficiència cardíaca. El descobriment del fenomen de condicionament, referit a la cardioprotecció adquirida després de l'aplicació de cicles alterns i breus d'isquèmia i reperfusió, ha revelat l'existència de programes cardioprotectors endògens. No obstant, s'han obtingut resultats decebedors en estudis clínics, assenyalant llacunes en la comprensió de les bases molecular de la cardioprotecció.

Primerament, explorem el programa cardioprotector desencadenat pel postcondicionament isquèmic (PostCo) en model porcí. Per a això analitzem el proteoma mitocondrial del miocardi en isquèmia, I/R i PostCo, mitjançant xarxes d'interacció proteïna-proteïna. Identifiquem el metabolisme cardíac i la inducció de DJ-1 com a actors importants en cardioprotecció. DJ-1 és una proteïna multifuncional implicada en la supervivència cel·lular en estrès, la funció molecular i regulació de la qual encara no s'han entès per complet. Proposem que la seva regulació proporcionaria una nova oportunitat terapèutica per a protegir el miocardi. Posteriorment, caracteritzem la dinàmica de DJ-1 en isquèmia i I/R. Usant un model *in vitro*, trobem que DJ-1 és escindit i secretat per cèl·lules endotelials (CEs) en isquèmia, i evidenciem un paper regulant l'activitat de l'ATP-sintasa ectòpica. Mentre la isquèmia va conduir a l'augment de gairebé 3 vegades en la generació d'ATP extracelul·lar, el silenciament de DJ-1 va anul·lar l'efecte. L'ATP-sintasa ectòpica es troba a la membrana plasmàtica de molts tipus cel·lulars i participa en la regulació del pH intracel·lular i senyalització purinèrgica, entre

altres. Usant DJ-1 recombinant, vam poder associar la seva presència extracelul·lar amb la regulació de la funció de CEs en reperfusió.

Per a explorar el potencial terapèutic de l'administració de DJ-1 recombinant, vam emprar un model murí d'IAM. L'administració intraperitoneal de DJ-1 recombinant abans de la inducció de l'IAM va reduir significativament la grandària de l'infart i va modificar el transcriptoma. Conjunts de gens associats amb la resposta immunitària i senyalització via receptors acoblats a proteïna G (RAPGs) es van detectar després de l'IAM en presència del tractament. Si bé la resposta immunitària és un actor conegut de la lesió per I/R, la senyalització per RAPGs participa en cardioprotecció. També, detectem la disminució de la infiltració leucocitària, estrès oxidatiu i apoptosi en el miocardi dels animals tractats.

Finalment, estudiem les implicacions funcionals de la capacitat de DJ-1 d'unir ARN en I/R. Amb aquesta finalitat, analitzem un conjunt de dades resultants de la identificació dels transcrits que uneix DJ-1 i descobrim la unió preferencial a transcrits codificades pel mitocondri. A més, la unió d'ARN a DJ-1 augmenta notablement en isquèmia, contribuint a l'estabilitat mitocondrial. Si bé la depleció de DJ-1 va resultar en un increment de l'expressió de proteïnes mitocondrials en normòxia, aquesta va caure significativament després d'I/R. De la mateixa manera, la depleció de DJ-1 va resultar en un reticle mitocondrial híper fusionat i sensible a I/R.

En conclusió, les nostres troballes destaquen la implicació de DJ-1 en les bases moleculars de la cardioprotecció. Proporcionem les primeres línies d'evidència respecte l'activitat extracelul·lar de DJ-1 i caracteritzem un aspecte addicional implicat en el manteniment de l'estabilitat mitocondrial.

Table of contents

Abbreviations

AC	Adenylate cyclase
acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine di-phosphate
ADP	Adenosine diphosphate
AGEs	Advanced glycation end-products
AIF	Apoptosis induction factor
AMI	Acute myocardial infarction
AMP	Adenosine mono-phosphate
AMP	Adenosine monophosphate
ANT	Adenosine nucleotide translocase
AP-1	Activator protein-1
apaf-1	Apoptotic protease activating factor 1
AR	Androgen receptor
ARE	Antioxidant response elements
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine mono-phosphate
Cas9	CRISPR asociated protein 9
cGMP	Cyclic guanosine mono-phosphate
CK	Creatinine kinase
CK-MB	Creatinine kinase-muscle brain
CLIP	cross-linked immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
CVDs	Cardiovascular diseases
DAG	Diacylglycerol
Daxx	Death-domain-associated protein 6
DJ-1ΔC	Cleaved protein DJ-1
DNA	Deoxyribonucleic acid
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
Erk	Extracellular signal-regulated kinases
Erk1/2	p44/p42 Extracellular signal-regulated protein kinase
ETC	Electron transport chain
FAD^+	Oxidized flavin adenine dinucleotide

$FADH_2$	Reduced flavin adenine dinucleotide
FAO	Fatty acid β-oxidation
GDP	Guanosine diphosphate
Glo-1	Glutathione-dependent glyoxalase 1
GLUT-1	Glucose transporter-1
GLUT-4	Glucose transporter-4
GPCR	G-protein coupled receptors
GRP75	Glucose-regulated protein 75
GSK-3β	Glycogen synthase kinase-3β
GTP	Guanosine triphosphate
HIF-1α	Hypoxia-inducible factor-1α
HK2	Hexokinase-2
HO-1	Hemeoxygenease-1
HSPA9	Mitochondrial stress-70 protein
I/R	Ischemia/Reperfusion
IL-1β	Interleukin-1β
IMM	Inner mitochondrial membrane
iNOS	Inducible nitic oxide synthase
IP_3	Inositol 1,4,5-trisphosphate
IPC	Ischemic pre-conditioning
IPostC	Ischemic post-conditioning
IS	Infarct size
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
Keap-1	Kelch-like ECH-associated protein-1
KO	Knockout
LDLR	Low density lipoprotein receptor
LVEF	Left ventricular ejection fraction
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase/ERK kinase
MMPs	Metalloproteinases
MnSOD	Mn^{2+}-superoxide dismutase
mPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
mRNP	Messenger ribonucleoprotein
MSI	Myocardial salvage index

MTND2	Mitochondria-encoded NADH:ubiquinone oxidoreductase core subunit 2
N	Sample size
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-κB
NO	Nitric oxide
Nrf-2	Nuclear erythroid 2-related factor-2
OMM	Outer mitochondrial membrane
OXA1L	Mitochondrial inner membrane protein
OXPHOS	Oxidative phosphorylation
PARK7	Parkinson diseases protein 7
PDKs	Phosphoinositide-dependent protein kinases
PI3K	Phosphatidylinositol 3'-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PP2A	Protein phosphatase 2A
PRAK	p38 Regulated/activated kinase
PTEN	Phosphatase and tensin homologue
RACK	Receptor for activated PKC
RAGE	Receptor of AGEs
RET	Reverse electron transport
RIC	Remote ischemic conditioning
RISK	Reperfusion injury salvage kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RREB1	Ras-responsive element (RRE)-binding protein-1
RTKs	Receptors tyrosine kinase
SAFE	Survival activating factor enhancement
sGC	Soluble guanylate cyclase
SGs	Stress granules
SOD1	Superoxide dismutase-1
SPECT	Single photon emision computed tomography
SREBP2	Sterol responsive element binding protein-2

STAT-1	Signal transducer and activator of transcription-1
STAT-3	Signal transducer and activator of transcription-3
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
TH	Tyrosine hydrolase
TLRs	Toll-like receptors
TNFR2	Tumor necrosis factor receptor 2
TNFα	Tumor necrosis factor-α
TnI	Troponin-I
TnT	Troponin-T
VDAC2	Voltage-dependent anion-selective channel 2
WT	Wild-type
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

List of figures

All figures included are original, and have been created by the author of this thesis.

1.

Introduction

1.- Introduction

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide [1–3]. According to the Global Burden of Diseases study, in 2019 CVDs accounted for 18.6 million deaths, representing a 31.5% of all global mortality [1]. Just in the European Union, CVDs have an annual cost of approximately 210 billion €, and are responsible for the 82% of disability-adjusted life years [3]. The global prevalence of CVDs has nearly doubled from 1990 to 2019, and is expected keep rising in the near future due to population growth and ageing [1]. New therapeutic approaches for the treatment and prevention of CVDs are needed to lower the burden that CVDs represent for health, quality of life of the population and economy.

Amongst all CVDs, acute myocardial infarction (AMI) and stroke are the most devastating, representing an 84.4% of all CVDs deaths worldwide [1]. AMI is defined as the sudden interruption of coronary blood supply to the myocardium, typically caused by the rupture of an atherosclerotic plaque and thrombus formation. Without immediate intervention, the interruption of blood supply (i.e. ischemia) quickly progress to necrosis [4]. The adult human heart retains a minimal regenerative capacity; thus, myocardium necrosis following AMI leads to an irreversible loss of functional tissue with subsequent adverse remodeling and non-contractile scar formation [5]. Infarct size is, therefore, the major predictor of AMI clinical outcomes [6–8].

Despite mortality due to AMI has dropped within the past decades, as a result of improvements of therapies and reperfusion interventions [9,10], the adverse heart remodeling and scar formation after AMI entails a high risk for the development of heart failure [11,12]. In this respect, cardioprotection is defined as any therapy or intervention that contribute to the preservation of heart function by reducing or preventing myocardial damage [13]. Many cardioprotective therapies aimed at reducing infarct size have been tested but proven inefficient in the clinical arena [14,15]. Therefore, cardioprotection is an unmet clinical need.

1.1.- Ischemia

Ischemia is defined as the stress resulting from the interruption of blood supply to a given tissue or organ. Atherosclerotic plaque rupture, thrombus formation and microvascular damage are the main causes for ischemia. In such event, the reduction in oxygen supply directly and promptly impacts on the cellular metabolism leading to ATP depletion and acidosis. The blood flow interruption also entails the accumulation of detrimental products. All effects combined result in an extensive cell death and subsequent organ dysfunction.

1.1.1.- Impact of ischemia in cell metabolism

Under aerobic conditions, most of the cellular ATP is produced within the mitochondria through oxidative phosphorylation (OXPHOS). This process relies on the ability of four protein complexes, termed the electron transport chain (ETC), to pump protons from the mitochondrial matrix to the intermembrane space. This generates an electrochemical gradient that will fuel the ATP production through the F_1F_0-ATP-synthase. In the course of the ETC, reducing equivalents (more specifically, NADH and $FADH_2$), arising from any catabolic reaction in the cell, are oxidized releasing protons to be pumped and electrons. The released electrons flow through the ETC, ultimately reaching the cytochrome-C oxidase (also known as complex IV) where oxygen acts as the final electron acceptor and H_2O is produced. The OXPHOS is an extremely efficient biological system being able to produce 36 molecules of ATP per molecule of glucose. The high performance achieved by cellular metabolism results from the close coupling of all metabolic reactions. The reducing equivalents needed for the course of the ETC are constantly produced throughout glycolysis, the fatty acid β-oxidation (FAO), and the tricarboxylic acid (TCA) cycle. Thus, all metabolic pathways ultimately converge in the OXPHOS, which strictly depends on the availability of oxygen supply. In the absence of O_2, the ETC is rapidly inhibited, as there is no final electron acceptor. The blockade of the ETC implies both the cessation of ATP production by the F_1/F_0-ATP synthase and the accumulation of reducing equivalents in their reduced form. The normal progression of all catabolic reactions depends on the availability of oxidized reducing equivalents, as they exist in a relatively low abundance and cannot be

imported from the extracellular space [16]. Thus, the accumulation of reducing equivalents in the reduced form greatly impacts in the normal progression of all metabolic routes.

Glycolysis takes place in the cytoplasm, and consists of a group of chemical reactions that sequentially oxidize glucose into pyruvate. Over the course of glycolysis, two molecules of ATP per molecule of glucose are produced through substrate-level phosphorylation, besides two molecules of NADH, and one molecule of pyruvate are produced. The resulting pyruvate is then imported to the mitochondria and converted to acetyl coenzyme A (acetyl-CoA), to join the TCA cycle. Over the course of ischemia, glycolysis switches to anaerobic glycolysis, which is virtually the only way to produce ATP in the absence of O_2. In this process, the reducing equivalents (i.e. NADH) generated over the sequential oxidation of glucose are re-oxidized throughout the conversion of pyruvate to lactate, thus allowing a continued progression, which is coupled to a minimal production of ATP by substrate-level phosphorylation. Given the blood supply interruption, extracellular glucose concentration rapidly diminishes. As a compensatory mechanism, ischemic cells firstly optimize glucose uptake throughout the up-regulation of the high affinity glucose transporters GLUT-1 and 4 [17,18], and secondly consume their intracellular glycogen stores [19,20].

The FAO occurs within the mitochondrial matrix, and consists in the sequential oxidation of fatty acids to acetyl-CoA, NADH, $FADH_2$, and GTP (through substrate-level phosphorylation). FAO yield is similar to glycolysis, as one molecule of NADH, $FADH_2$, and acetyl-CoA are produced within each FAO round. However, several rounds of FAO are needed to fully catabolize one fatty acid molecule. Again, the resulting acetyl-CoA enters the TCA cycle. The FAO strictly depends on the availability of oxidized reducing equivalents; hence, it is rapidly inhibited during ischemia.

The TCA cycle (also known as Krebs cycle or citric acid cycle) is a central metabolic pathway for both the aerobic catabolism and anabolism. It consists in a series of oxidation-reduction reactions by which acetyl-CoA is sequentially oxidized, generating two molecules of CO_2, three molecules of NADH, one molecule of $FADH_2$,

and one molecule of GTP, after the complete oxidation of one molecule of acetyl-CoA. The TCA cycle is the main source for reducing equivalents and constitutes a hub for anabolic reactions providing multiple precursors for the synthesis of aminoacids. Whilst the normal TCA cycle progression is no longer possible in ischemia, a non-canonical flow through the TCA cycle is established to guarantee a second source of GTP in the absence of O_2; however, as the ETC lasts inhibited, succinate accumulates within the mitochondrial matrix (**Figure 1**) [21–23].

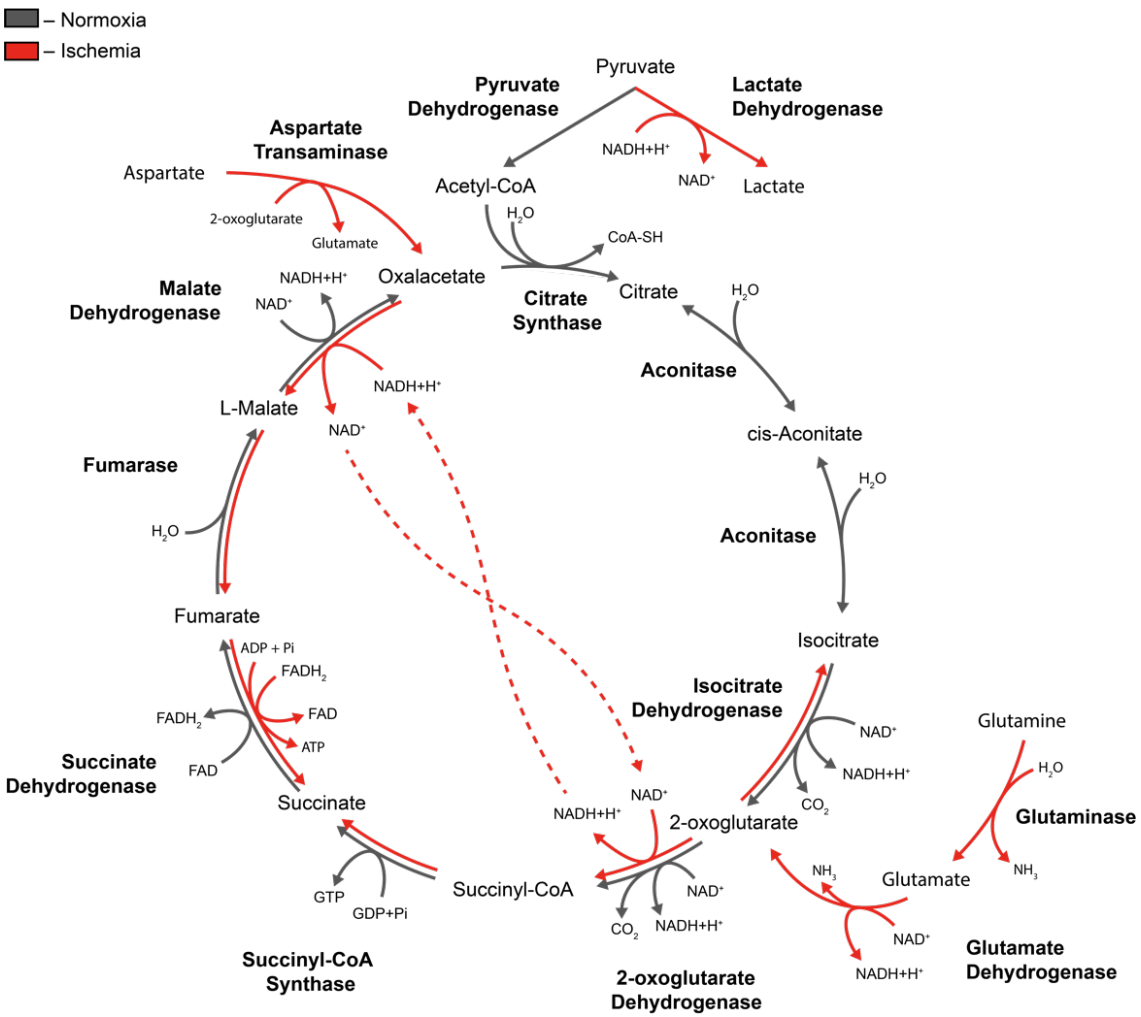

Figure 1 – Reactions composing the tricarboxylic acid cycle in normoxia (gray arrows) and ischemia or hypoxia (red arrows). The enzymes responsible of each reaction are also indicated (bold labels).

1.1.2.- ATP depletion

During ischemia, the whole cellular ATP production is sustained by the anaerobic glycolysis and the non-canonical adaptation of the TCA cycle. However, during a blood-flow restriction, both the extracellular glucose supply and intracellular glycogen stores are finite. Furthermore, anaerobic glycolysis yields 2 molecules of ATP per molecule of glucose, which is minimal compared to the 36 molecules of ATP per glucose molecule acquired by the aerobic metabolism. Hence, ATP consumption rapidly exceeds production, and the intracellular ATP pools fall. Another factor contributing to the ATP depletion is, paradoxically the mitochondrial F_1F_0-ATP synthase itself, as a result of a reverse activity. When the mitochondrial potential falls below a certain level, due to a compromised ETC, the F_1F_0-ATP synthase reverses its function hydrolyzing ATP to keep a proton gradient across the inner mitochondrial membrane (IMM) [24–26]. Indeed, some pharmacological strategies aimed at the reversible inhibition of the mitochondrial F_1F_0-ATP synthase have been proposed for ischemia [26,27].

Ischemia and the consequent ATP depletion have a number of deleterious effects upon cell biology, starting with the disturbance of the cell ionic balance. The increased proton production coupled to the anaerobic glycolysis activates the Na^+/H^+ exchangers. This leads to the intracellular accumulation of Na^+, which activates the Na^+/Ca^{2+} exchangers. As the ATP pools fall, the Na^+/K^+ pumps inactivates, progressively leading to the intracellular accumulation of Na^+ and Ca^{2+}. Additionally, the lack of ATP diminishes the active Ca^{2+} efflux, as well as its reuptake by the sarcoplasmic reticulum, thereby leading to cytosolic Ca^{2+} overload [28]. Due to the osmotic effect, the cytosolic ion accumulation induces water to flood the cell, causing cellular edema, and eventually promoting necrosis [29].

The ATP depletion, and the consequent accumulation of adenosine di-phosphate (ADP) and adenosine mono-phosphate (AMP), stimulates purine catabolism. Therefore, the enzymes 5′-nucleotidase, adenosine deaminase, and purine nucleoside phosphorylase are activated and sequentially catabolize AMP into adenosine, inosine and hypoxanthine [30].

1.2.- Reperfusion

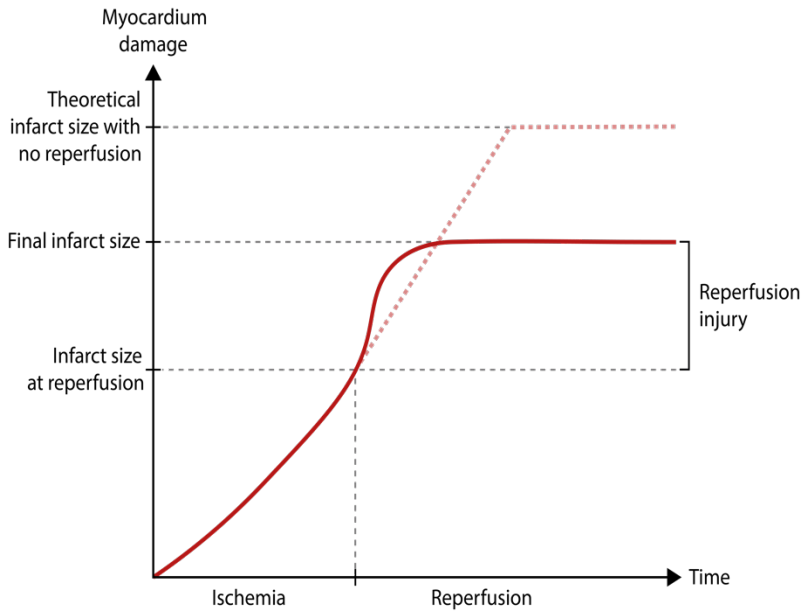

Figure 2 – Schematic representation of ischemia/reperfusion injury over time. Whilst in the absence of reperfusion, the theoretical infarct size would cover the entire myocardium at risk (dashed red line), reperfusion halts infarct expansion, but causes additional damage, leading to larger infarct size than the associated to the ischemic insult alone.

Given the absolute requirement of oxygen for the correct coupling of all metabolic reactions, both the magnitude and duration of ischemia are the major determinants of the damage extent. Thereby, the early and complete restoration of blood flow is the definitive treatment for ischemia. However, the reperfusion of an ischemic tissue paradoxically entails the potential to exacerbate damage in a process known as ischemia and reperfusion (I/R) injury (**Figure 2**). I/R injury is believed to happen during the very first minutes of blood reflow and accounts for a significant part of the final infarct size [31,32]. The generation of reactive oxygen species (ROS), and a further disturbance of the ionic balance are the main triggers of I/R damage.

1.2.1.- Reactive Oxygen Species

ROS arise from the the reduction of the oxygen molecule. Being continuously produced as by-product of cell metabolism, ROS are eliminated by the endogenous

antioxidant systems. Despite playing a role as second messengers in cell signaling [33], their accumulation (termed, oxidative stress) is toxic, causing lipid peroxidation, protein misfolding, and DNA damage. Being ROS central in reperfusion injury, several antioxidant therapies have been considered for cardioprotection, but clinical data is either lacking or controversial regarding effects upon infarct size and heart function [34,35].

The first source of ROS at reperfusion arise from the accumulation of succinate and NADH during ischemia, as a result of an inhibited ETC. When oxygen is reintroduced by revascularization, the electron flux through the ETC is restored and, therefore, all the accumulated succinate and NADH are oxidized. Consequently, the ubiquinone pool is reduced and the mitochondrial potential abruptly increases. However, as purine catabolism is favored during ischemia, the ADP stores are low at the time of reperfusion, limiting the activity of the mitochondrial F_1F_O-ATP synthase, and further favoring the mitochondrial hyperpolarization. A high mitochondrial potential together with a highly reduced ubiquinone pool, are signs of a saturated ETC, which ends up promoting the reverse electron transport (RET) from ubiquinone to the ETC complex I, that is coupled to the generation of ROS [36]. The competitive inhibition of succinate dehydrogenase (at the ETC) with a cell-permeable derivate of malonate have been reported to protect the heart against I/R injury limiting mitochondrial hyperpolarization and RET upon reperfusion [37,38].

The second source of ROS at reperfusion results from purine catabolism and xanthine dehydrogenase (XDH) conversion to xanthine oxidase (XO) in ischemia. The intracellular accumulation of Ca^{2+} during ischemia entails the activation of Ca^{2+}-dependent proteases, which cleave XDH into XO. XO catalyzes the conversion of hypoxanthine, arising from purine catabolism, to xanthine, in an oxygen-dependent reaction coupled to the production of ROS [30]. Xanthine is later converted to uric acid by the remaining XDH. The inhibition of XO may be a tentative strategy to limit myocardial damage upon reperfusion. However, no benefits where reported in animal models [39].

1.2.2.- Ionic unbalance and Ca^{2+} overload

Another consequence of reperfusion arises from the clearance of the extracellular media and the recovery of physiologic pH. During ischemia, the acidification of the cytoplasm, due to anaerobic metabolism, combined with the lack of ATP lead to a dysregulation of the cell ionic balance. It is initiated by the extrusion of H^+ through the Na^+/H^+ passive exchangers, which causes the extracellular media to also acidify while in ischemia. The recovery of blood flow at reperfusion entails the washout of the extruded protons, thus favoring the activity of the Na^+/H^+ passive exchangers, and aggravating the intracellular accumulation of Na^+. The rise of intracellular Na^+ concentration, in turn, favors the activity of the Na^+/Ca^{2+} exchangers, consequently aggravating the intracellular accumulation of Ca^{2+} [31]. Given that Ca^{2+} is a second messenger, the consequences of Ca^{2+} overload go beyond the osmotic mobilization of water, volume increase and necrosis. Hence, once the intracellular concentration

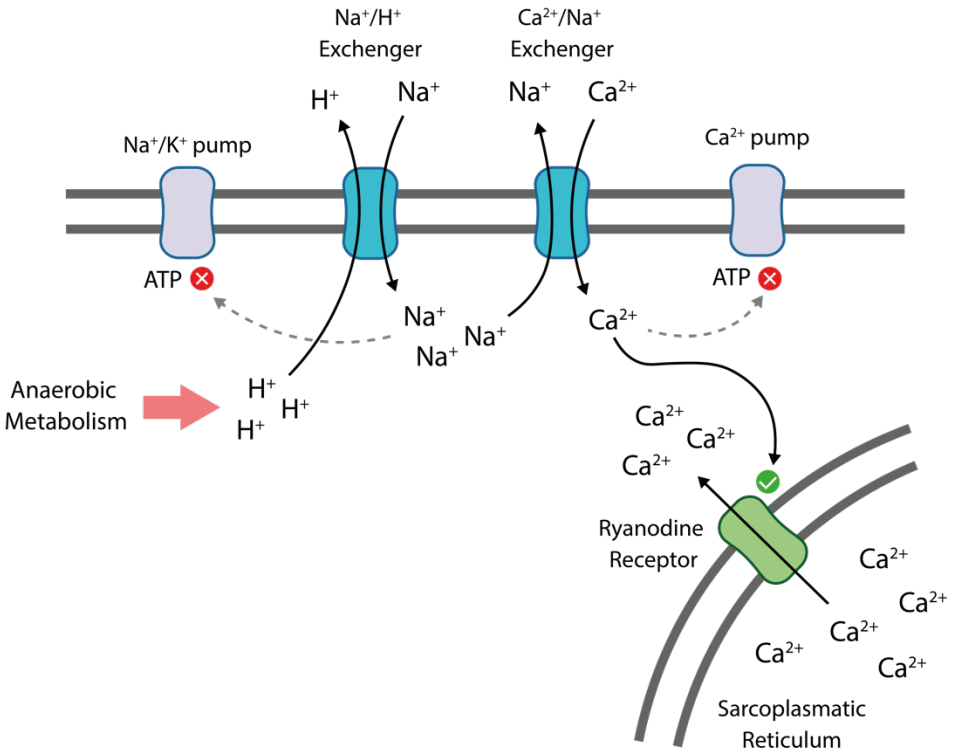

Figure 3 – Schematic representation of ischemia/reperfusion-induced ionic unbalance leading to Ca^{2+} overload.

of Ca^{2+} reaches a certain level, it stimulates the opening of ryanodine receptors from the sarcoplasmic reticulum, which liberates more Ca^{2+} to the cytoplasm (**Figure 3**). This Ca^{2+}-induced Ca^{2+}-release aggravates the Ca^{2+} overload, causing myofibers hypercontraction, and further activating of Ca^{2+}-dependent proteases [28]. This is the case of calpain-1, which once active it migrates to the mitochondrion, primes the apoptosis induction factor (AIF), and promotes the opening of the mitochondrial permeability transition pore (mPTP), collectively promoting apoptosis during reperfusion [40,41]. Oppositely to necrosis, which is a passive and uncoordinated event, apoptosis requires ATP to proceed. Thus whilst necrosis plays an important role in cell death during ischemia and reperfusion, apoptosis is more likely to occur in reperfusion.

1.2.3.- Mitochondrial permeability transition pore

The mitochondrial permeability transition pore (mPTP) is a voltage-dependent and high-conductance channel, that is located within the IMM. It opens under some pathologic conditions, and drives both a profound mitochondrial dysfunction and apoptosis. Under physiologic conditions, the IMM is highly impermeable, which allows the establishment of a proton gradient fueling the OXPHOS. Once open, the mPTP has an apparent diameter of about 3 nm, which allows the passive diffusion across the IMM with an exclusion size of 1.5 kDa. Thus, the mPTP opening completely disrupts the mitochondrial potential. Protein concentration within mitochondria is higher than that of the cytosol. However, due to the exclusion size of the mPTP, proteins are retained within the mitochondria after permeability transition, entailing a high colloidosmotic pressure and promoting water to flood the mitochondrial matrix. As a result, mitochondria swell upon mPTP opening. Whilst the IMM is protected from rupture by the unfolding of cristae upon an increased hydrostatic pressure, the outer mitochondrial membrane (OMM) is not, and will eventually break liberating the intermembrane space content to the cytoplasm [42–44]. As a result, cytochrome-C is released to the cytoplasm where it binds the apoptotic protease activating factor 1 (apaf-1), which cleaves procaspase-9 into active caspase-9 and initiates the intrinsic apoptosis pathway [45].

The factors contributing to the opening of the mPTP prevail at the time of reperfusion [42–44]. Those are divalent cations, inorganic phosphate, ROS accumulation, mitochondrial depolarization, and decreased ATP levels [42]. Whilst Ca^{2+} overload was firstly hypothesized to be sufficient to induce the opening of the mPTP, recent studies highlighted that the Ca^{2+} concentration needed is massive, and indeed, not physiological [40,41]. Hence, permeability transition of the IMM is more likely to result from a combination of factors. Several approaches targeting mitochondrial permeability transition have been proven to limit infarct size in animal models, but clinical studies are currently lacking [46].

The identity of the mPTP is still in debate, which convolutes its pharmacological targeting. It has been hypothesized that mPTP results form the interaction of three mitochondrial proteins. Those are cyclophilin-D (in the mitochondrial matrix), the adenine nucleotide translocase (in the IMM), and the voltage-dependent anion channel (in the OMM). However, genetic models lacking some of this hypothesized components still exhibit mPTP activity, and this composition may not explain the effects of some experimental mPTP regulators [42].

1.3.- Pathophysiology of myocardial infarction

The heart is mostly composed of cardiomyocytes, fibroblasts, endothelial cells (ECs), and leukocytes, each one contributing in its particular way to the correct functioning of the heart. Likewise, their response following AMI significantly contributes to the outcome. From a time point perspective, the infarct healing response can be subdivided into four partially overlapping phases: inflammatory, proliferative, maturation, and remodeling, in which each cell type carries out an specific role (**Figure 4**) [47].

During AMI, there is an extensive myocardial necrosis, which causes the release of intracellular contents to the extracellular media, serving as damage signals, and activating the immune response. This initiates the inflammatory phase, in which proinflammatory pathways are activated, via toll-like receptors (TLRs), in all cell types present within the infarcted myocardium. Consequently, chemokines and cytokines are produced and released, and circulating immune cells are recruited to

the injured tissue [48,49]. The main goal of the inflammatory phase is the clearance of infarcted myocardium from death cells and matrix debris, as well as the activation of the following healing program. Although an appropriate immune response is necessary for the correct healing of the infarcted myocardium, the over-activation may compromise tissue viability [50,51]. Hence, a coordinated and well-balanced immune response following AMI is key to maintain cardiac function. Once necrotic cells and matrix debris have been cleared, an anti-inflammatory response is promoted both passively, through the removal of danger signals (i.e. necrotic products), and actively, through the production of anti-inflammatory cytokines [47–49]. This initiates the proliferative phase of cardiac repair.

During the proliferative phase, activated myofibroblasts and vascular cells infiltrate the infarcted myocardium to build up a collagen scar. Throughout this phase, resident fibroblasts, are activated and acquire a myofibroblast phenotype, which includes a high proliferative activity, contractility, and elevated synthetic capacity [52]. Although still poorly understood, during this phase, beyond cardiac fibroblasts, several cell types have been found to transdifferentiate into myofibroblasts. This is the case of ECs, vascular smooth muscle cells, and pericytes, among others [53]. The proliferative phase is characterized by a rapid synthesis and deposition of extracellular matrix, parallel to the formation of an immature vascular network to support the high metabolic demand. Very dynamic changes of cell and matrix composition of the scar are observed during the proliferative phase [54]. The early vascular network of the scar displays a hyperpermeable and pro-inflammatory phenotype, and lacks a pericyte coat [48].

The formation of a fully vascularized collagen matrix sets the start for the maturation phase, in which both the extracellular matrix and the vascular network undergo structural modifications and stabilization. Over the course of this phase, the number of myofibroblasts is notably reduced and the synthetic activity is greatly reduced. Collagen fibers are cross-linked leading to a more stable extracellular matrix [47,52]. While some myofibroblasts become quiescent, decreasing their synthetic activity and contractility, many others undergo apoptosis [52].

Angiogenesis is also inhibited during the maturation phase. Some neovessels acquire a pericyte coat and become stable, while uncoated vessels degenerate [48].

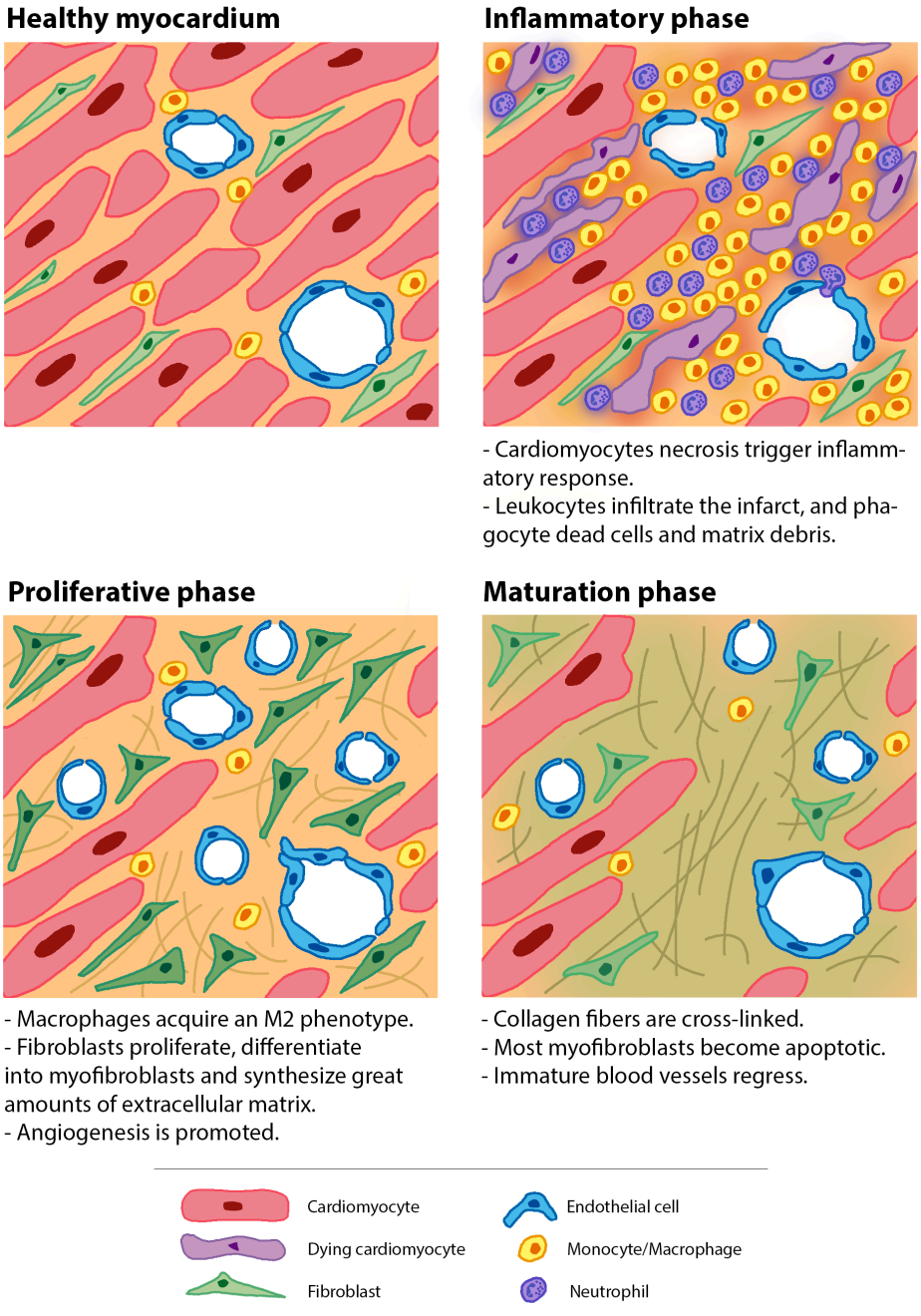

Figure 4 – Overview of the cardiac repair process following an acute myocardial infarction.

The physiologic cardiac repair process ends with the maturation phase and the establishment of a stable and vascularized scar. However, the loss of functional myocardium entails the overload of the remaining non-infarcted organ, which then leads to structural modifications of the ventricle, known as cardiac remodeling [47]. Myocardium overload promotes cardiomyocyte hypertrophy, as a compensatory mechanism, thus reducing cardiac wall stress and allowing the maintenance of cardiac output. Over time, the heart undergoes architectural and geometrical changes as a result of an increasing physical demand. Heart chambers dilate and become more spherical, and the heart wall gets thinner. These alterations are associated to a progressive systolic dysfunction, a higher incidence of arrhythmias, and an increased risk of heart failure [55]. As initiated by compensatory mechanisms, the degree of cardiac remodeling is directly dependent on infarct size [56,57].

1.3.1.- Cardiomyocytes

Cardiomyocytes are the functional unit of the heart carrying the generation of the contractile force. Due to their high metabolic demand, cardiomyocytes are particularly susceptible to ischemia. However, as highly specialized and terminally differentiated cells they retain a minimal proliferative capacity within the adult human heart [58]. Therefore, after cardiomyocyte loss, there is no functional replacement [5]. As particularly sensitive and functionally irreplaceable cells, the necrosis of cardiomyocyte serves as major stimulus to initiate the inflammatory phase of the cardiac repair program [48]. Also, as functional units of the myocardium, cardiomyocytes become critical players of cardiac remodeling [55].

1.3.2.- Leucocytes

Upon myocardial injury, resident immune cells are activated and initiate an acute immune response. Likewise, following cardiomyocyte necrosis, most cell types present in the myocardium acquire a pro-inflammatory phenotype, further contributing to the recruitment of immune cells to the damaged tissue. Neutrophils are first to infiltrate the infarcted tissue [59]. They contribute to the clearance of dead cells and matrix debris. However, evidence support that neutrophils may also

aggravate damage through direct cytotoxic effects upon nearby viable cardiomyocytes [50,51].

Due to their relative abundance and plasticity, monocytes and macrophages play a central role in regulating the events of cardiac repair [48]. During the inflammatory phase, both resident and infiltrating macrophages display a pro-inflammatory M1 phenotype, with a marked phagocytic activity. Thereafter, as the infarct is cleared, macrophages switch to an anti-inflammatory M2 phenotype setting the start of the proliferative phase [59,60]. Macrophages, then, contribute to the clearance of the injured tissue and the orchestration of the inflammatory phase, providing multiple cytokines and growth factors to mediate the inflammatory state, fibroblast activation, and angiogenesis [61].

Additional leukocyte subsets infiltrate the infarct alongside neutrophils and macrophages during the inflammatory phase [59]. Amongst them, cytotoxic T lymphocytes are predominant, and have been reported to both play a role in the orchestration of the immune response, and to aggravate damage through the cytotoxic activities upon viable cardiomyocytes [62].

1.3.3.- Fibroblasts

The human heart contains abundant fibroblasts [63]. Following AMI, resident cardiac fibroblasts contribute to the initiation of the inflammatory phase of cardiac repair, producing pro-inflammatory cytokines in response to cardiomyocyte necrosis. During the inflammatory phase, fibroblasts act as a source of interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), and metalloproteinases (MMPs) [64]. Once necrotic cells and matrix debris have been cleared, immune cells produce anti-inflammatory cytokines, including transforming growth factor-β (TGF-β). TGF-β stimulation activates the differentiation process of cardiac fibroblasts into myofibroblasts, which exhibit a contractile and synthetic phenotype [65]. During the proliferative phase, myofibroblasts are responsible for the synthesis and deposition of extracellular matrix to organize the scar [52]. Although scar formation is necessary to keep organ integrity after myocardial injury, an excessive fibroblast activation can lead to pathologic cardiac fibrosis [65].

1.3.4.- Endothelial cells

The myocardium has a high metabolic demand, therefore, it contains an extensive vascular network. ECs line the entire cardiovascular system, including all kinds of blood vessels and the endocardium, thus representing the most abundant cell type in the heart [66]. Rather than a barrier between bloodstream and the surrounding tissue, ECs play pivotal roles in regulating heart homeostasis and disease. They provide multiple factors involved in cardiomyocyte growth, metabolism, and contractility [67,68]. Following AMI, ECs acquire a pro-inflammatory phenotype and express surface adhesion molecules such as P- and E-selectins, that allow the interaction with circulating leukocytes and their extravasation to the injured tissue [48,68]. Microvascular rarefaction occurs in the acute phase of AMI reducing the blood perfusion in the infarcted tissue [69]. Activated ECs are also an important source of pro-inflammatory cytokines and chemokines during the inflammatory phase of cardiac repair.

During the proliferative phase, ECs proliferate and migrate towards the necrotic core to form a new vascular network. Indeed, ischemia is a potent stimulus for angiogenesis, which is the process of neovessels formation from pre-existing blood vessels [70]. Thus, the angiogenic process starts very early following coronary occlusion [71]. However this ECs response is not sufficient to compensate for the tissue needs. The vascularization of the scar is essential to meet the metabolic demand of the cardiac repair process, and play a role in the stabilization of the scar. Initial neovessels display a pro-inflammatory phenotype. They lack a pericyte and smooth muscle coat, and are hyperpermeable, which facilitate the extravasation of circulating leukocytes. Over time, newly formed blood vessels mature and acquire a mural coat. During the maturation phase of cardiac repair, uncoated vessels degenerate [47,49]. The establishment of a functional vascular network is fundamental to the stabilization of the scar, and it is associated with better outcomes [70,72]. Angiogenesis further provides a mechanism to restore microvascular dysfunction resulting from AMI, that is also an important factor affecting the outcome [69].

1.4.- Conditioning phenomena

The conditioning phenomena propose the existence of a possible protection to the myocardium arising from the application of transient cycles of ischemia and reperfusion before a prolonged ischemic insult (ischemic pre-conditioning; IPC) [73], after ischemia and before reperfusion (ischemic post-conditioning; IPostC) [74], or even in a remote organ before, during or after an ischemic insult (remote ischemic conditioning, RIC) [75–78] (**Figure 5**). The discovery of the conditioning phenomena supported the existence of an endogenous protective response against I/R injury.

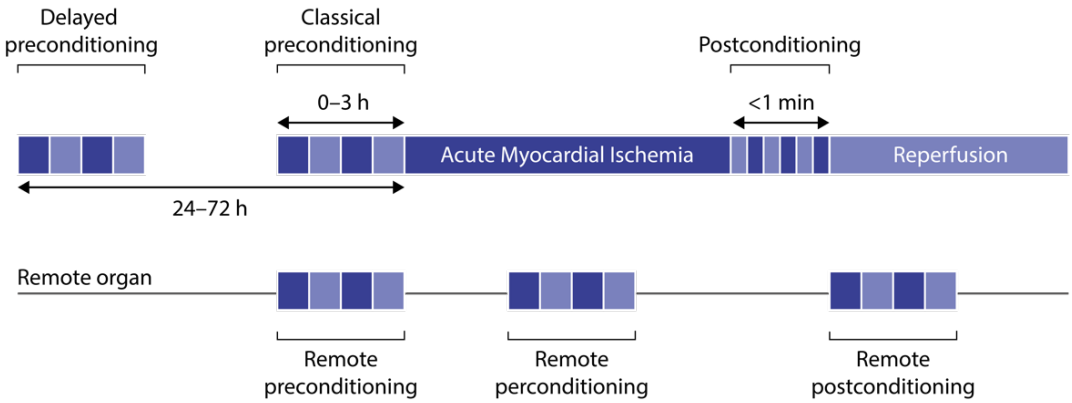

Figure 5 – Schematic representation of the different forms of ischemic conditioning.

IPC was the first to be described, back in 1986 by Murray *et al.* [73], who experimentally reported four cycles of alternated 5 min ischemia and reperfusion, applied immediately before a period of sustained ischemia, to greatly reduce infarct size and to improve heart function in a pre-clinical animal model. However, given the unpredictability of AMI, the clinical use of IPC is fairly limited. Thus, after the initial observation, several variations of the protocol have been explored. This is the case of IPostC, which is more clinically relevant, as the conditioning protocol is performed at the time of reperfusion [74]. It also proved effective in animal models as means of infarct size reduction, and the amelioration of post-ischemic endothelial dysfunction and blood flow defects [74,79,80].

Although offering interesting therapeutic opportunities to AMI patients, the need of direct manipulation of the heart is a major disadvantage of the local conditioning protocols (i.e. IPC and IPostC). In this context, Przyklenk *et al.* [75] made the intriguing discovery that an IPC stimulation at the circumflex coronary artery also protected the heart from I/R from the left anterior descending coronary artery, thus suggesting that the IPC signal is diffusible. Later on, the same effect was seen for kidney [78], small intestine [76], and limb [77], where the application of IPC protocol remotely protected the heart from I/R. This phenomenon was termed remote ischemic pre-conditioning, and has proven effective also when applied during ischemia (remote per-conditioning) [81], and at the time of reperfusion (remote post-conditioning) [82].

Whilst the benefits of ischemic conditioning have been broadly proven in animal models, clinical trials are controversial [83–85]. Differences in the conditioning protocols, comorbidities and patient medications may be important sources of variation for the clinical study of ischemic conditioning [86–88]. The elucidation of the molecular mechanisms behind the conditioning phenomena is essential to improve translation, as well as to develop pharmacologic strategies to stimulate the endogenous cardioprotective program. For IPC and IPostC, the current understanding of the molecular mechanisms at play suggests that the conditioning stimuli induce cardiomyocytes to release autacoids (such as adenosine, bradykinin, endothelin, and opioids), which then bind and activate their specific G-protein coupled receptors (GPCR), triggering a number of signaling pathways that mostly converge in the mitochondria. For RIC, although the cellular mediators and end effectors are thought to be the same, the activation of a neuro-humoral pathway appears to be a key factor as a trigger [89–91].

1.4.1.- Local conditioning triggers

The first evidence that the cardioprotective effects of ischemic conditioning result from a signal transduction program arises from the observation that the coronary infusion of either adenosine or A1-adenosine receptor agonists, prior a period of sustained ischemia, limits infarct size [92,93]. Conversely, the administration of A1-adenosine receptor antagonists abrogates both IPC and IPostC effects [92,93]. A1-

adenosine receptor is a GPCR coupling both G_i and G_q heterotrimeric $G_{\alpha\beta\gamma}$ proteins [94]. Signaling downstream G_i results in the inhibition of adenylate cyclase and the activation of mitogen-activated protein kinases (MAPK) pathway, whereas G_q-coupled receptors signaling activate phospholipase C (PLC). Later, PLC catalyzes the cleavage of phosphatidylinositol 4,5-biphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), that serve as second messengers to induce a rise of the intracellular Ca^{2+} concentration and the activation of protein kinase C (PKC) (**Figure 6**).

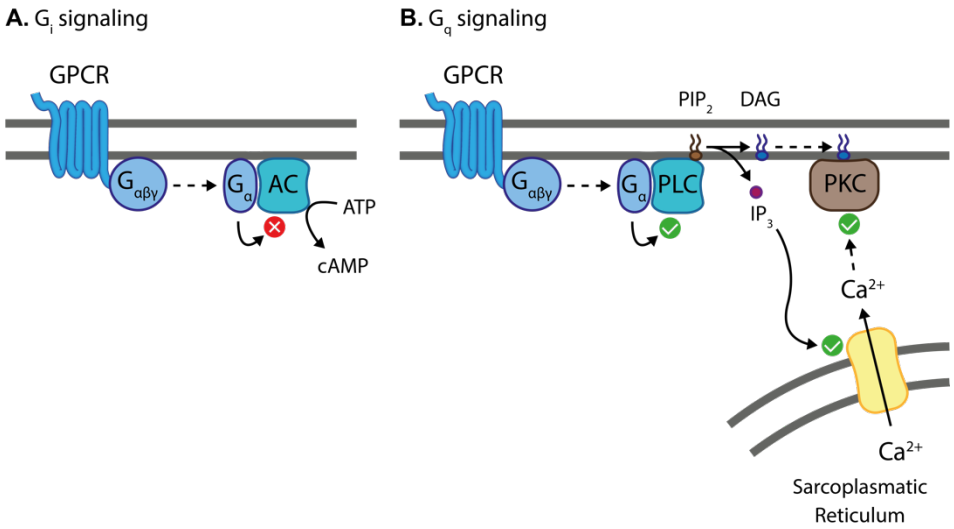

Figure 6 – Schematic representation of G-protein coupled receptors (GPCRs) activation signaling, for GPCRs coupling G_i (**A.**) and G_q (**B.**) heterotirmeric $G_{\alpha\beta\gamma}$. GPCR, G-protein coupled receptor; AC, adenylate cyclase; cAMP, cyclic adenosine mono-phosphate; PLC, phospholipase C; PKC, protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate.

Several G_i and G_q-coupling receptor agonists have been proposed as IPC and IPostC triggers. This is the case of bradykinin [95,96], opioids [97,98], acetylcholine [99], catecholamines [100], angiotensin II [101], and endothelin-1 [102]. All these molecules (i.e. autacoids) are produced by resident cells during the conditioning cycles and act in an additive fashion eventually reaching a threshold for protection. Hence, while each of the inductors triggers different signaling pathways, they must

eventually converge to elicit cardioprotection [91]. Protection can also be achieved through the exogenous administration of agonists [103].

Receptors tyrosine kinase (RTKs) may also play a role in cardioprotection [89]. The exogenous administration of growth factors, which mainly bind RTKs, has proven to limit infarct size [104,105]. Likewise, the treatment with broad spectrum tyrosine kinase inhibitors abolishes IPC protective effects [106]. However, no evidence currently support a causal relation between growth factors and the conditioning phenomena; hence, cytosolic tyrosine kinases may also be at play [90].

ROS have also been found to play a role in the conditioning phenomena [89–91]. Although the over-production and accumulation of ROS is toxic, small amounts of ROS play important roles in cell signaling [33]. The IPC cycles cause a transient burst of ROS prior to myocardial ischemia [107], and antioxidant administration abrogates both IPC and IPostC effects [108,109]. Also, low doses of ROS have been shown to mimic IPC in the absence of conditioning cycles [110].

Although cardiomyocytes being the functional unit of the myocardium are central to cardioprotection, other cell types present in the heart have been described to play pivotal roles in triggering the cardioprotective response. This is the case of ECs, whose unique location, between the blood-stream and tissue, makes them active players sensing and responding to heamodynamic changes and ischemia [68]. Therefore, ECs act as an important source of autacoids during the conditioning I/R cycles [67,111].

1.4.2.- Local conditioning mediators

A number of signaling pathways have been reported to mediate the cell response to ischemic conditioning. PKC was the first downstream mediator to be identified through inhibition and stimulation studies in IPC models [112]. PKC is activated downstream GPCR-mediated PLC activation. Several PKC isoforms are expressed in the heart, each one believed to interact with a specific receptor for activated PKC (RACK) upon activation. RACKs locate into specific organelles, bringing each PKC isoform to its target's proximity [113]. Thus, cardioprotective effects arising from

PKC activation are isoform-specific. Although some disparities have been reported across species, IPC was found to activate PKC ε-isoform, and to inhibit PKC ∂-isoform. Upon activation, PKC ε-isoform is imported to the mitochondria, where it activates the mitochondrial ATP-sensitive K^+ channels (mitochondrial K_{ATP} channels), and to the sarcoplasmic reticulum, where it limits Ca^{2+} release [90]. PKC ε-isoform has also been causally involved in the cardioprotection afforded by IPostC [114].

A role for phosphatidylinositol 3'-kinase (PI3K)/Akt pathway limiting infarct size upon ischemic conditioning has also been proposed [115,116]. When active, PI3K is recruited to the plasma membrane, where it phosphorylates phosphatidylinositol 4,5-bisphosphate, generating phosphatidylinositol 3,4,5-triphosphate (PIP_3). Accumulation of PIP_3 recruits Akt to the membrane, where it is activated by phosphoinositide-dependent protein kinases (PDKs). Akt then phosphorylates downstream targets both in the cytoplasm, and mitochondria, such as the adenosine nucleotide translocase (a suspected member of the mPTP), the glycogen synthase kinase-3β (GSK-3β) and the hexokinase-2 [115]. The endothelial nitric oxide synthase (eNOS) is also a target for Akt [117]. Upon phosphorylation eNOS produces nitric oxide (NO), activating soluble guanylate cyclase which then produces cGMP that activates protein kinase G (PKG). Mitochondrial K_{ATP} channels, and the Na/H^+-exchanger, are PKG targets [96]. PKG have proven to be causally involved in the conditioning phenomena [118,119]. The activation of PI3K/Akt and downstream targets, together with the activation of the p44/p42 extracellular signal-regulated protein kinase (Erk1/2) at reperfusion, have been collectively designated as the reperfusion injury salvage kinase (RISK) pathway (**Figure 7**) [120]. Interestingly, it shows a biphasic activation during the IPC cycles and at the time of reperfusion [121,122]. Whether the activation of PI3K and the RISK pathway upon conditioning stimuli is downstream GPCRs, RTKs, or independent of receptors (e.g. through redox signaling), remains to be elucidated.

The mitogen-activated protein kinase (MAPK) pathway may also be involved in IPC signal transduction [89–91]. MAPKs are a group of protein kinases involved in signal transduction for a wide spectrum of cellular stimuli. They can be classified into three

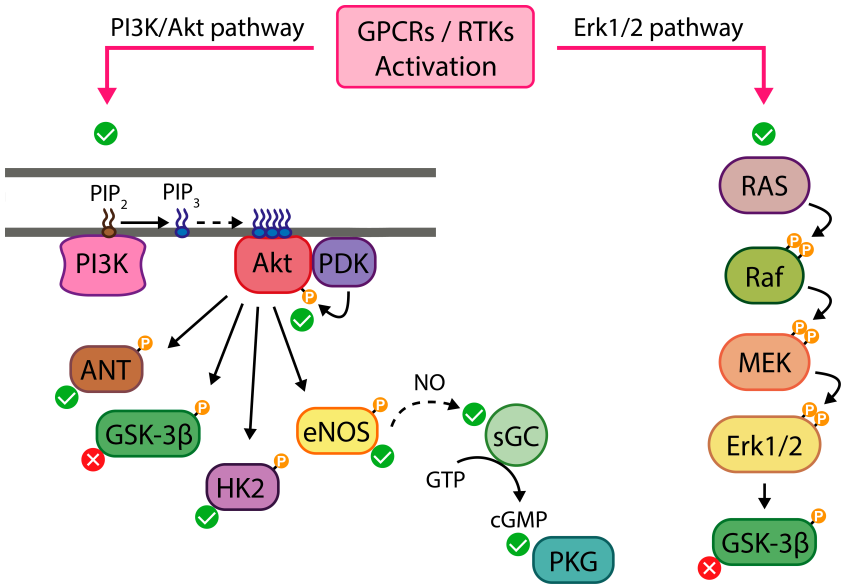

Figure 7 – Overview of the reperfusion injury salvage kinase (RISK) pathway components. GPCRs, G-protein coupled receptors; RTKs, receptors tyrosine-kinase; PI3K, phosphatidylinositol 3 kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate; PDK, phosphoinositide-dependent protein kinase; ANT, adenosine nucleotide translocase; GSK-3β, glycogen synthase kinase-3β; HK2, hexokinase 2; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylate cyclase; cGMP, cyclic guanylate mono-phosphate; PKG, protein kinase G; MEK, mitogen-activated protein kinase/ERK kinase; Erk1/2, p44/p42 extracellular signal-regulated protein kinase.

subfamilies, which are: the extracellular signal-regulated kinases (Erk), the c-jun N-terminal kinases (JNK), and the p38 MAPKs. There are studies linking each MAPKs subfamily to cardioprotection [89,123]. However, there is not yet an agreement regarding their specific roles, and controversial data have been raised. While some studies report Erk1/2 activation following IPC stimulation, other report no changes [124]. Likewise, the administration of Erk1/2 specific inhibitors abrogated infarct limiting effects of IPC in a pig model [125], but no effects were reported for rat and rabbit [126,127]. The pharmacological activation of Erk1/2 at the time of reperfusion appeared to be protective, suggesting a role in IPostC [120]. We find a similar scenario for the JNK subfamily, with reports both evidencing and denying a causal relation to the conditioning phenomena [128,129]. As a matter of fact, transgenic knockout (KO) mice models for JNK appear to be protected against I/R

in vivo, but the overexpression of MAPK kinase 7, which is the JNK activator, also confers protection [130]. The role of p38 is also surrounded by controversial data. Completely different patters of activation upon ischemia, reperfusion and IPC cycles, have been reported for different species, as well as different effects of specific inhibitors upon infarct size [89,123]. Whilst at least five isoforms of p38 MAPK have been described, the heart mainly express the α and β-isoforms, reportedly displaying opposite effects in cardioprotection [131]. The relative balance between isoforms in each species, as well as the isoform specificity of inhibitors may be the factors behind results disparities. MAPK pathway plays an unresolved role in cardioprotection, as multiple effects have been reported from MAPK pathway manipulation upon I/R. Also, PKC-ε, which is causally involved in IPC has been found to form a signaling complex with Erk1/2, p38 MAPK and JNK in the mitochondria [132].

Another mediator of the cardioprotection afforded by ischemic conditioning is the designated survival activating factor enhancement (SAFE) pathway [133]. This entails signaling through the TNFα receptor 2 (TNFR2) and the downstream activation of Janus kinase (JAK) and the signal transducer and activator of transcription-3 (STAT-3), during reperfusion (**Figure 8**) [134]. As a transcription factor, STAT-3 is involved in the mid-term up-regulation of proteins involved in cardioprotection [135]. The SAFE pathway is believed to be parallel to the RISK pathway, but necessary both for IPC and IPostC [89,90,133].

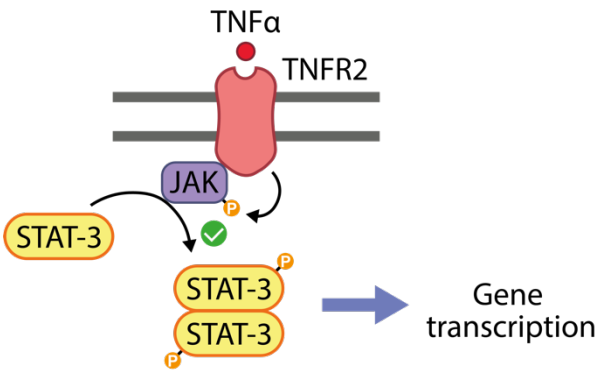

Figure 8 – Overview of the survival activating factor enhancement (SAFE) pathway. TNFα, tumor necrosis factor-α; TNFR2, tumor necrosis factor receptor 2; JAK, Janus kinase; STAT-3, signal transducer and activator of transcription-3.

1.4.3.- Local conditioning effectors

Cell signaling following ischemic conditioning is complex and diverse. Multiple pathways, simultaneously triggered, coordinate a response to offset and counteract the deleterious effects of I/R. Being mitochondria the major O_2 consumer organelle and a metabolic hub, they firstly sense the effects of ischemia and lead ROS production and apoptosis at reperfusion. Functional mitochondria guarantee the maintenance of cellular ATP pools, which further allow keeping the ionic balance. Whereas mitochondrial dysfunction compromise ATP production, promoting cellular ionic unbalance, the opening of mPTP, and apoptosis. Mitochondria are, therefore, central to the cell response to I/R and, not surprisingly, virtually all pathways involved in cardioprotection converge there [16,136,137].

The opening of the mPTP is a feature of I/R injury leading to mitochondrial swelling, OMM rupture, and apoptosis upon reperfusion, which significantly contributes to the final infarct size [42–44]. Pharmacological strategies targeting the opening of mPTP have proven cardioprotective in animal models [46]. While factors contributing to mPTP opening prevail at reperfusion [42–44], both IPC and IPostC inhibit the opening [138,139]. Interestingly, conditioned mitochondria, isolated at the end of reperfusion, exhibit a reduced mPTP Ca^{2+} sensitivity, which suggests a certain degree of inhibition following ischemic conditioning [138]. Multiple molecular mechanisms have been postulated to explain the mPTP desensitization afforded by ischemic conditioning. Ischemic conditioning mediators Akt, Erk1/2, PKC, and PKG, phosphorylate GSK-3β at Ser9, resulting in its inhibition [115]. GSK-3β has been found to target all known mPTP components, whose phosphorylation (by GSK-3β) primes the opening [140,141].

Paradoxically, the mPTP opening may also serve as trigger for ischemic conditioning signaling. As previously discussed, the mPTP opening is early promoted at reperfusion. A transient mPTP opening, during ischemic conditioning cycles, has been suggested to produce small amounts of ROS, which may then trigger a cardioprotective program via redox signaling [137,142]. For instance, small amounts of ROS are known to activate PKC-ε [143].

Mitochondrial K_{ATP} channels are also believed to play a role downstream the endogenous cardioprotective signaling. Although the molecular identity is still a matter of debate, studies with pharmacological inhibitors have demonstrated a role in IPC and IPostC mediated cardioprotection [144,145]. The mitochondrial K_{ATP} channel is a target for NO, PKC and PKG, all involved in the signaling program described for ischemic conditioning [146–148]. The opening of mitochondrial K_{ATP} channels is coupled to a small production of ROS, which in turn trigger further signaling eventually preventing mPTP opening [149]. The mechanism by which the opening of mitochondrial K_{ATP} channels generates ROS is also not yet fully understood.

Although ischemic conditioning cycles induce a transient burst of ROS, which seems to play a role in the settlement of protection [107,110], ischemic conditioning reportedly offsets ROS accumulation at reperfusion [150]. However, the mechanism at play remains to be elucidated. A role for the endogenous antioxidant response, which comprises the activation of the nuclear erythroid 2-related factor-2 (Nrf-2) and the downstream transcription of the antioxidant response elements, has been proposed [151]. But the need of protein expression makes it only feasible for the delayed protection afforded by ischemic conditioning. Also, whilst the accumulation of succinate during ischemia and its oxidation at reperfusion is a major source of ROS, IPC failed at modifying these kinetics [152].

Another effector of ischemic conditioning are the Na^+/H^+ and the Na^+/Ca^{2+} exchangers, involved in the cellular ionic unbalance of I/R. Interestingly, IPC was found to inhibit the activity of both exchangers, limiting Ca^{2+} and Na^+ overloads during ischemia [153]. Likewise, the activation of PKG, following IPostC, has proven to delay the recovery of physiologic pH at reperfusion throughout the inhibition of the Na^+/H^+ exchangers [118].

1.4.4.- Second window of protection

While infarct size limiting effect of IPC disappears within 2-3 hours after the IPC stimulation, cardioprotection spontaneously re-appears around 12-24 hours later and lasts 72 hours [154]. IPC, thus, follows a biphasic response, offering two

windows of protection. Whilst the first window of protection (also known as classic conditioning) results from the interaction of mediators with end effectors (i.e. mPTP components, mitochondrial K_{ATP} channel, and ion channels), the second window of protection (also known as delayed conditioning) entails the activation of transcription factors and subsequent *de novo* protein synthesis. STAT-3, Nrf-2, activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and the hypoxia-inducible factor-1α (HIF-1α) are known transcription factors activated following ischemic conditioning. The downstream transcriptional program coordinates the expression of distal mediators (i.e. proteins involved in delayed cardioprotection). Known examples of these *de novo* synthesized proteins are the mitochondrial Mn^{2+}-superoxide dismutase (MnSOD) and hemeoxygenease-1 (HO-1), both members of the antioxidant response, heat-shock proteins, involved in protein folding, and the inducible nitic oxide synthase (iNOS) [155].

1.4.5.- Remote ischemic conditioning

Without a need of manipulating the heart, RIC offers a more clinically relevant strategy to stimulate the endogenous cardioprotective program. However, whilst both mediators and effectors of RIC afforded cardioprotection are essentially the same as for local conditioning methods, the mechanism by which conditioning stimuli are remotely transferred to the myocardium is currently unclear [89,90]. The initial hypothesis involved the release of a humoral factor and the diffusion from the conditioned organ to the heart. This hypothesis was supported by the observation that blood taken from rabbits subjected to IPC could protect the heart and limit infarct size of untreated animals after whole blood transfusion [156]. Theoretically, autacoids released from the remote organ in response to ischemic conditioning cycles could enter the bloodstream eventually reaching the myocardium to elicit cardioprotection. Experiments with non-specific antagonists have raised evidence for adenosine [157], opioids [158], bradykinine [159], and endocannabinoids [160] as triggers for RIC induced cardioprotection. Whilst these autacoids resemble the known triggers for local conditioning, RIC induction appears to be more complex. A different hypothesis, involving a neural pathway, was raised after the observation that a treatment with the ganglion blocker hexamethonium

inhibited RIC protective effects [76]. The involvement of a neural pathway in triggering RIC effects was further demonstrated by dissection of the renal nerve [161], and resection of the heart's vagal innervation [162], both abrogating RIC effects. Although initially conceived as mechanistically different, these two hypotheses are not mutually exclusive. Indeed, both humoral and neural pathways have proven co-dependent to elicit cardioprotection following RIC stimulation [163]. Further research is needed in order to understand the interconnection between both in the induction of RIC cardioprotection.

1.4.6.- The endogenous cardioprotective program

The discovery of the conditioning phenomena has evidenced the existence of an endogenous cardioprotective program. Ischemic conditioning techniques have proven to work in a number of animal models, and evidence has also been provided for the human [164,165]. This is the case of pre-infarction angina, in which patients with AMI preceded by angina exhibit smaller infarcts and better outcomes, and the warm up phenomenon, referring to the better outcomes of AMI patients who underwent a brief period of exercise before the AMI onset [165]. However, clinical trials on conditioning techniques have brought mixed results, with both positive and neutral effects reported (**Figure 9**) [83–85]. Differences in the conditioning protocols (i.e. the number and duration of I/R cycles), comorbidities, and patient medications, may be the main factors contributing to results disparities [86–88]. Also, different responses to conditioning could be expected from patients presenting fully or partially occluded coronary arteries, as well as for different AMI durations [166].

Beyond ischemic conditioning, the investigation of the molecular mechanism behind cardioprotection has provided multiple targets susceptible to pharmacologic manipulation. The exogenous stimulation of cardioprotective programs (i.e. pharmacologic conditioning) has also been an intensive field of research over the past years. However, whilst hundreds of compounds have proven cardioprotective in animal models, most drugs failed at demonstrating clinical benefits [14,15]. Importantly, pre-clinical research is usually performed in healthy animals, lacking of atherosclerosis, risk factors, comorbidities and medications, all common in

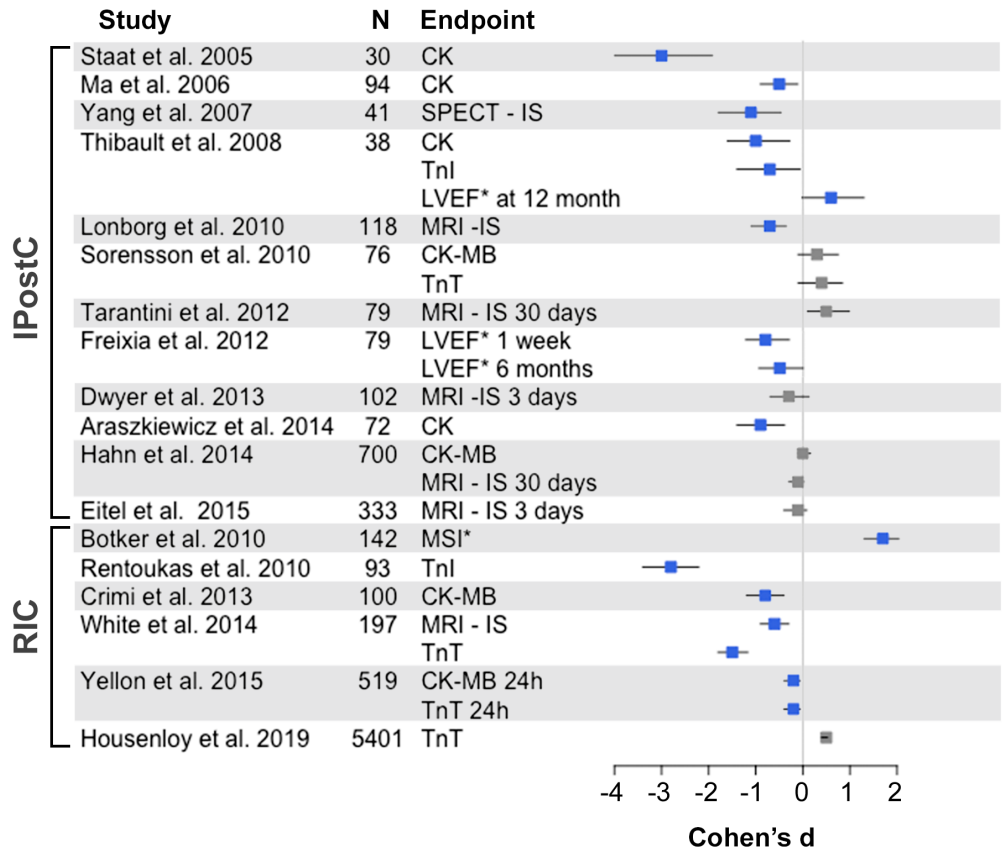

Figure 9 – Forest plot of major clinical studies on ischemic post-conditioning and remote ischemic conditioning in acute myocardial infarction patients. Squares and horizontal bars represent the mean effect size and 95% confidence interbal (calculated as Cohen's d statistic). Blue coloring represents significance. Based on Heusch, 2015 [90] and Housenloy and Yellon, 2016 [166]. IPostC, ischemic post-conditioning; RIC, remote ischemic conditioning; N, sample size; CK, creatinine kinase; CK-MB, creatinine kinase-muscle brain; SPECT, single photon emision computed tomography; IS, infarct size; TnI, troponin-I; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; MSI, myocardial salvage index; TnT, troponin-T. * These endpoints reflect myocardium salvage instead of damage.

patients undergoing AMI. Furthermore, the most robust endpoint for cardioprotection in pre-clinical research is infarct size, as it is the major predictor of clinical outcomes [6–8], but mid and long-term effects, with regard to adverse remodeling and heart function, are usually overlooked. Thus, whilst a certain

intervention may result in acutely reduced infarct size, collateral effects of the same intervention may affect the repair process diluting the effect over time [15]. More robust animal models, multi-center cooperation, and long term follow up, are needed to improve pre-clinical research translation.

Furthermore, the disappointing results from clinical trials highlight knowledge gaps within the understanding of the endogenous cardioprotective program. Although, the schematic view of ischemic conditioning as the sum of triggers, mediators, and effectors has helped the understanding of the operating mechanisms, it may be too simplistic. Rather than linear and unidirectional pathways, cell signaling more likely results from a complex network of protein-protein interactions. Hence, all identified mechanisms may be simultaneously active, interacting with each other, and concomitant to a major stressing situation such as ischemia and reperfusion. Moreover, in AMI patients, those interactions and signaling pathways are also affected by an underlying pathology, adding an extra layer of complexity. Further research, which must consider the identity and nature of these interactions, is needed to conceive the whole picture of the endogenous cardioprotective program and to improve clinical translation.

1.5.- Protein DJ-1

The early-onset Parkinson's disease associated protein DJ-1 (also known as PARK7) is a ubiquitously expressed homodimeric small protein (of about 20 kDa) belonging to the DJ-1/pfpI superfamily, with conserved homologues from bacteria to human [167]. Structurally, DJ-1 exhibits an α/β-fold composed of 11 β-strands (β1-β11) and 8 α-helices (αA-αH). It displays a central β-sheet composed of 7 β-strands, 6 of them parallel, wraped by the eight α-helices. The αH chain of DJ-1 is the only α-helix that does not directly contact the central β-sheet, instead it projects away and contacts α-helices αA and αG. Beyond the central β-sheet, there is a β-harpin (formed by β3 and β4) that locates in the dimerization surface, and a β-α-β motif (formed by β8, αF and β9) (**Figure 10**) [168,169].

Initially identified as an oncogene [170] DJ-1 has been further related to several pathologic conditions such as Parkinson's diseases [171,172], Alzheimer's disease [173,174], and heart failure [175], among others. Due to its role in pathology, DJ-1 has been extensively studied over the past decades, and several functions have been proposed, including chaperone [176] and protease activities [177], transcriptional regulator [178], redox sensor [179] and mitochondrial homeostasis keeper [180,181]. Also, a role in cell signaling has been suggested [182]. However, the exact molecular function and its regulation remain elusive. From all proposed functions, cell protection against oxidative stress induced damage is the most consistently described role for DJ-1.

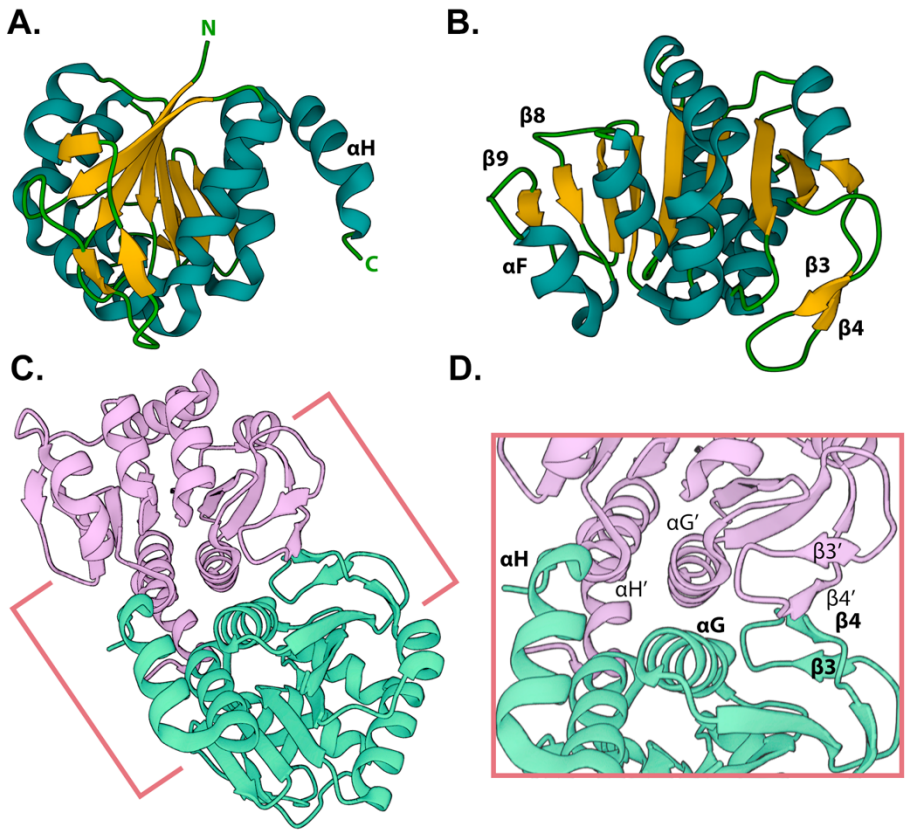

Figure 10 – Protein structure of DJ-1 monomer (**A.** and **B.**), dimer (**C.**), and detail of the dimerization interface (**D.**). **A.** and **B.** show different views of the DJ-1 monomer. 3D protein structure model was obtained from Protein Data Bank [301].

1.5.1.- Redox sensing and ROS quenching

Cysteine residues are known to play crucial roles in redox signaling. They contain a thiol group (-SH) which is susceptible to oxidation into sulfenic (-SOH), sulfinic (-SO_2H), and sulfonic acid (-SO_3H), that constitute important post-transcriptional modifications for redox signaling [183]. The oxidation of cysteine residues drives the formation of disulfide bounds, involved in the regulation of protein interaction, activation, localization, and stability [183]. DJ-1 has three highly conserved cysteine residues (Cys53, Cys46 and Cys106) thus allowing redox sensing and conferring the ability to eliminate small amounts of ROS through sporadic self-oxidation [184–187].

From all cysteine residues of DJ-1, Cys106 has been suggested as a key regulator of DJ-1 activity [179]. Mutations at Cys106 lead to an impaired protection against stress [188–192], which highlights the association existing between redox signaling and DJ-1. Indeed, ROS has been linked to the activation of DJ-1 and enhanced cell protection throughout the induction of a carboxyl-terminal 15 amino acid cleavage (DJ-1ΔC), corresponding to αH [177,193]. This mechanism might be complementary or dependant to Cys106 oxidation. Despite the process leading to DJ-1 activation remains unclear, there is a consensus regarding redox signaling, either through the direct oxidation of Cys106, the cleavage of the carboxyl-terminal α-helix, or both [177,185,193].

1.5.2.- Antioxidant defense

DJ-1 is known to play an important role in the anti-oxidant defense. Loss of DJ-1 entails an enhanced susceptibility to oxidative stress both *in vitro* and *in vivo*, whereas its overexpression is protective [194–196]. The three cysteine residues of DJ-1 allow the scavenging of ROS in small amounts [184]. However, as there is not a known mechanism by which this residues could be reduced after oxidation, the ROS-scavenging activity is believed a minor aspect of DJ-1 [197]. Therefore, a role in regulating downstream mediators of the antioxidant repose through redox sensing seems more reasonable [197]. In such a manner, DJ-1 has been reported to regulate

the Nrf-2 response to oxidative stress, and to aid the superoxide dismutase-1 (SOD1) activation.

Nrf-2 is a master transcription factor driving the cell response to oxidative stress. It plays a role in the establishment of the delayed cardioprotection conferred by ischemic conditioning. At basal conditions, Nrf-2 is constantly expressed, repressed by the Kelch-like ECH-associated protein-1 (Keap-1), and targeted for degradation through the proteasome system [198]. In an oxidative stress condition, Nrf-2 dissociates from Keap-1, avoiding degradation, and accumulating fast in the cell cytoplasm [199]. Then it translocates to the nucleus, where it interacts with other transcription regulators and activates transcription of the antioxidant response elements (ARE) which coordinate a detoxifying response [198–200]. DJ-1 has been found to directly interact with Nrf-2, preventing the association with Keap-1, and facilitating nuclear translocation and downstream effects upon oxidative stress [201,202]. The possibility of tissue-specificity for the DJ-1/Nrf-2 interaction has also been discussed, as Nrf-2 activation was found independent of DJ-1 in brain [203].

Additionally, DJ-1 has been reported to act as a copper chaperone for SOD1, which is a known ARE [204,205]. SOD1 catalyzes the conversion of the superoxide anion (O^-) to molecular oxygen and hydrogen, in a reaction that is dependent on copper and zinc. DJ-1 was shown to interact with copper ions and to transfer them to SOD1 following activation [205]. Further roles of DJ-1 affecting cell response to oxidative stress arise from the regulation of signaling pathways involved in cell fate decisions.

1.5.3.- Mitochondrial homeostasis

Despite mitochondria being the main source of ROS, they are particularly sensitive to oxidative stress [206]. ROS damage to the mitochondria may result in mitochondrial dysfunction, ATP depletion, mPTP opening, and cell death. Thus, mitochondria have proven central in the pathophysiology of several diseases, such as Parkinson's diseases, and I/R injury, among others [207,208]. DJ-1 is known to play a role in regulating mitochondrial homeostasis. The lack of DJ-1 results in mitochondria fragmentation, reduced mitochondrial complex-I activity, and mitochondrial depolarization after the exposure to cell stressors, both *in vitro* and

in vivo [209–212]. Despite, little amounts of DJ-1 are located in the mitochondria at basal conditions [213], Cys106 oxidation drives its translocation to healthy mitochondria through a mechanism dependent on the glucose-regulated protein 75 (GRP75; also known as mitochondrial stress-70 protein; HSPA9) [190,214]. Within the mitochondria, DJ-1 has been described to bind and preserve the mitochondrial complex-I function under stress [180], to bind and regulate the activity of the mitochondrial F_1F_O-ATP synthase [215], and to prevent excessive mitochondrial fission following I/R [216].

1.5.4.- Transcriptional regulation

DJ-1 has the ability to regulate transcription for several genes through the interaction with various transcription factors [178]. This is the case of Nrf-2, the androgen receptor (AR) [217,218], tyrosine hydrolase (TH) [219], p53 [220,221], sterol responsive element binding protein-2 (SREBP-2) [222], Ras-responsive element (RRE)-binding protein-1 (RREB-1) [223], and signal transducer and activator of transcription-1 (STAT-1) [224]. Altogether, placing DJ-1 in the regulation of a wide range of cellular processes.

Of particular interest for I/R injury and CVDs are the regulation of Nrf-2 and the antioxidant response elements, and SREBP-2, which is as a positive regulator of the low density lipoprotein receptor (*LDLR*) gene. The lack of DJ-1 leads to a reduced *LDLR* expression, and consequently, higher levels of circulating LDL. Again, Cys106 oxidation appears to be essential for the enhancement of *LDLR* gene expression by DJ-1, as the C106S mutant of DJ-1 fails to activate *LDLR* transcription [222].

1.5.5.- RNA-binding activity

Despite DJ-1 lacks a canonical RNA-binding domain, it has the ability to bind RNA, as demonstrated by UV-cross-linked immunoprecipitation (CLIP) studies of DJ-1 [225–227]. Yet in 1999, DJ-1 was identified as a regulatory member for a large RNA-binding complex [225]. Since then, multiple transcripts have been identified to interact with DJ-1 at RNA level [226]. However, no functional characterization has been provided, and thus the nature and purpose of such interaction is not

completely understood. Interestingly, the oxidation status of DJ-1 appears to be the regulator of its RNA-binding activity; thereby, DJ-1 dissociates from interacting transcripts under oxidative stress, which may allow their translation [226,227]. Moreover, DJ-1 reportedly interacts with messenger ribonucleoprotein (mRNP) granules during stress [228], also involved in gene translation [226].

1.5.6.- Cell signaling

DJ-1 is believed to be implicated in the regulation of cell signaling either directly, through the interaction and modulation of pathway mediators, or indirectly interacting with inhibitors or co-activators [182]. Erk1/2, p53, Akt, and the apoptosis signal-regulating kinase 1 (ASK1) are the four major pathways in which DJ-1 reportedly plays a role.

The Erk1/2 pathway is a classical MAPK signaling pathway involved in many cellular functions such as cell growth, autophagy and differentiation, that has been reported to be affected by DJ-1. The overexpression of DJ-1 resulted in a higher activation of Mek1/2 and Erk1/2 *in vitro*, and the inhibition of Erk1/2 signaling abrogates DJ-1 protective effects [229]. Evidence for direct interaction between DJ-1 and Erk1/2 has also been provided by co-immunoprecipitation studies both *in vivo* and *in vitro*, and a role promoting Erk1/2 translocation to the nucleus under oxidative stress has been proposed [230]. However, there is not yet a robust consensus on the mechanism by which DJ-1 regulates Erk1/2 signaling, and a role for DJ-1 interacting with Erk1/2 upstream factors has also been reported [229,231,232]. In such a manner, c-Raf [232], an upstream activator of Mek1/2, and protein phosphatase 2A (PP2A) [229], a negative regulator of Erk1/2, have also been found to interact with DJ-1.

p53 is a major tumor-suppressor protein in the cell. It acts as a transcription factor which is activated in response to several cell stresses and promotes apoptosis, in order to preserve genome stability. A direct interaction of DJ-1 with p53 was proven by co-immunoprecipitation studies, in which Cys106 was shown crucial [221]. Functionally, DJ-1 has been suggested to inhibit p53 transcriptional activity through

the interaction with its DNA-binding domain in an oxidative stress-dependent manner [221,233].

The Akt pathway is one of the central signaling pathways controlling cell growth and survival, that has been implicated in the cell protection conferred by DJ-1. The most accepted mechanism by which DJ-1 promotes Akt signaling is through the inhibition of the phosphatase and tensin homologue (PTEN), a known antagonist of Akt signaling [234–237]. More specifically, DJ-1 has been reported to directly interact and inhibit PTEN activity through transnitrosylation [238]. This interaction, again, is dependent on the oxidation state of DJ-1; at least half of the total amount of DJ-1 has to be on its reduced state to successfully inhibit the activity of PTEN [239]. Additionally, during oxidative stress, DJ-1 has been reported to promote Akt recruitment to the membrane, a feature needed for Akt priming and activation [240].

The ASK1 pathway is a MAPK signaling pathway involved in the apoptosis commitment. Under stress, ASK1 is activated and builds up a signalosome that will trigger the intrinsic apoptosis pathway. DJ-1 reportedly inhibits ASK1 signaling at multiple levels. DJ-1 physically interacts with ASK1, in an oxidative stress-dependant manner [191,241,242]. Such interaction inhibits ASK1 activation, disrupting the signalosome assembly. In parallel, DJ-1 has been reported to repress ASK1 activation through the direct interaction and nuclear sequestration of the death-domain-associated protein 6 (Daxx), which needed for the activation of ASK1 [243]. DJ-1 lacks a nuclear importing signal; therefore, an additional interaction with p38 regulated/activated kinase (PRAK) is required to allow DJ-1 localization to the nucleus and Daxx sequestration [244].

1.5.7.- Enzymatic activity.

Although DJ-1 has been related to a wide range of cellular processes and signaling pathways, whether it displays an enzymatic activity is still a matter of debate, and a clear active center has not been identified so far. Structurally DJ-1 resembles to other members of the PfpI protein family with proven protease activity [168]. However, it lacks the characteristic catalytic triad. It has been hypothesized that the

conserved Cys106 residue could form a catalytic diad with His126, conferring some protease activity [177], but no evidence for this happening *in vivo* has been provided.

Furthermore, although still in debate, DJ-1 has been reported to display both deglycase [245–247] and glyoxalase [248–250] activities. Glycation is a non-enzymatic reaction whereby free reducing sugars combine with macromolecules, specially proteins, forming Amadori products (early glycation products). Over time, early glycation products undergo a series of reactions and rearrangements leading to the formation of advanced glycation end-products (AGEs) [251]. Their formation alter protein structure and function, and promote protein aggregation. Also, extracellular AGEs activate the receptor of AGEs (RAGE) which promotes inflammation [252]. Glycation is a fundamental mechanism involved in aging, and plays a significant role in pathology. Reducing sugars are barely reactive; thus, glycation and AGEs formation are slow rate reactions under physiologic conditions. However, the exposure of sugars, early glycation products, or lipids to oxidants results in the formation of reactive dicarbonyls, such as glyoxal and methylglyoxal, that are about 20.000 times more reactive than glucose and greatly speed up AGEs formation [253,254]. Deglycase activity defines the ability of repairing glycation products, whereas glyoxalase activity refers to the capacity of metabolizing glyoxal and methylglyoxal into non-reactive molecules.

A glyoxalase activity for DJ-1 has been consistently proved and reproduced across laboratories [248–250]. However, it displayed a considerably lower kinetic efficiency than that of the classical glutathione-dependent glyoxalase 1 (Glo-1), which is the main glyoxalase system of the cell. Regarding the deglycase activity, controversial data have been raised, and some reports suggest it a secondary effect of the glyoxalase activity [255,256].

1.6 - DJ-1 and I/R injury

DJ-1 regulates a wide variety of proteins and transcription factors, some of them directly involved in the endogenous cardioprotective program. This is the case of Akt and Erk1/2, core members of the RISK pathway, or Nrf-2, involved in the second

window of protection following ischemic conditioning [257,258]. Also the mitochondrial activities found for DJ-1 upon oxidative stress make possible a role for DJ-1 as a cardioprotection effector. Indeed, DJ-1 has been suggested as a promising therapeutic target for myocardial I/R injury [259]. In such a way, animals lacking of DJ-1 exhibits larger infarcts, increased mitochondrial fission, and worse left ventricular function as compared to wild-type littermates [216,257,260–262], whilst DJ-1 overexpression is protective [257,261]. Similar results have been raised for stroke models, in which the loss of DJ-1 resulted in larger infarcts *in vivo*, and enhanced cell death *in vitro* [263]. Moreover, although primarily conceived intracellular, it also has been found to be secreted under some specific conditions, such as, breast cancer [264], Parkinson's disease [265], and stroke [266], and the exogenous administration of DJ-1 has proven protective in stroke models [267,268].

2.

Hypothesis

2.- Hypothesis

Although revascularization is the definitive treatment for obstructive ischemic heart disease events, the blood reperfusion process paradoxically aggravates the damaged myocardium. Given the limited regenerative capacity of the adult human heart, there is little opportunity to recover the functional myocardium lost after acute myocardial infarction. Therefore, limiting infarct size is the best strategy to minimize re-events and to improve quality of life. The discovery of the conditioning phenomena, referring to the acquired cardioprotection arising from the application of transient cycles of ischemia and reperfusion, has evidenced the existence of endogenous cardioprotective programs. However, disappointing results have been obtained in clinical trials trying to promote cardioprotection.

The endogenous cardioprotective programs have been classically conceived linearly as trigger-mediator-effector processes. A view that has helped the identification of multiple players. But again, most of the pharmacological strategies targeting these players have failed to prove clinical benefits. Considering the complexity of the biological processes, and the presence of an underlying pathology, the classical view of cardioprotection may be too simplistic. A more holistic comprehension of the molecular basis of endogenous cardioprotective programs may help to improve translation.

DJ-1 is a multifunctional protein playing intriguing roles in cell survival for a wide variety of cellular insults and stresses. The existing consensus encompasses the maintenance of mitochondrial homeostasis upon oxidative stress. Despite the many functions reported for DJ-1, its exact molecular role and regulation have not been elucidated so far. Some of the reported molecular activities may result from a yet-to-be-understood upstream function of DJ-1.

Considering the molecular basis of ischemia and reperfusion injury, and the currently proposed molecular activities of DJ-1, we hypothesized that DJ-1 participates in the endogenous cardioprotective programs and that its regulation would provide a new therapeutic opportunity for myocardium salvage.

3.

Objectives

3.- Objectives

In order to test our hypothesis, we have set the following objectives:

Objective 1.- To explore the mitochondrial response of the myocardium to ischemic post-conditioning.

Objective 2.- To characterize the dynamics and functional implications of protein DJ-1 upon ischemia and reperfusion.

Objective 3.- To evaluate the therapeutic potential of recombinant protein DJ-1 for minimizing myocardium damage following acute myocardial infarction.

Objective 4.- To characterize the functional implications of the RNA-binding activity of DJ-1 upon ischemia and reperfusion.

4.

Materials and methods

4.- Materials and methods

The employed materials and methods have been extensively detailed within each of the scientific publications that comprise the results section of this thesis. Please refer to section 5.- Results.

4.1.- Animal care statement

All animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committees (CEEA-IR Sant Pau) of ICCC-Hospital de la Santa Creu i Sant Pau, and authorized by the Animal Experimental Committee of the local government in accordance to the Spanish law (RD 53/2013) and the European Directive 2010/63/EU. All experimental procedures were conformed to the Guide for the Care and Use of Laboratory Animals from the US National Institutes of Health [269], and were committed to the principal of the 3Rs (replacement, reduction and refining) for laboratory animals research:

- **Replacement:** To substitute the use of animals by alternative methods.
- **Reduction:** To minimize the number of animals employed in research, or to maximize the amount of information obtained from each animal.
- **Refining:** To minimize the pain, suffering, distress or lasting harm that laboratory animals might experience.

All animals were allowed to acclimate for a minimum of 7 days upon arrival, and housed in appropriate cages, in a light and temperature controlled environment. After the experimental procedures, animals were not allowed to recover from the anesthesia.

5.

Results

5.- Results

5.1.- Article 1

Network-Assisted Systems Biology Analysis of the Mitochondrial Proteome in a Pre-Clinical Model of Ischemia, Revascularization and Post-Conditioning

Alex Gallinat, Gemma Vilahur, Teresa Padró, and Lina Badimon

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Objective 1.- To explore the mitochondrial response of the myocardium to ischemic post-conditioning.

Highlights:

- The topology of protein-protein interaction networks is tightly correlated with cellular functions.
- Network systems biology provide a powerful approach towards the understanding of complex biological processes such as ischemia, reperfusion and cardioprotection.
- Ischemic post-conditioning promotes the metabolic reprogramming of the myocardium at risk together with the up-regulation of the cardioprotective proteins DJ-1 and VDAC2.
- Cardiac metabolism is an important driver of the cardioprotection conferred by ischemic post-conditioning.

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Network-Assisted Systems Biology Analysis of the Mitochondrial Proteome in a Pre-Clinical Model of Ischemia, Revascularization and Post-Conditioning

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Abstract: Infarct size is the major risk predictor for developing heart failure after an acute myocardial infarction (AMI). The discovery of the conditioning phenomena (i.e., repetitive brief cycles of ischemia applied either before or after a prolonged ischemic insult) has highlighted the existence of endogenous protective mechanisms of the heart potentially limiting infarct size after revascularization. However, most cardioprotective strategies, aiming at infarct size reduction, have failed in clinical studies. Thus, cardioprotection is an unmet clinical need. In the present study, we took a network-assisted systems biology approach to explore the mitochondrial proteomic signature of the myocardium after ischemia, ischemia with direct revascularization, and ischemia with re-establishment of blood flow by post-conditioning in a swine model of AMI. Furthermore, network extension with the ENCODE project human regulatory data allowed the prediction of potential transcription factors at play in the response to post-conditioning of the myocardium. Collectively, our results identify cardiac metabolism as a driver of cardioprotection, highlighting a dual role for post-conditioning promoting metabolic reprogramming of the myocardium, and a protective response mediated by VDAC2 and DJ-1 in the mitochondria.

Keywords: post-conditioning; ischemia; cardioprotection; mitochondria; proteomics; network biology

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1. Introduction

Despite a drop in mortality of acute myocardial infarction (AMI) in the past decades due to the application of primary percutaneous coronary intervention (PPCI) [1,2], AMI survivors are at high risk of developing heart failure (HF) [3,4], which is a cause of a high morbidity and mortality worldwide [5] and of huge global burden on healthcare and economic resources [6]. AMI is an ischemic event caused by the sudden interruption of coronary blood supply to the myocardium. From the onset of AMI, a wave-front of necrosis grows within the at-risk myocardium [7]. Due to the minimal regenerative capacity of the adult human heart, there is no recovery of the lost functional tissue after an AMI [8] and thus, infarct size is the major predictor of AMI clinical outcomes [9,10]. The early and successful restoration of coronary blood flow by PPCI is the most effective strategy to limit infarct size. However, this process that occurs within the very first minutes of reflow paradoxically has the potential to exacerbate damage and accounts for a significant part of the final infarct size [11,12]. A vast number of cardioprotective therapies aimed at reducing infarct size have failed to demonstrate clinical benefits [13,14]. Thus, cardioprotection is currently an unmet clinical need. A better understanding of the mechanisms at play in the ischemic myocardium is needed in order to both explore novel cardioprotective strategies and to improve translation.

In 1986, Murray et al. [15] first described how the application of repetitive brief cycles of ischemia and revascularization prior to a prolonged ischemic insult greatly limits infarct size and improves post-AMI heart function. This practice was called ischemic pre-conditioning and has demonstrated the existence of endogenous cardioprotective mechanisms that are worth exploring. However, the clinical application of pre-conditioning is limited because AMI is a sudden event and cannot be accurately predicted. Later on, Zhao et al. [16] reported that ischemic conditioning also results in significant protection when applied at the time of the re-establishment of coronary blood flow after a prolonged ischemic insult. This practice was called post-conditioning, and markedly reduced infarct size, endothelial dysfunction, and post-ischemic blood flow defects [16–18]. Despite broadly proven benefits of ischemic conditioning in animal models, it turned controversial in clinical trials [19–21], possibly due to differences in the conditioning protocols, patient medications, and comorbidities [22–24]. Thus, a better understanding of the mechanisms at play is needed to improve translation. Many studies have focused on the identification of single molecules and apoptosis-related pathways at play [25], but a global characterization of the coordinated changes in the myocardium following post-conditioning is still lacking.

Network medicine is a rapidly growing field combining systems biology with network science to understand the relationship between molecular signatures and disease pathogenesis [26,27]. Based on the assumption that biological processes are not primarily constrained by single proteins or discrete pathways, but rather a network of complex molecular interactions, network medicine provides powerful tools to understand the driving mechanisms of disease [28].

Mitochondria play a central role in the generation and maintenance of cellular ATP pools. They also provide a hub for cell metabolism, programmed cell death, and have multiple roles in signaling. These features make them central organelles for cellular adaptation to ischemia and revascularization, and for cardioprotection [29–31]. Nearly all identified signaling pathways in the cardioprotective response triggered by the conditioning phenomena converge in the mitochondria; thus, mitochondria are believed to be the effector organelles for cardioprotection [32].

The present work is the continuation of a previous study in which we took a proteomic approach to identify regulators of post-conditioning and their signaling pathways [33]. In the first part of the study, a beneficial effect of post-conditioning over the global heart function, infarct size, and cell survival was found (Supplemental Figure S1), and an effect on the canonical aryl-hydrocarbon receptor pathway was shown [33]. Now, we have investigated whether a network-assisted systems biology approach focusing on the mitochondrial proteome evolution all along ischemia, revascularization, and post-conditioning, would shed additional light on the understanding of the molecular basis of cardioprotection beyond signaling, and how post-conditioning modifies the response of the myocardium to the re-establishment of coronary blood flow.

2. Results

2.1. Ischemia, Revascularization, and Post-Conditioning Impacts on the Swine Mitochondrial Proteome

The proteomic characterization of the myocardium at risk revealed at least 26 mitochondrial proteins to be differentially regulated as a result of ischemia, revascularization, and post-conditioning in the swine heart (Supplemental Table S1). In order to depict an overall functional characterization, we built a physical protein–protein interaction (PPI) network according to the STRING [34] database with 26 differentially regulated proteins detected across all conditions (Figure 1). The resulting network revealed a significant PPI enrichment, indicating that the differentially expressed proteins are biologically connected as a group [26]. Then, we applied the Markov clustering (MCL) [35,36] strategy and ran a functional overrepresentation analysis upon Gene Ontology biological process terms and pathways from the Wikipathways [37] database. The MCL algorithm identified three PPI-enriched clusters within the network, tightly correlating with the significantly overrep-

resented pathways. More specifically, the tricarboxylic acid (TCA) cycle (WP78; FDR ≈ 0) and the oxidative phosphorylation (OXPHOS) system (WP111; FDR ≈ 0) (Figure 1—see Supplemental Table S2 for a complete list of the overrepresented pathways). Accordingly, strongly significant enrichments were found for Gene Ontology biological process terms related to the energy metabolism and cellular respiration (Table 1), collectively highlighting the pivotal role of the energy metabolism in organ resilience during ischemia and revascularization.

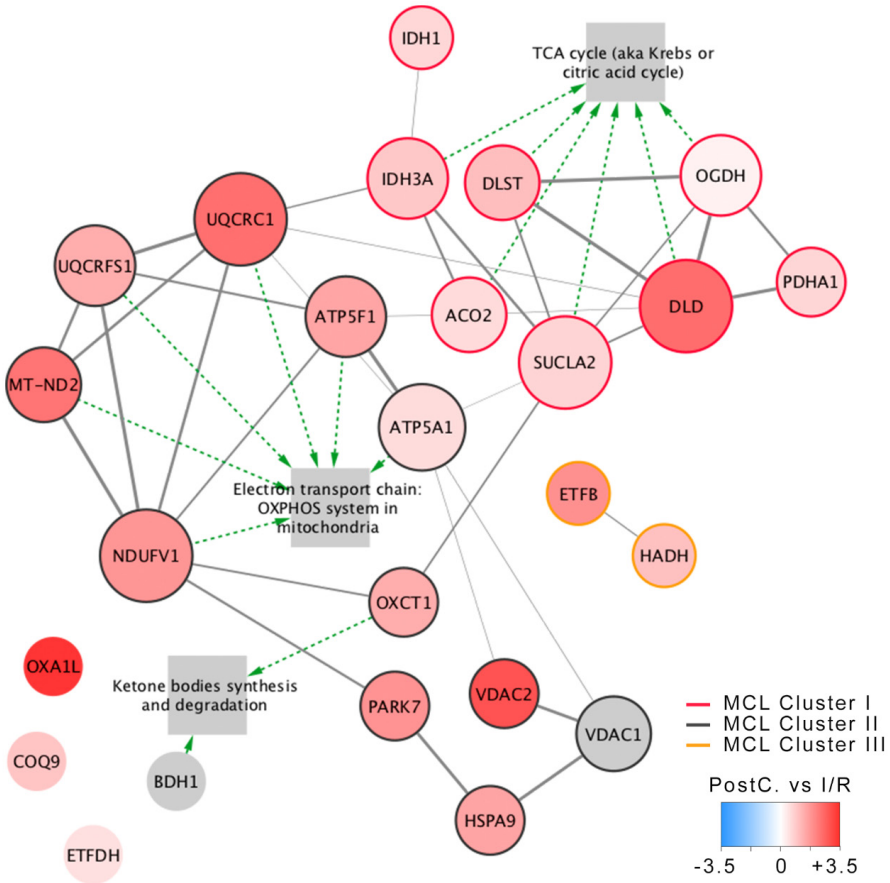

Figure 1. Mitochondrial physical PPI network analysis. All proteins detected to be differentially expressed (absolute logFC > 0.5) in at least one condition were included. PPIs were retrieved from the STRING database, and the Markov clustering (MCL) strategy with an inflation parameter of 1.4 was applied to identify protein-protein interaction (PPI) clusters. The PPI network of differentially regulated proteins, including the most relevant enriched pathways, was detected in the analysis. Green arrows connect pathways with their matching proteins in the network. Node color represents the logFC between post-conditioning and direct revascularization, size represents node degree within the network, and edge thickness the confidence of interaction between nodes. Abbreviations—I/R: Ischemia/revascularization group; PostC: Post-conditioning group; ACO2: Heart aconitase; ATP5A1:

ATP synthase subunit alpha; ATP5F1: ATP synthase beta subunit; BDH1: D-beta-hydroxybutyrate dehydrogenase; COQ9: Ubiquinone biosynthesis protein COQ9; DLD: Dihydrolepolyl dehydrogenase; DLST: Dihydrolipollysin-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; ETFB: Electron transfer flavoprotein subunit beta; ETFDH: Electron transfer flavoprotein-ubiquinone oxidoreductase; HADH: Hydroxyacyl-coenzyme A dehydrogenase; HSPA9: Stress-70 protein, mitochondrial; IDH2: Isocitrate dehydrogenase [NADPH]; IDH3A: Isocitrate dehydrogenase [NAD] subunit alpha; MT-ND2: NADH-ubiquinone oxidoreductase; NDUFV1: NADH dehydrogenase [ubiquinone] flavoprotein 1; OGDH: 2 oxoglutarate dehydrogenase; OXA1L: Mitochondrial inner membrane protein; OXCT1: Succinyl-CoA:3-ketoacid-coenzyme; PARK7: Protein DJ-1; PDHA1: Pyruvate dehydrogenase; SUCLA2: Succinyl-CoA ligase [ADP-forming] subunit beta; UQCRC1: Cytochrome b-c1 complex subunit 1; UQCRFS1: Cytochrome b-c1 complex subunit Rieske; VDAC1: Voltage-dependent anion-selective channel protein 1; VDAC2: Voltage-dependent anion-selective channel protein 2.

Table 1. Top 10 most significant Gene Ontology biological terms detected.

GO_Term	Description	Strength	FDR
GO:0006091	Generation of precursor metabolites and energy	1.59	2.88×10^{-25}
GO:0045333	Cellular respiration	1.9	1.24×10^{-23}
GO:0055114	Oxidation-reduction process	1.2	6.17×10^{-17}
GO:0009060	Aerobic respiration	2	2.41×10^{-14}
GO:0006099	Tricarboxylic acid cycle	2.27	6.75×10^{-13}
GO:0044281	Small molecule metabolic process	0.92	4.85×10^{-11}
GO:0022904	Respiratory electron transport chain	1.77	2.40×10^{-9}
GO:0006119	Oxidative phosphorylation	1.72	4.49×10^{-9}
GO:0046034	ATP metabolic process	1.54	5.51×10^{-9}
GO:0019752	Carboxylic acid metabolic process	1.08	1.14×10^{-8}

2.2. Tricarboxylic Acid Cycle

A total of 8 proteins involved in the TCA cycle were detected to be differentially regulated across all conditions (Figure 2). In ischemic hearts, both the succinyl-CoA ligase (SUCLA2) and the dihydrolepolyl dehydrogenase (DLD), a component of the oxoglutarate dehydrogenase complex, were found to be down-regulated. After the re-establishment of coronary blood flow, there was no recovery of SUCLA2 or DLD, but instead a further down-regulation of the TCA cycle member isocitrate dehydrogenase subunit alpha (IDH3A) and the isocitrate dehydrogenase 2 (IDH2) was detected. The oxoglutarate dehydrogenase complex component OGDH was up-regulated after revascularization. When post-conditioning was performed, a wide up-regulation of enzymes involved in the progression of the TCA cycle was detected, with the recovery of IDH3A and all the oxoglutarate dehydrogenase complex members. Heart aconitase (ACO2), the enzyme responsible for the first two reactions of the TCA cycle, was up-regulated following post-conditioning.

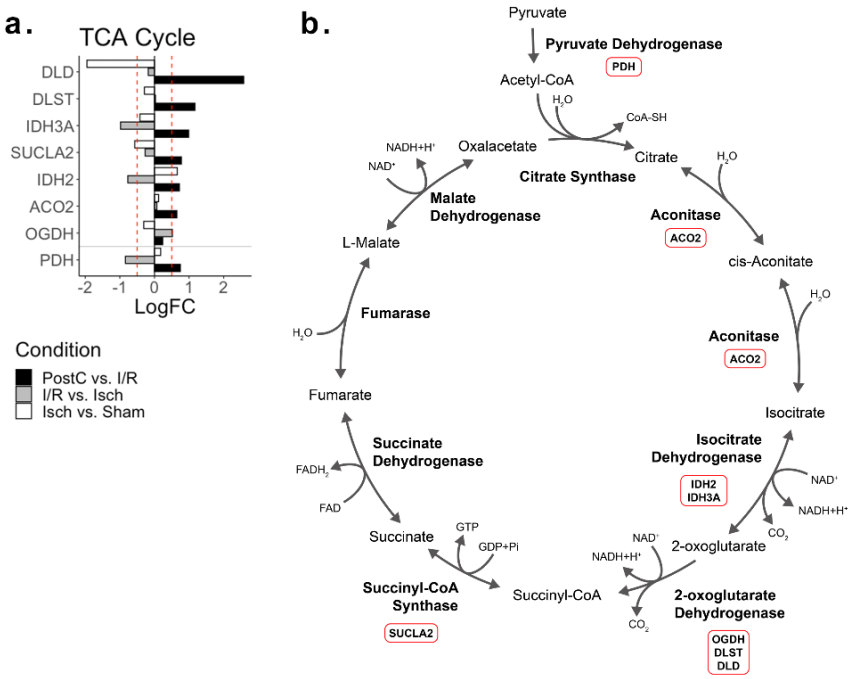

Figure 2. Tricarboxylic acid (TCA) cycle related differentially regulated proteins.(**a**) LogFC plot of TCA cycle related proteins across ischemia, reperfusion, and post-conditioning. The red dashed line indicates the threshold at absolute logFC > 0.5. (**b**) TCA cycle pathway scheme with the differentially regulated proteins detected highlighted within red boxes. Abbreviations—Isch: Ischemia group; I/R: Ischemia/revascularization group; PostC: Post-conditioning group; ACO2: Heart aconitase; DLD: Dihydrolepolyl dehydrogenase; DLST: Dihydrolipollysin-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; IDH2: Isocitrate dehydrogenase [NADPH]; IDH3A: Isocitrate dehydrogenase [NAD] subunit alpha; OGDH: 2 oxoglutarate dehydrogenase; PDHA1: Pyruvate dehydrogenase; SUCLA2: Succinyl-CoA ligase [ADP-forming] subunit beta.

2.3. Oxidative Phosphorylation

A total of 6 proteins belonging to the electron transport chain complexes I, III, and V were affected by ischemia, direct revascularization, and post-conditioning (Figure 3). Ischemic hearts exhibited a down-regulation of proteins belonging to both complexes I and V. After the re-establishment of coronary blood flow, further down-regulations of complex I and V were seen, together with a strong down-regulation of the complex III core catalytic subunit (cytochrome b-c1 oxidoreductase complex subunit Rieske; UQCRFS1). Conversely, post-conditioned hearts exhibited a strong up-regulation of the electron transport chain complexes I, III, and V. Additionally, the electron-transfer flavoprotein oxidoreductase (ETFDH) and the electron-transfer flavoprotein subunit beta (ETFB), which are not part of the electron transport chain complexes themselves but a crucial link between the fatty acids beta-oxidation and the oxidative phosphorylation, were also up-regulated in the post-conditioned hearts.

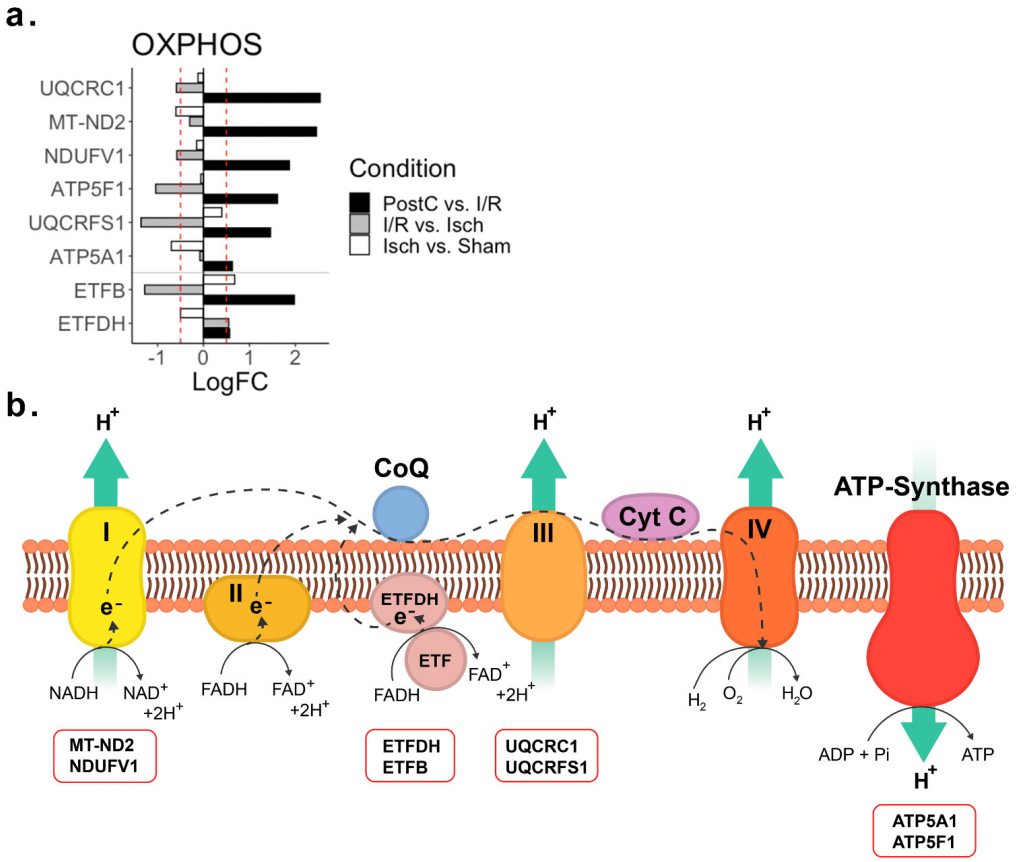

Figure 3. Oxidative phosphorylation (OXPHOS)-related differentially regulated proteins. (**a**) LogFC plot of the OXPHOS related proteins across ischemia, revascularization, and post-conditioning. The red dashed line indicates the threshold at absolute logFC > 0.5. (**b**) OXPHOS scheme with the differentially regulated proteins detected highlighted within red boxes. Abbreviations—Isch: Ischemia group; I/R: Ischemia/ revascularization group; PostC: Post-conditioning group; ATP5A1: ATP synthase subunit alpha; ATP5F1: ATP synthase beta subunit; ETFB: Electron transfer flavoprotein subunit beta; ETFDH: Electron transfer flavoprotein-ubiquinone oxidoreductase; MT-ND2: NADH-ubiquinone oxidoreductase; NDUFV1: NADH dehydrogenase [ubiquinone] flavoprotein 1; UQCRC1: Cytochrome b-c1 complex subunit 1; UQCRFS1: Cytochrome b-c1 complex subunit Rieske.

2.4. Non Metabolic Proteins

Between the differentially regulated proteins detected across all conditions, seven proteins with non-metabolic functions were detected to be strongly up-regulated following post-conditioning (Figure 4). Amongst them, we found that the mitochondrial inner membrane protein (OXA1L), the voltage-dependent anion-selective channel 2 (VDAC2), and protein DJ-1 (PARK7) were greatly up-regulated. Also, the mitochondrial stress-70 protein (HSPA9, also known as mortalin), which is functionally related to PARK7, was found to be up-regulated in the post-conditioned hearts.

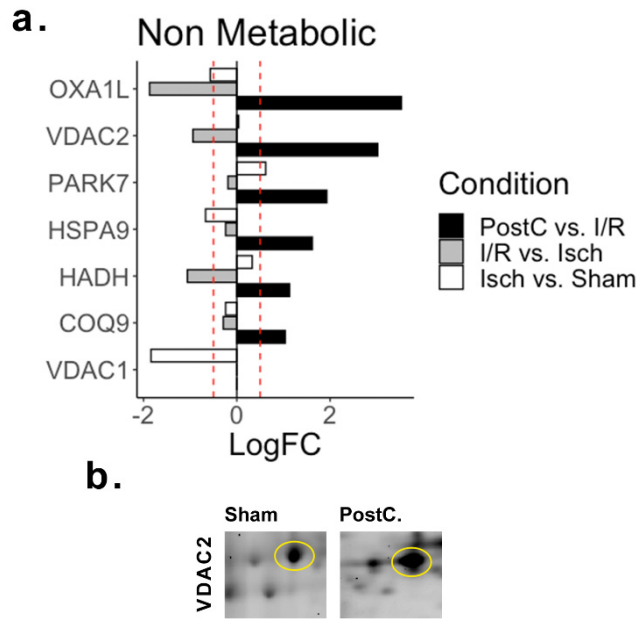

Figure 4. Non-metabolic differentially regulated proteins.(**a**) LogFC plot of the non-metabolic proteins detected across ischemia, reperfusion, and post-conditioning. The red dashed line indicates the threshold at absolute logFC > 0.5. (**b**) Detail of VDAC2 spot across conditions. Abbreviations—Isch: Ischemia group; I/R: Ischemia/revascularization group; PostC: Post-conditioning group; COQ9: Ubiquinone biosynthesis protein COQ9; HADH: Hydroxyacyl-coenzyme A dehydrogenase; HSPA9: Stress-70 protein, mitochondrial; OXA1L: Mitochondrial inner membrane protein; PARK7: Protein DJ-1; VDAC1: Voltage-dependent anion-selective channel protein 1; VDAC2: Voltage-dependent anion-selective channel protein 2.

2.5. Prediction of Ischemic Post-Conditioning Mitochondrial Regulatory Network

In order to better understand the gene regulation at play following post-conditioning, we expanded the PPI network built upon the post-conditioning differentially expressed genes compared to direct revascularization with the human regulatory transcription factor network data retrieved from the ENCODE [38] database. As a result, 20 transcription factors were predicted to be at play (Figure 5a). From them all, the CCCTC-binding factor (CTCF), the glucocorticoid receptor (NR3C1), and the nuclear respiratory factor 1 (NRF1) were connected to nodes exhibiting opposite regulation at revascularization and post-conditioning versus ischemia (Figure 5b,c).

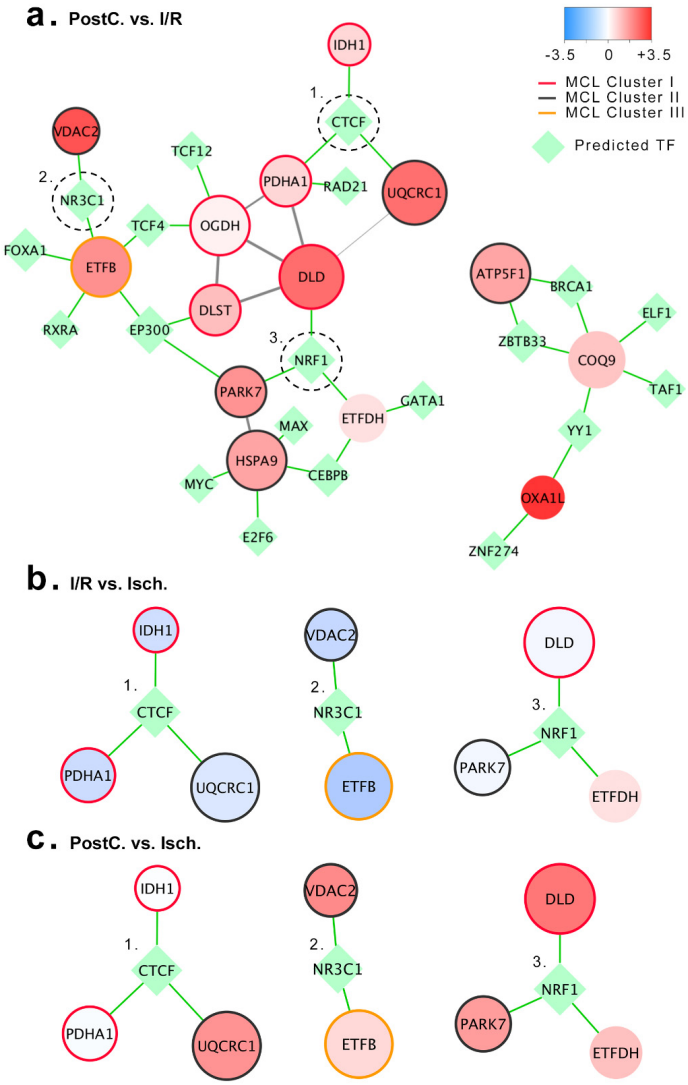

Figure 5. Extension of the post-conditioning mitochondrial protein-protein interaction (PPI) network with the ENCODE human regulatory data. Only proteins exhibiting an absolute logFC > 0.5 at post-conditioning versus I/R, were included. (**a**) Mitochondrial post-conditioning associated PPI network including regulatory elements retrieved from the ENCODE project human regulatory data. Nodes with no prediction of regulatory elements were hided, to simplify visualization. Nodes fill color represents logFC between post-conditioning and I/R and size represents node degree within the complete network Predicted regulatory elements are indicated as green diamonds. (**b**) Regulatory sub-network of CTCF, NR3C1, and NRF1 at ischemia/reperfusion compared to ischemia. (**c**) Regulatory sub-network of CTCF, NR3C1, and NRF1 at post-conditioning compared to ischemia. Abbreviations—Isch: Ischemia group; I/R: Ischemia/revascularization group; PostC: Post-conditioning group; TF: Transcription factor; ATP5F1: ATP synthase beta subunit; BRCA1: Breast cancer type 1 susceptibility

protein; CEBPB: CCAAT/enhancer-binding protein beta; COQ9: Ubiquinone biosynthesis protein COQ9; CTCF: CCCTC-binding factor; DLD: Dihydrolepolyl dehydrogenase; DLST: Dihydrolipollysin-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; E2F6: Transcription factor E2F6; ELF1: ETS-related transcription factor Elf-1; EP300: Histone acetyltransferase p300; ETFB: Electron transfer flavoprotein subunit beta; ETFDH: Electron transfer flavoprotein-ubiquinone oxidoreductase; FOXA1: Hepatocyte nuclear factor 3-alpha; GATA1: Erythroid transcription factor; HSPA9: Stress-70 protein, mitochondrial; IDH2: Isocitrate dehydrogenase [NADPH]; MAX: Protein max; MYC: Myc proto-oncogene protein; NR3C1: Glucocorticoid receptor; NRF1: nuclear respiratory factor 1; OGDH: 2 oxoglutarate dehydrogenase; OXA1L: Mitochondrial inner membrane protein; PARK7: Protein DJ-1; PDHA1: Pyruvate dehydrogenase; RAD21: Double-strand-break repair protein rad21 homolog; RXRA: Retinoic acid receptor RXR-alpha; TAF1: Transcription initiation factor TFIID subunit 1; TCF12: Transcription factor 12; TCF4: Transcription factor 4; UQCRC1: Cytochrome b-c1 complex subunit 1; VDAC2: Voltage-dependent anion-selective channel protein 2; YY1: Transcriptional repressor protein YY1; ZBTB33: Transcriptional regulator Kaiso; ZNF274: Neurotrophin receptor-interacting factor homolog.

3. Discussion

3.1. Mitochondrial Response to Ischemia in the Pig Heart

In aerobic conditions, heart mitochondria rely on three main metabolic pathways to satisfy the ATP demand. These are the fatty acid β-oxidation (FAO), the TCA cycle, and the OXPHOS system. During FAO, fatty acids are sequentially oxidized and ultimately converted to reducing equivalents (NADH and FADH2), acetyl-CoA, and GTP (through substrate-level phosphorylation). Then, acetyl-CoA enters the TCA cycle where it is further oxidized, thereby producing more reducing equivalents, GTP, and CO_2. In the course of OXPHOS, all reducing equivalents are utilized by the electron transport chain (ETC) complexes to pump protons into the inter-membrane space, generating an electrochemical gradient that will drive the main ATP production, supported by the F_1F_o-ATP-synthase (complex V). During OXPHOS, electrons released from the oxidation of reducing equivalents flow through the ETC, ultimately reaching the cytochrome-c oxidase (complex IV), where O_2 acts as the final electron acceptor, and H_2O is produced.

During ischemia, O_2 deprivation rapidly inhibits the electronflow through the ETC, leading to the accumulation of reducing equivalents. The catabolic metabolism strictly depends on the re-oxidation of reducing equivalents to progress, as they exist in a relatively low abundance in the cell and cannot be imported [31]. The ETC inhibition directly impacts the TCA cycle progression and FAO pathway. In the absence of O_2, ATP can no longer be produced by OXPHOS; instead, metabolism switches to anaerobic glycolysis, which is virtually the only way of producing ATP in the absence of O_2. Whilst the lack of oxidized reducing equivalents disrupts the normal TCA cycle progression, some TCA cycle intermediates accumulate during a period of oxygen deprivation as a result of non-canonical TCA cycle activity [39–41]. This non-canonical flow through the TCA cycle leads to the accumulation of succinate but also guarantees a second source of ATP in the absence of O_2. Only a few changes in the mitochondrial proteome were found following ischemia; these were the down-regulation of the TCA cycle enzymes DLD and SUCLA2 and the ETC members MT-ND2 and ATP5A1.

3.2. Mitochondrial Response to Revascularization in the Pig Heart

At the onset of revascularization, O_2 availability restores the normal ETC activity. When this happens, succinate that has been accumulating during ischemia is oxidized at ETC complex II, reducing the ubiquinone pool and increasing the mitochondrial potential. These two conditions, together with a compromised ATP-synthase activity due to a low ADP availability following ischemia, promote reverse electron transport (RET) through complex I, ending up in the generation of reactive oxygen species (ROS) [42,43]. Despite ROS being continuously produced during metabolic activity, there is a balance with the endogenous antioxidant mechanisms, as they have the potential to induce several macromolecular

alterations ranging from protein misfolding to DNA damage. Yet, ROS have a central role in myocardial damage following revascularization [44,45]. Within complex I, the mitochondrial encoded subunit ND2 (MT-ND2) and flavin mononucleotide-binding subunits are the most susceptible to oxidative damage, as they comprise the main superoxide generation site during RET [46]. Oxidative damage to complex I following revascularization is reflected in the swine mitochondrial proteome as the down-regulation of both MT-ND2 and the NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFS1). RET through complex I is dependent on a high mitochondrial potential and a reduced ubiquinone pool and is thus transitory [47]. Yet, complex III has been identified as the second major source for ROS generation at the mitochondria as a result of electron leakage [48,49]. In contrast to the RET-driven ROS generation by complex I, complex III ROS generation occurs even under physiologic conditions [49] and is promoted following the re-establishment of coronary blood flow [47]. Consistently, a strong down-regulation of complex III was found following revascularization, which may be a consequence of oxidative damage to the ETC [50].

In addition to a compromised ETC, the expression of several enzymes belonging to the TCA cycle was found to be affected by ischemia and revascularization. This is the case of SUCLA2 and DLD, which were down-regulated during ischemia and not recovered after revascularization, and the isocitrate dehydrogenase components IDH3A and IDH2, which were found to be down-regulated after the re-establishment of coronary blood flow. The oxoglutarate dehydrogenase complex component OGDH is the only TCA cycle enzyme that was up-regulated at revascularization.

3.3. Mitochondrial Response to Post-Conditioning in the Pig Heart

The application of post-conditioning at the onset of revascularization had a great impact on the mitochondrial proteome of the myocardium at risk. When post-conditioning was performed, no down-regulation of complex III was detected, but robust up-regulation of complexes I, III, and V was found. These differences may result from reduced oxidative damage or de novo protein synthesis. When compared to the sham group, all the complex I members MT-ND2, NDUFSV1, and the complex III subunit UQCRC1, were seen to be strongly up-regulated, suggesting de novo synthesis (Supplemental Figure S2). Consistently, OXA1L, a protein involved in the insertion and correct assembling of inner mitochondrial membrane integral proteins, was greatly up-regulated following post-conditioning (both compared to direct revascularization and sham groups). Conversely, the complex III subunit UQCRFS1, and the complex V subunits ATP5A1 and ATP5F1, remained unaltered when compared to sham but were down-regulated after direct revascularization. These results are in line with previous studies performed in ex vivo Langerdoff-perfused rodent hearts [51,52], thus confirming the pivotal implication of the ETC complexes in the cardioprotection conferred by post-conditioning in a pre-clinical animal model of myocardial infarction. Moreover, ETFB and ETFDH, the links between the ETC and FAO, were found to be up-regulated in the post-conditioned hearts, indicating a higher implication of FAO in the post-ischemic cardiac metabolism in the post-conditioned hearts.

It is worth noting that whilst revascularization led to the down-regulation of the isocitrate dehydrogenase components IDH3A and IDH2, together with the non-recovery of SUCLA2 and DLD, post-conditioning induced a wide up-regulation of the enzymes involved in the progression of the TCA cycle, suggesting that post-conditioning promotes a canonical TCA cycle progression.

Changes in the mitochondrial proteome reflect the balance existing between metabolic adaptation and damage. Amongst the non-metabolic differentially expressed proteins detected in the post-conditioned hearts, both VDAC2 and DJ-1 have been previously related to cardioprotection [53–55]. Furthermore, HSPA9, a protein necessary for the mitochondrial import of DJ-1 [56] was also up-regulated. Additionally, VDAC2, DJ-1, and HSPA9 exhibited strong up-regulation when compared to the sham group, supporting the induction of a cardioprotective program following post-conditioning (Supplemental Figure S3). Yet

the exact role that these proteins play in the response to ischemia and revascularization remains to be investigated.

Network biology provides an intuitive and powerful approach to characterize and understand cellular mechanisms and pathology. As shown here, network topology tightly correlates with functional modules, and collectively highlights the great implications of the energy metabolism in the cardioprotection conferred by post-conditioning. Furthermore, networks can be expanded by adding new pairs of scientifically proven interactions arising from pre-built interaction networks, allowing the identification of protein complexes and regulatory elements. In such a way, the ENCODE project includes the information of validated chromatin immunoprecipitation sequencing data sets for over 119 distinct transcription factors [38]. The post-conditioning associated PPI mitochondrial network extension through the human regulatory data retrieved from the ENCODE project revealed 20 transcription factors putatively at play in the regulation of the post-conditioning response. Amongst them, CTCF, NR3C1, and NRF1 exhibited the most consistent topology with the post-conditioning proteomic response and may be new targets for cardioprotective strategies. Further research is needed in order to elucidate the transcriptional regulation at play and upstream signaling.

4. Materials and Methods

4.1. Experimental Model

Twenty-one regular farm pigs were randomized into four experimental groups: (I) closed-chest 90 min left anterior descending (LAD) coronary artery balloon occlusion with no revascularization (ischemia group; N = 7); (II) closed-chest 90 min LAD occlusion followed by 2.5h of revascularization (ischemia-revascularization group; N = 5); (III) closed-chest 90 min LAD occlusion followed by post-conditioning and 2.5 h of revascularization (post-conditioning group; N = 5); and (IV) sham-operated animals, which underwent the same surgical procedure without balloon inflation (sham group; N = 4). To avoid thrombotic complications due to catheter manipulation, a loading dose of clopidogrel was administered to all animals 12h before the experimental procedure. Closed-chest LAD occlusion was performed as previously described under angiographic monitoring [33]. Briefly, anesthesia was administered by an intramuscular injection of zoletil® (7mg/Kg), domtor® (7mg/Kg), and atropine (0.03mg/Kg). Then, animals underwent endotracheal intubation, and anesthesia was maintained by isofluorane inhalation (2%). Continuous infusion of amiodarone (300mg, 75mg/h) was initiated at the beginning of the procedure in all pigs as prophylaxis for malignant ventricular arrhythmias. These amiodarone doses do not alter hemodynamic parameters [57]. Angiography was employed both to guide angioplasty balloon placement (below the first diagonal branch) and to corroborate the successful occlusion of the LAD coronary artery (no flow downstream balloon position upon contrast injection). Animals were then randomized to one of the four groups described (Supplemental Figure S1). The post-conditioning protocol consisted of six cycles of 20 s of balloon disinflation (revascularization) and 20 s of re-occlusion (ischemia) at the onset of revascularization [58,59]. Animals were not allowed to recover from the anesthesia.

Heart rate and electrocardiogram were monitored throughout the experimental procedure. Left ventricular ejection fraction (LVEF) was assessed at baseline, 90 min post-LAD occlusion (before revascularization), and at the end of revascularization time (sacrifice). All echocardiographic measurements were taken blindly by the same technician.

4.2. Sample Collection and Protein Extraction

Evan's blue dye was injected in anesthetized animals to outline the area at risk, immediately after hearts were arrested, rapidly excised, and sliced. Slices were alternatively collected for infarct size analysis with triphenyl tetrazolium chloride (TTC) and sample collection. Heart samples from the inner border zone of the area at risk were collected, snap-frozen, smashed to powder, and homogenized in urea/thiourea buffer for proteomic

analysis. The protein concentration was then quantified with 2D-Quant Kit (GE Healthcare, Chicago, IL, USA).

4.3. Proteomic Analysis

Protein extracts were separated by two-dimensional gel electrophoresis as previously described [53]. Gels were then labeled with Flamingo Fluorescent Gel Stain (Bio-Rad, Hercules, CA, USA), scanned in a Typhoon 9400 (GE Healthcare, Chicago, IL, USA), and analyzed for spot variations using PD-Quest 8.0 (Bio-Rad, Hercules, CA, USA). Three animals from each group were analyzed. Spots of interest were then excised and identified by matrix-assisted laser desorption/ionization-time-of-flight using an AutoFlex III Smartbeam MALDI-ToF/ToF (Bruker Daltonics, Billerica, MA, USA).

4.4. In Silico Analysis

Spot intensities in each gel were normalized by the intensity of the albumin spot. Intensity medians were then used to calculate the log-fold change (logFC) between groups, and only those proteins exhibiting an absolute logFC higher than 0.5 were considered to be differentially expressed. Differentially expressed proteins were employed to build a PPI network according to the STRING [34] database. The resulting network was then imported to Cytoscape 3.0 [60] for further analysis and visualization. The MCL strategy with an inflation parameter of 1.4 was applied in order to predict functional clusters [35,36]. Seed nodes were analyzed for functional enrichment in Gene Ontology biological process terms and pathways from Wikipathways [37] database. In order to predict putative transcription factors at play, the differentially expressed PPI network was expanded according to the human regulatory network derived from the ENCODE [38] database using the Cytoscape app CyTargetLinker [61].

4.5. Statistical Analysis

A bootstrapping approach was employed to calculate the effect size of each condition amongst the identified proteins. Briefly, a random sampling with replacement step was performed upon normalized spot intensities before logFC calculations [62]. An effect size threshold for significance was set at 0.5 absolute logFC. All statistical analyses were performed with RStudio (RStudio, Boston, MA, USA). Unpaired multi-two group Gardner–Altman estimation plots were generated with the R package 'dabester'.

5. Conclusions

Our systems biology analysis results collectively highlight a dual role for post-conditioning both for promoting metabolic reprogramming and a protective response potentially mediated by VDAC2 and DJ-1 in the mitochondria. Therapies targeting cardiac metabolism may thus allow the pharmacological emulation/recapitulation of post-conditioning general changes, potentially overcoming the limitations found in the clinical studies. Additionally, hallmarks of the metabolic re-adaptation were found in the post-conditioning cytoplasmic proteome as illustrated previously by the regulation of the mammalian target of rapamycin (mTOR) following post-conditioning [33]. Indeed, cardiac metabolism has been suggested as a driver for cardioprotection, and several metabolic approaches have been proposed as beneficial within the AMI framework [63]. Our results describe the complex mitochondrial proteomic signature triggered by post-conditioning. Further research is needed in order to explore the upstream regulation and the link existing between VDAC2, DJ-1, and the post-ischemic metabolic adaptation to revascularization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23042087/s1.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committees (CEEA-IR Sant Pau) of ICCC-Hospital de la Santa Creu i Sant Pau (protocol code #022, approved in 2012) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Data Availability Statement: The data presented in this study are available in supplementary material.

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Table S1. Changes in mitochondrial proteins detected in the swine at-risk myocardial proteome.

Protein name	Symbol	Isch vs. Sham	I/R vs. Isch	PostC vs. Isch	PostC vs. I/R
Voltage-dependent anion-selective channel protein 1	VDAC1	-1.84	N.D.	3.45	N.D.
D-beta-hydroxybutyrate dehydrogenase	BDH1	0.41	N.D.	2.48	N.D.
Mitochondrial inner membrane protein	OXA1L	-0.57	-1.87	1.66	3.53
Voltage-dependent anion-selective channel protein 2	VDAC2	0.04	-0.94	2.08	3.02
Dihydrolepolyl dehydrogenase	DLD	-1.95	-0.18	2.40	2.58
Cytochrome b-c1 complex subunit 1	UQCRC1	-0.12	-0.59	1.95	2.54
NADH-ubiquinone oxidoreductase	MT-ND2	-0.60	-0.30	2.16	2.46
Electron transfer flavoprotein subunit beta	ETFB	0.68	-1.28	0.70	1.98
Protein DJ-1	PARK7	0.62	-0.19	1.74	1.93
NADH dehydrogenase [ubiquinone] flavoprotein 1	NDUFV1	-0.15	-0.58	1.30	1.87
Stress-70 protein, mitchondrial	HSPA9	-0.67	-0.24	1.37	1.62
ATP synthase beta subunit	ATP5F1	-0.06	-1.04	0.58	1.61
Succinyl-CoA:3-ketoacid-coenzyme	OXCT1	0.01	-0.39	1.08	1.47
Cytochrome b-c1 complex subunit Rieske	UQCRFS1	0.40	-1.36	0.10	1.46
Dihydrolipollysin-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	DLST	-0.29	0.03	1.21	1.17
Hydroxyacyl-coenzyme A dehydrogenase	HADH	0.33	-1.06	0.07	1.13
Ubiquinone biosynthesis protein COQ9	COQ9	-0.24	-0.29	0.75	1.04
Isocitrate dehydrogenase [NAD] subunit alpha	IDH3A	-0.42	-0.98	0.01	0.99
Succinyl-CoA ligase [ADP-forming] subunit beta	SUCLA2	-0.57	-0.27	0.51	0.78
Pyruvate dehydrogenase	PDHA1	0.18	-0.84	-0.08	0.75
Isocitrate dehydrogenase [NADPH]	IDH2	0.66	-0.77	-0.05	0.72
Heart aconitase	ACO2	0.12	0.07	0.72	0.65
ATP synthase subunit alpha	ATP5A1	-0.70	-0.08	0.54	0.63
Electron transfer flavoprotein-ubiquinone oxidoreductase	ETFDH	-0.50	0.55	1.13	0.57
Superoxide dismutase	SOD1	0.01	0.15	0.52	0.37
2 oxoglutarate dehydrogenase	OGDH	-0.31	0.52	0.77	0.24

Table S2. Wikipathways enrichment analysis result of the differentially regulated proteins detected across ischemia, revascularization, and post-conditioning.

WP_id	Term description	Strength	FDR
WP2453	TCA cycle and deficiency of pyruvate dehydrogenase complex (PDHc)	2.47	2.90E-10
WP78	TCA cycle (aka Krebs or citric acid cycle)	2.42	2.90E-10
WP3925	Amino acid metabolism	1.78	1.64E-08
WP111	Electron transport chain: OXPHOS system in mitochondria	1.66	1.83E-06
WP4932	7q11.23 copy number variation syndrome	1.58	8.26E-05
WP4290	Metabolic reprogramming in colon cancer	1.87	9.45E-05
WP623	Oxidative phosphorylation	1.72	0.00031
WP311	Ketone bodies synthesis and degradation	2.5	0.0071
WP4236	Krebs cycle disorders	2.35	0.0109
WP4742	Ketogenesis and ketolysis	2.29	0.0122
WP4297	Thiamine metabolic pathways	2.24	0.0136
WP5037	Riboflavin and CoQ disorders	2.05	0.027
WP4921	Mitochondrial complex III assembly	1.99	0.0318

Figure S1. Cardioprotection conferred by post-conditioning. **A.** Left ventricular ejection fraction (LVEF) at baseline, at 90 min post AMI, and at sacrifice. (90 min post AMI and sacrifice are the same for Isch. group) **B.** Infarct size measurement expressed as a percentage of the area at risk (AAR). **C.** Scheme illustrating the experimental procedure timeline for each group. Red arrows indicate sacrifice. Data is presented as mean ± standard error. (* $p < 0.05$).

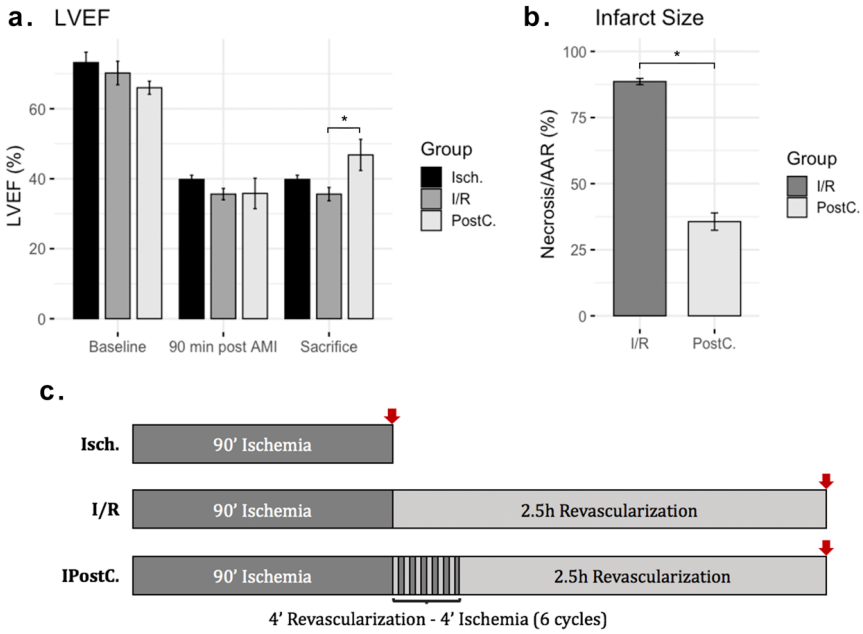


Figure S2. Multi two-group Gardner-Altman estimation plots of the ETC identified members across all conditions. The bootstrap distribution of the unpaired mean difference between each condition and the sham is depicted (gray shadow). **A.** ETC complex I. **B.** ETC complex III. **C.** ETC complex V.

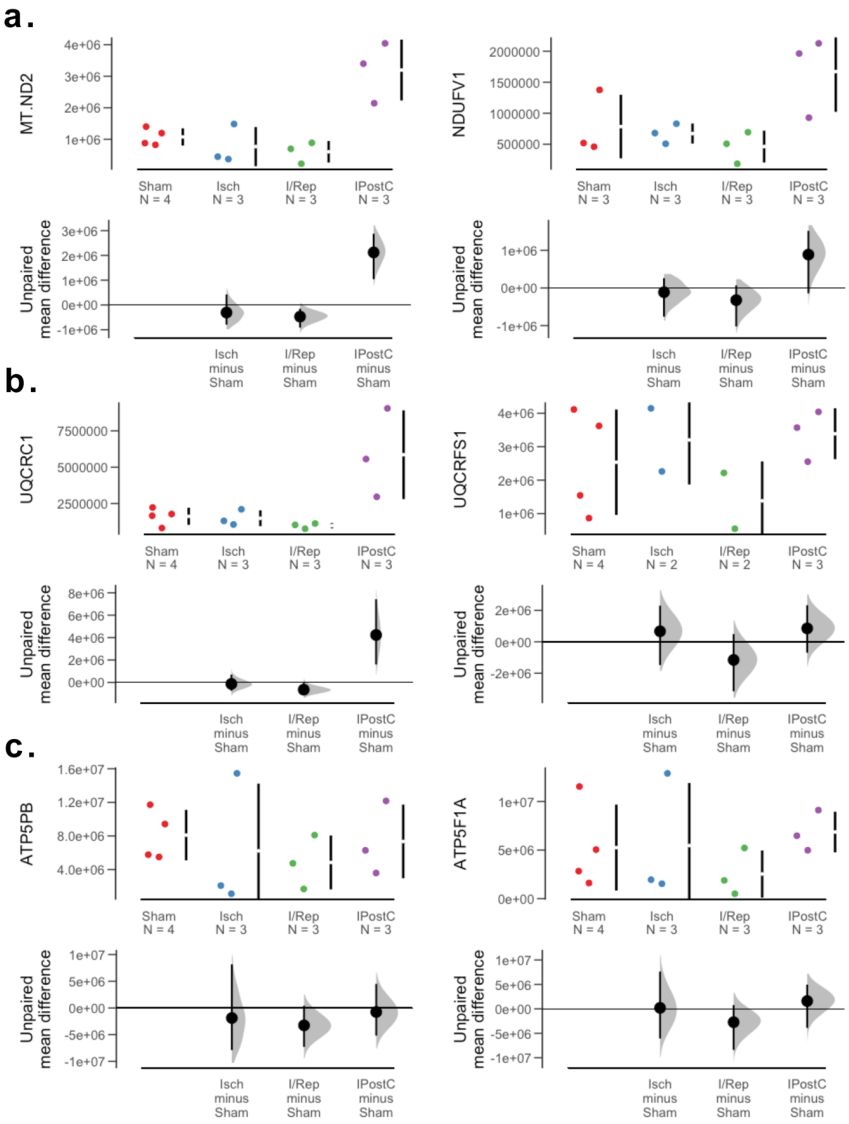
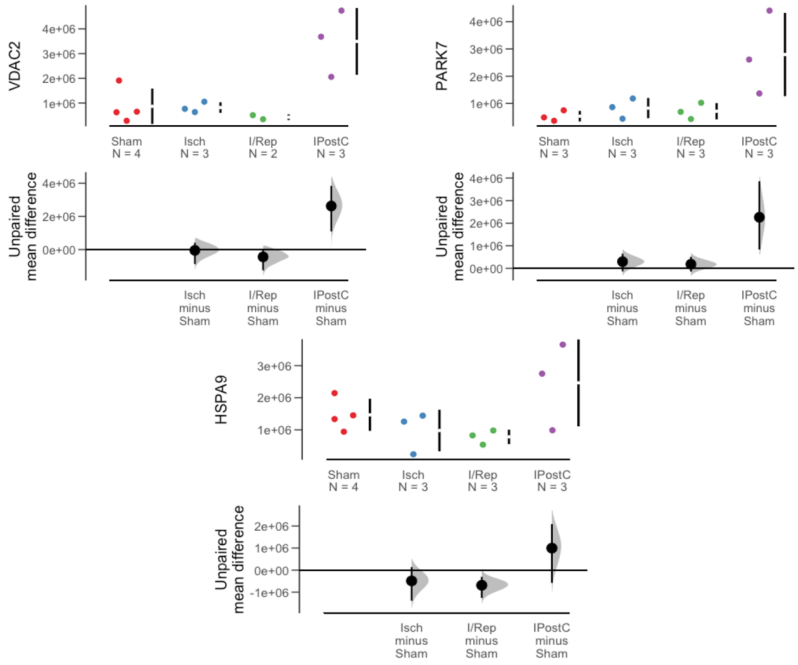


Figure S3. Multi two-group Gardner-Altman estimation plots of VDAC2, DJ-1, and HSPA9.



5.2.- Article 2

DJ-1 interacts with the ectopic ATP-synthase in endothelial cells during acute ischemia and reperfusion

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Objective 2.- To characterize the dynamics and functional implications of protein DJ-1 upon ischemia and reperfusion.

Highlights:

- Both full-length DJ-1 and DJ-1ΔC are secreted by endothelial cells during ischemia and reperfusion.
- DJ-1 secretion occurs in a soluble form, not bound to extracellular vesicles.
- Extracellular DJ-1 of either form binds and regulates the ectopic ATP-synthase activity during ischemia.
- DJ-1 stimulation of the ectopic ATP-synthase during ischemia contributes to the maintenance of endothelial function after reperfusion.

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DJ-1 interacts with the ectopic ATP-synthase in endothelial cells during acute ischemia and reperfusion

Alex Gallinat[1,2] & Lina Badimon[1,3,4]✉

Endothelial cells (ECs) play a central role in ischemia. ATP-Synthase is now recognized to be ectopically expressed in the cell surface of many cell types, with putative roles described in angiogenesis, proliferation, and intracellular pH regulation. DJ-1 is a multifunctional protein, involved in cell protection against ischemia, ischemia–reperfusion (I/R), and oxidative stress, that regulates mitochondrial ATP-synthase. Here we focused on the characterization of the endothelial dynamics of DJ-1, and its implication in the regulation of the ectopic ATP-synthase (ecATP-S) activity, during acute ischemia and I/R in ECs. We found that DJ-1 is secreted from ECs, by a mechanism enhanced in ischemia and I/R. A cleaved form of DJ-1 (DJ-1ΔC) was found only in the secretome of ischemic cells. The ecATP-S activity increased following acute ischemia in ECs, coinciding with DJ-1 and DJ-1ΔC secretion. The inhibition of DJ-1 expression inhibited the ecATP-S response to ischemia by ~ 50%, and its exogenous administration maximized the effect, together with an enhanced Akt phosphorylation and angiotube-formation potential at reperfusion. Immunoprecipitation studies showed direct interaction between DJ-1 and the ecATP-S. Altogether suggesting that DJ-1 is actively cleaved and released from ischemic ECs and plays an important role in the regulation of the ecATP-S activity during acute ischemia and reperfusion.

Ischemia is defined as the stress resulting from the restriction of blood supply to a given tissue or organ. It is mainly caused by the capillary obstruction due to either microthrombus formation or microvascular damage. When this happens, oxygen unavailability disrupts the cellular metabolism leading to ATP depletion, acidosis, and the accumulation of detrimental products, ultimately resulting in an extensive cell death and organ dysfunction. Ischemia is a common hallmark of many diseases, as is the case of myocardial infarction, stroke, and kidney, limb, or intestinal ischemia, all of them responsible of a high morbidity and mortality worldwide. Furthermore, ischemia is also found in the core of most solid tumours, and is believed to play a role in the malignant cell progression[1].

Endothelial cells (ECs), lining the circulatory system, play a pivotal role in regulating homeostasis and disease. Rather than a barrier, their key location between blood stream and the surrounding tissue, makes the endothelium an active player sensing and responding to hemodynamic changes and ischemic damage[2,3]. From normal embryonic development to tumour progression and the onset of an ischemic event, ECs provide multiple autocrine and paracrine signals supporting organ function[4], and modulate the immune response[2].

The F_OF_1 ATP-synthase is the enzyme responsible for the formation of ATP from ADP and inorganic phosphate, driven by the electrochemical gradient established through the electron transport chain in the mitochondria. Despite it was originally believed to exclusively locate in the inner mitochondrial membrane, its ectopic expression in the cell surface have been proven for a wide spectrum of cell types, including vascular ECs[5–7], hepatocytes[8,9], adipocytes[10], lymphocytes[11], keratinocytes[12], muscle[13], and neural cells[14,15], in both tumour and normal conditions[16]. It is known to act as a receptor for angiostatin[6], and apolipoprotein A-I[7,8], and to promote tumour-recognition by the immune system[16–18]. In ECs, ectopic ATP-synthase (ecATP-S) have been recognized to play a role in angiogenesis, proliferation, and regulating intracellular pH[7,19,20]. Furthermore, its location within the lipid rafts and caveolae makes plausible a functional connection with purinoreceptors, modifying the local

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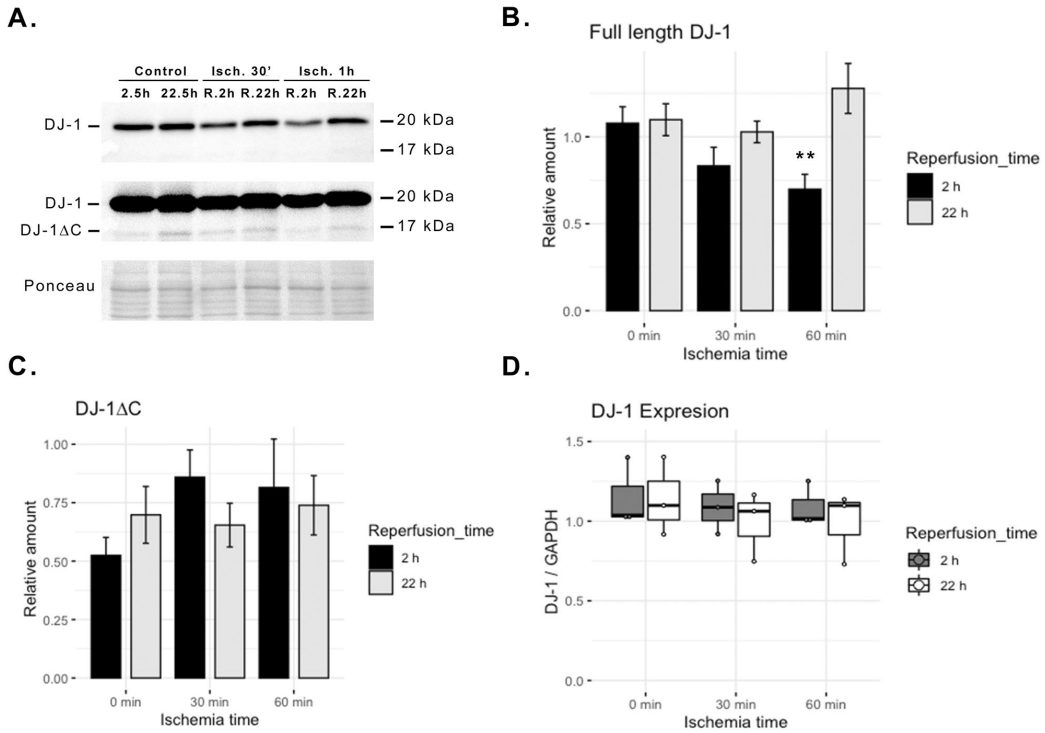

Figure 1. Endothelial DJ-1 content declines during I/R injury. ECs cultures were subjected to either 30 min or 1 h in vitro ischemia followed by 2 or 22 h of reperfusion, and both protein and RNA extracts were probed for DJ-1. (**A**) Representative western blot. (**B**) Quantification of endothelial full length DJ-1 upon I/R. (n = 5; $^{**}p < 0.01$). (**C**) Quantification of endothelial DJ-1ΔC upon I/R (n = 5).; and, (**D**) *park7* gene expression upon I/R in ECs (n = 3). Data presented as mean ± SEM. *ECs* endothelial cells, *I/R* ischemia–reperfusion. Corresponding uncropped western blot acquisitions can be found in Supplemental Fig. 2.

ATP/ADP concentrations, and thus promoting downstream signalling[9,11,21]. Since the ecATP-S highly increases its activity under acidic and hypoxic cell culture conditions[22], a role in regulating cell function under ischemia could be presumed.

The early-onset Parkinson's disease associated protein DJ-1 (also known as PARK7) is a protein with several pleiotropic functions including chaperone[23] and protease[24], deglycase[25,26], transcriptional and translational regulator[27,28], redox sensor[29] and mitochondrial homeostasis keeper[30,31], that has been shown to bind and regulate the mitochondrial F_OF_1 ATP-synthase activity[32]. A cleaved form of DJ-1, corresponding to a 15 carboxyl-terminal amino acids deletion (referred to as DJ-1ΔC), has been suggested as the active form in in vitro testing, and cell protection effects have been proven[24,33,34]. Despite the exact role of DJ-1 and DJ-1ΔC has not yet been elucidated, it is widely accepted to play a role in cell protection against ischemia, ischemia–reperfusion (I/R) and oxidative stress[35]. Here we have focused on the characterization of the endothelial dynamics of protein DJ-1 and DJ-1ΔC, and their implication in the regulation of the ec-ATP-S activity, in a model of acute ischemia and I/R.

Results

Endothelial DJ-1 content declines during I/R. We investigated whether ECs subjected to I/R injury show a dysfunctional regulation of DJ-1 and/or its cleavage. Different periods of in vitro I/R were tested, and both the full-length and cleaved forms of DJ-1, as well as DJ-1 expression, were measured. A short period of reperfusion (i.e. 2 h) following ischemia led to a significant decline in intracellular full-length DJ-1 ($p < 0.01$; Fig. 1A–C). A significant effect was found in the interaction between duration of ischemia and duration of reperfusion ($p < 0.05$), meaning that DJ-1 decline at reperfusion was dependent on the severity of ischemia. Interestingly while after 2 h of reperfusion the levels of full-length DJ-1 were reduced from baseline (cells without ischemia), after 22 h of reperfusion, DJ-1 basal level was restored (Fig. 1A–C). DJ-1ΔC was found intracellularly at much lower levels than DJ-1 and although these levels seemed to rise at reperfusion, differences did not reach significance at initially measured time-points (Fig. 1B,C). The assessment of DJ-1ΔC dynamics at different reperfusion times after 1 h of ischemia revealed that while DJ-1ΔC intracellular content falls during ischemia,

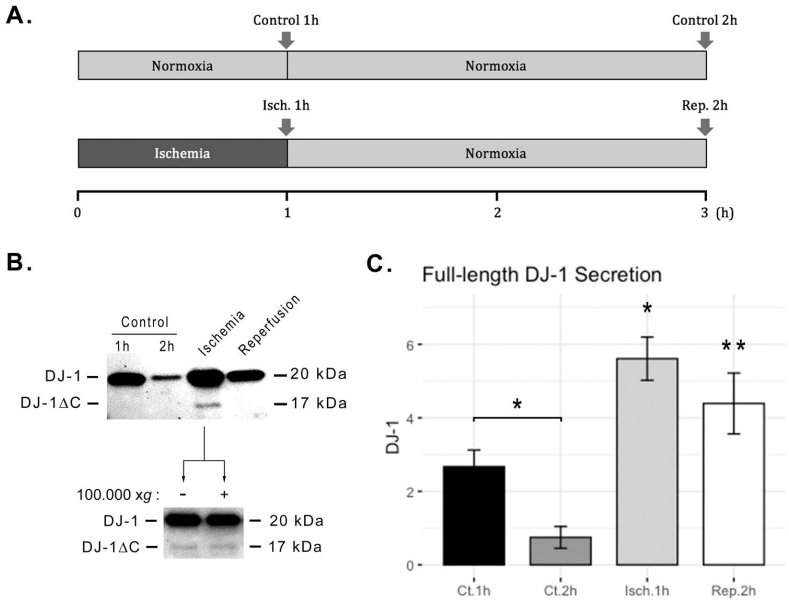

Figure 2. In vitro I/R promotes DJ-1 and DJ-1ΔC endothelial secretion. ECs cultures were subjected to 1 h in vitro ischemia followed by 2 h of reperfusion, and supernatants from ischemia, reperfusion, and control cultures, were probed for DJ-1. (**A**) Timeline scheme representing the experimental work-flow and group definitions for the analysis of the secretome. Arrows represent supernatant collection for analysis and medium exchange. (**B**) Representative western blot. (**C**) Western blot quantification of secreted full length DJ-1 (n = 5; $^{*}p < 0.05$; $^{**}p < 0.01$; n.s., not significant). Data presented as mean ± SEM. *ECs* Endothelial cells. Corresponding uncropped western blot acquisition can be found in Supplemental Fig. 3.

it increases with time after reperfusion (Supplemental Fig. 1). Regardless of the effects of I/R on DJ-1 protein content, no differences in DJ-1 gene transcription were found (Fig. 1D).

Acute ischemia and I/R promote DJ-1 and DJ-1ΔC endothelial secretion. The reduced levels of DJ-1 in ECs after I/R may be explained by its release from the cell. Thus, we analysed the presence of DJ-1 forms in the secretome of ECs subjected to I/R. Interestingly, the full-length form of DJ-1 was detectable in the cell secretome in each of the conditions tested, with a significantly higher abundance after ischemia ($p < 0.05$) and after I/R ($p < 0.01$; Fig. 2), suggesting an active release of DJ-1 by cells under ischemic stress. Moreover, DJ-1ΔC was found to be present just in the secretome of cells during ischemia alone (Fig. 2B) and occasionally during reperfusion, but never in the controls. These results suggest that DJ-1 is cleaved into DJ-1ΔC and actively released by ECs during ischemia. In order to check whether DJ-1 and DJ-1ΔC are secreted in a soluble form or within extracellular vesicles (EVs), we removed EVs by ultracentrifugation (1 h at 100.000 × *g*) of the supernatants collected after 1 h of ischemia prior protein precipitation. There were no differences on DJ-1 and DJ-1ΔC levels (Fig. 2B), indicating that both DJ-1 forms are found in the soluble secretome.

DJ-1 down-regulation impairs ectopic ATP-synthase activity rise after ischemia. Given that DJ-1 has been previously reported to regulate the mitochondrial ATP-synthase activity[32], and that ECs extracellular ATP generation increases after a period of incubation under ischemia-like conditions[22], we sought to analyse whether ECs DJ-1/DJ-1ΔC secretion under ischemia has an impact on the ecATP-S activity. Thus, we analysed the extracellular ATP generation of DJ-1 knocked-down ECs cultures exposed to normoxia and ischemia, compared to untransfected controls. After 1 h of ischemia, untransfected cultures exhibited nearly threefold increase in the extracellular ATP generation. Such increase after ischemia was significantly reduced in DJ-1 knocked-down cultures ($p < 0.05$; Fig. 3), indicating that protein DJ-1 is needed for the ecATP-S regulation after ischemia.

Extracellular DJ-1 boosts ectopic ATP-synthase activity after ischemia. In order to test whether the reported effect of DJ-1 upon the activity of the cell surface ATP-synthase is dependent on the extracellular form, we tested the extracellular ATP generation in ECs cultures subjected to ischemia or normoxia, in the presence and the absence of extracellular recombinant full-length DJ-1 and DJ-1ΔC (at 100 nM). The administration

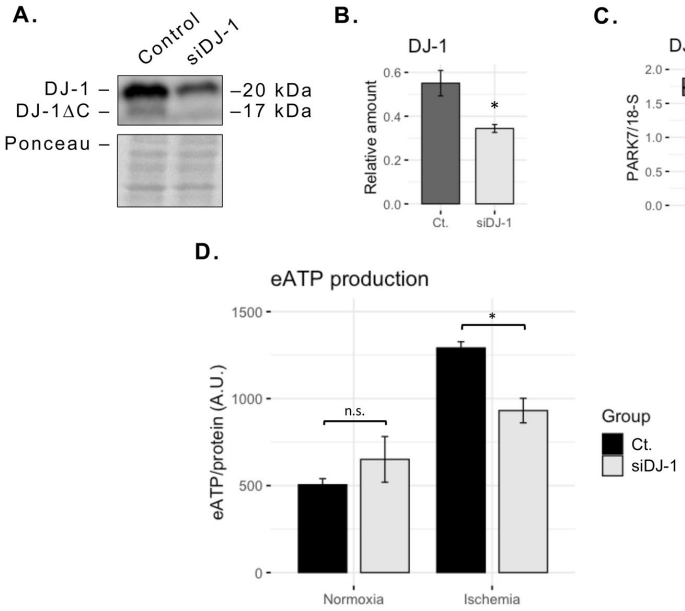

Figure 3. Extracellular ATP generation after ischemia is dependent on DJ-1. ECs cultures were transfected with a siRNA targeted to DJ-1, and subjected to 1 h in vitro ischemia. Extracellular ATP generation was then evaluated. (**A**) Representative western blot showing DJ-1 inhibition 72 h post-transfection. (**B**) Western blot quantification of DJ-1 72 h post-transfection (n = 4; $*p < 0.05$). (**C**) *park7* gene expression of control and knock-down cultures 72 h post-transfection (n = 3; $**p < 0.01$). (**D**) Extracellular ATP generation of DJ-1 knock-down and control cultures after 1 h in vitro ischemia compared to normoxic controls (n = 4; $*p < 0.05$; *n.s.* not significant). Data presented as mean ± SEM. *ECs* endothelial cells. Corresponding uncropped western blot acquisition can be found in Supplemental Fig. 4.

of DJ-1 resulted in a highly significant increase of the extracellular ATP generation after ischemia ($p < 0.01$), independently of the DJ-1 form employed (Fig. 4A). This effect could be explained either by an increase of the ecATP-S activity or localization to the cell surface. After measuring the ectopic expression of the ATP-synthase following ischemia and I/R in the presence and the absence of exogenous DJ-1 and DJ-1ΔC, no differences were detected across conditions (Fig. 4B,C), meaning the reported changes in the extracellular ATP generation are a consequence of an activity increase rather than localization.

Extracellular DJ-1 interacts with the ectopic ATP-synthase. ECs were cultured for 1 h under ischemic or normoxic conditions, in the presence and the absence of exogenous DJ-1 and DJ-1ΔC. Thereafter, cells were thoroughly rinsed, lysed, and immunoprecipitated against ATP-synthase. Immunocaptures were then assayed by western blot for both ATP-synthase and DJ-1. While exogenous full length DJ-1 was detectable in both normoxic and ischemic cell immunocaptures, interaction with DJ-1ΔC was only found in cells under ischemia (Fig. 5A,B). In order to check whether DJ-1/DJ-1ΔC association with the ATP-synthase is preserved over the course of reperfusion, the same experiment was performed in cultures subjected to ischemia and reperfusion in the presence and the absence of DJ-1 and DJ-1ΔC during the ischemic stimuli. As a result, after 2 h of reperfusion some traces of DJ-1ΔC remained detectable in the ATP-synthase immunocapture, while the full-length DJ-1 association with the ATP-synthase was lost. After 24 h of reperfusion, no association was detectable for DJ-1 of either form.

Extracellular DJ-1 enhances Akt phosphorylation and angiotube formation. Because the extracellular ATP generation through the ecATP-S has been reported to activate downstream signalling involved in cell survival via purinoreceptors[20]. We analysed the Akt phosphorylation status in normoxia, ischemia, and I/R, in the presence and the absence of exogenous full-length DJ-1 or DJ-1ΔC (at 100 nM). While no differences were found between treated and untreated cultures in normoxia or ischemia, an enhanced Akt activation during reperfusion was found for the cultures treated with DJ-1 in either form ($p < 0.01$; Fig. 6A,B). Within the endothelial cell, Akt is involved in a number of processes ranging from cell survival and inhibition of apoptosis to angiogenesis[36–38]. In order to test whether the Akt activation at reperfusion after the exposure to DJ-1 is involved in cell survival or angiogenesis, we analysed both the cleavage of Casp-3, as a surrogate of apoptosis,

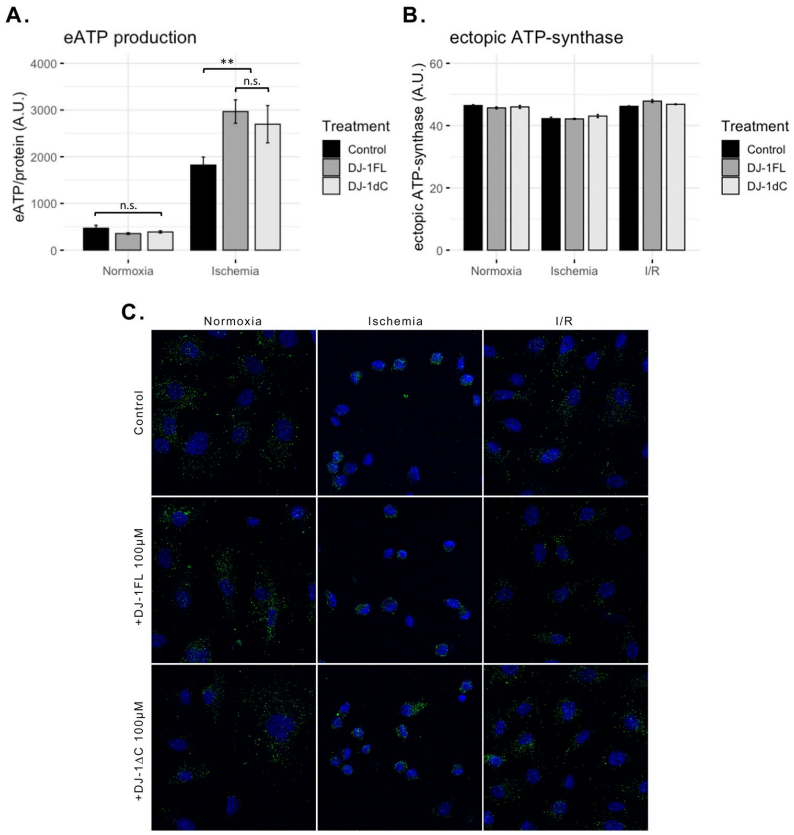

Figure 4. Extracellular DJ-1 promotes ectopic ATP-synthase activity following acute ischemia. ECs cultures were subjected to 1 h in vitro ischemia in the presence and the absence of full length DJ-1 and DJ-1ΔC (100 nM), and both the extracellular ATP generation and the ecATP-S expression were evaluated. (**A**) Extracellular ATP generation after 1 h in vitro ischemia in the presence and the absence of full length DJ-1 and DJ-1ΔC (n = 4; $^{**}p < 0.01$; *n.s.* not significant). (**B**) ecATP-S expression measured by immunohistochemistry across conditions. (**C**) Representative images of the ecATP-S immunohistochemistry across conditions. Green signal corresponds to ecATP-S. *ECs* endothelial cells, *ecATP-S* ectopic ATP-synthase.

and tube-formation capacity at reperfusion, as a surrogate of the angiogenic potential. As a result, no cleavage of Casp-3 was detectable in any condition (Fig. 6C), but a significantly faster formation of capillary-like structures during reperfusion was seen for the treated cultures (Fig. 6D,E). The sub-lethal nature of the assayed ischemic protocol, proven by the absence of Casp-3 cleavage, further confirms that all reported effects following DJ-1 administration are indeed dependent on DJ-1 and independent of cell death.

Discussion

In the present study we characterized the endothelial dynamic of DJ-1 within the context of I/R injury, and explored its implications in the regulation of the ecATP-S activity following acute ischemia. We demonstrated that DJ-1 is actively cleaved and released by ECs, and depicted an autocrine effect upon the ecATP-S activity, extracellular ATP generation, and angiogenesis, in a human umbilical-vein endothelial cells (HUVEC) model of sub-lethal ischemia and I/R.

Ever since their first isolation by Jaffe *et al.* back in 1973[39], to current days, HUVECs have become a valuable model for the in vitro study of vascular physiology and disease. As a non-immortalized human cellular model of ECs, HUVECs faithfully reproduce human ECs behaviour, and have been largely employed to study a broad array of biological processes and diseases[40]. Consequently, there are a number of standardized assays for the analysis of endothelial physiology and angiogenesis in HUVEC, facilitating the comprehension and reproducibility of results.

It is widely accepted that protein DJ-1 is implicated in cell survival following I/R and oxidative stress, as previously proven both in vitro and in vivo by loss-of-function models of myocardial infarction and stroke[34,41–47].

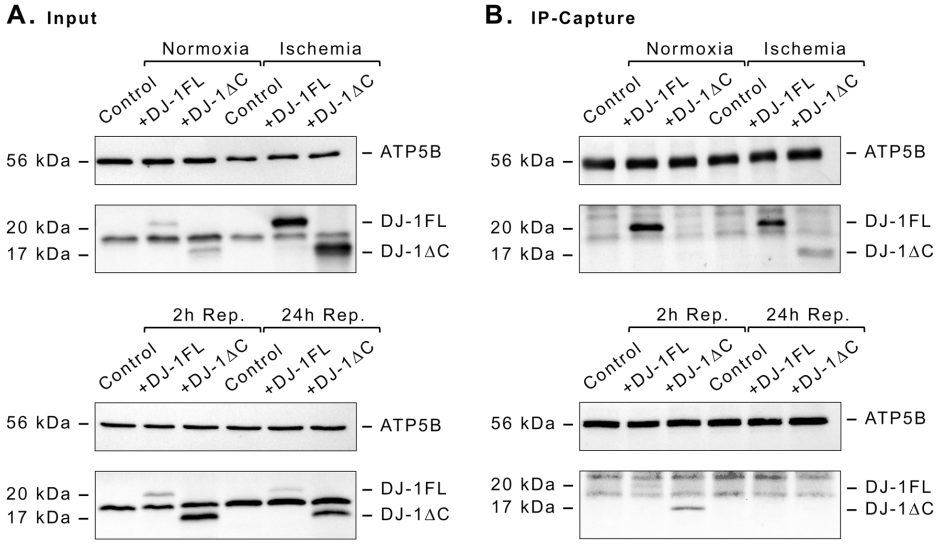

Figure 5. ATP-synthase co-immunoprecipitation with exogenous DJ-1 and DJ-1ΔC in ischemia and I/R. ECs cultures were exposed to either 1 h ischemia or normoxia, in the presence and the absence of exogenously administrated DJ-1 or DJ-1ΔC at 100 nM, with and without a reperfusion period of 2 h and 24 h (whithout exogenous DJ-1/DJ-1ΔC). After indicated treatment, cells were thoroughly rinsed, lysed, and immunoprecipitated against ATP-synthase. (**A**) Western blot analysis of ATP synthase (ATP5B) and DJ-1/DJ-1ΔC in whole cell lysates (immunoprecipitation input). (**B**) Western blot analysis of ATP synthase (ATP5B) and DJ-1/DJ-1ΔC in the IP-captures. *ECs* endothelial cells, *IP* immunoprecipitate, *I/R* ischemia/reperfusion. Membranes were split in two before incubation. Corresponding uncropped western blot acquisitions can be found in Supplemental Fig. 5.

However, although many molecular functions have been attributed to DJ-1, there is not yet a consensus on its exact molecular function. DJ-1ΔC arises from a 15 amino acid carboxyl-terminal deletion of DJ-1, resulting from a specific cleavage in response to mild oxidative stress, and have been purposed as a mechanism triggering its cytoprotective activity[24,33,48]. We evaluated the impact of I/R upon the DJ-1 reservoir of ECs, and found a significant and ischemia-dependent decline of the full-length form after reperfusion. This observation may be explained by protein degradation or release to the extracellular space. We further demonstrated that both forms were present in the cell secretome. Interestingly, DJ-1ΔC secretion was found to be associated to ischemia, suggesting that DJ-1 is actively cleaved and released by ECs during ischemia. Moreover, we demonstrated DJ-1 to be secreted in a soluble form (i.e. not bound to EVs), as the ultracentrifugation of the cell culture media prior sample processing had no effect upon the detected extracellular DJ-1 and DJ-1ΔC.

In order to avoid protein interference in the analysis of secretomes, we performed the secretion experiments under serum-free conditions, which may have affected the secretion process. Thus, two different controls were employed for ischemia and reperfusion, and so while both ischemia and first control underwent a transition from basal culture conditions to serum-free, both reperfusion and second control were kept in the absence of serum. Therefore, the observed differences in the DJ-1 secretion levels between the secretomes of the two controls arise from the acute cell stress induced by the transition from basal culture conditions to serum-free media.

Given the current consensus about a protective role for DJ-1, and the endothelial dynamic upon I/R injury[2,4,49], the reported release of DJ-1 and DJ-1ΔC may behave as a cell sensor for damage or as an autocrine/paracrine cell function modulator. Lacking of a conventional secretory signal peptide, DJ-1 has been previously suggested to be secreted through the autophagy-based unconventional secretion pathway, as proven by experiments with autophagy inhibitors and autophagy-related knockout models[50]. Being ischemia and I/R known stimuli to promote autophagy, this is could be a tentative and feasible mechanism. Yet, DJ-1 have been previously reported to be secreted under some pathologic conditions such as breast cancer[51], Parkinson's disease[52,53] and stroke[54], and a protective role upon ischemia[42] and I/R[55] have been proven for the extracellular form in neuronal cells. However, the mechanism by which extracellular DJ-1 confers protection remains to be clarified.

The ATP-synthase ectopic expression in the cell surface is now recognized for a number of cell types, and is known to display several functions ranging from angiogenesis to cholesterol uptake[6–8,21]. Being essentially a H^+ channel, a role in the regulation of the intracellular pH is presumable, as shown by its inhibition with monoclonal antibodies targeted to the ectopic ATP-synthase, which resulted in a dysregulation of the intracellular pH[20]. This mechanism would be especially relevant in ischemia, which rapidly leads to acidosis. Indeed, the ecATP-S has been reported to highly increase its activity under chronic ischemic conditions[22], and has been proposed as a mechanism of ischemia tolerance for ECs[56]. Additionally, the ecATP-S has been proposed to affect downstream

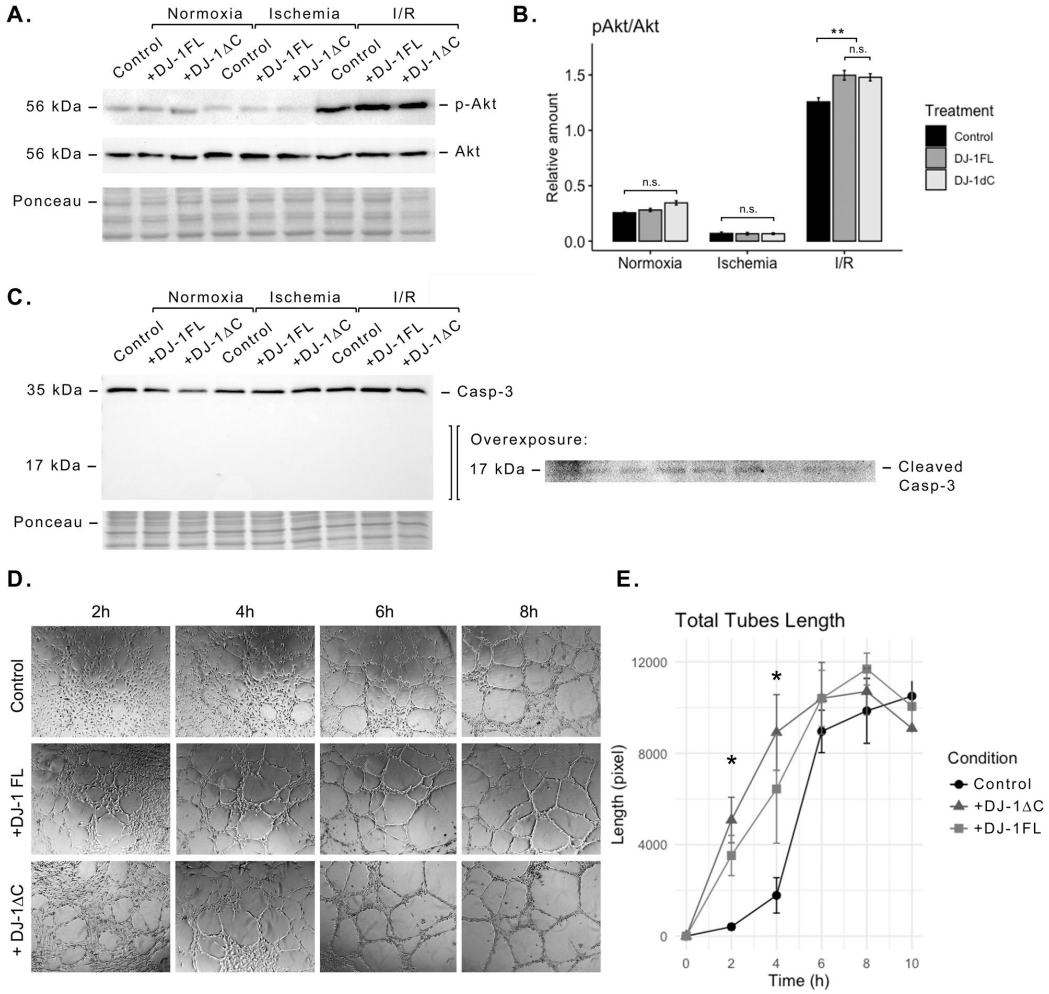

Figure 6. Effect of DJ-1 and DJ-1ΔC ischemic exposure in signalling and in vitro angiogenesis. ECs cultures were exposed to either 1 h ischemia or normoxia, in the presence and the absence of exogenously administrated DJ-1 or DJ-1ΔC at 100 nM, with and without a reperfusion period of 2 h, and both the phosphorylation status of Akt and the cleavage of Casp-3 were analysed upon cell lysates. (**A**) Representative western blot of phospho-Akt (Ser473) and total Akt in normoxia, ischemia, and I/R, in the presence and the absence of full-length DJ-1 or DJ-1ΔC. (**B**) Akt relative phosphorylation status quantification (n = 4; $^{**}p < 0.01$, *n.s.* not significant). (**C**) Representative western blot of Casp-3 in normoxia, ischemia, and I/R, in the presence and the absence of full-length DJ-1 or DJ-1ΔC. No cleavage of Casp-3 was detectable in any condition, an overexposed acquisition of the 17 kDa surrounding region is included. (**D**) Representative acquisitions of tube-formation assay of ECs during reperfusion after the exposure to 1 h of ischemia, in the presence and the absence of exogenously administrated DJ-1 or DJ-1ΔC at 100 nM. No exogenous DJ-1/DJ-1ΔC were added in the reperfusion media. (**E**) Total tubes length quantification over reperfusion time (n = 3; $^{*}p < 0.05$). *Casp-3* Caspase-3, *ECs* endothelial cells, *I/R* ischemia/reperfusion. Corresponding uncropped western blot acquisitions can be found in Supplemental Figs. 6 and 7.

signalling. Despite not yet elucidated, its location within the lipid rafts and caveolae allows it to modify the local ATP/ADP concentrations, potentially inducing purinergic signalling[9,11,21]. Treatments with monoclonal antibodies targeted to the ectopic ATP synthase were shown to antagonize Akt and Erk1/2 signalling, and to activate JNK and MAPK-p38, in ECs[20]. Also, the accumulation of extracellular ATP have been reported to protect endothelial barrier integrity following I/R injury[57].

Here we report a great increase in the activity of the ecATP-S following acute ischemia in ECs, coinciding with DJ-1 and DJ-1ΔC secretion. After 1 h of ischemia, control cultures exhibited nearly threefold increase in the extracellular ATP generation without changes in the relative amount of the cell-surface ATP-synthase. Such increase appeared to be dependent on DJ-1, as the inhibition of DJ-1 expression also inhibited the ecATP-S response to ischemia by ~50%, and the administration of exogenous DJ-1 in either its full-length or cleaved form, maximized the effect. Noteworthy, the exogenous administration of DJ-1 in normoxia had no effect upon ecATP-S activity, possibly due to a lack of a proton gradient to drive its activity in the absence of acidosis. Hence, rather than activate the ecATP-S, extracellular DJ-1 seems to optimize its performance, as previously described in the mitochondria[32]. Furthermore, the exogenously administrated DJ-1 was proven to physically interact with the ATP-synthase in the same fashion seen for secretion. And so, interaction with ATP-synthase was found both under normoxia and ischemia for the full-length form of DJ-1, and exclusively under ischemia for the cleaved form. Such association was lost over the course of reperfusion in the absence of exogenous DJ-1.

Whilst ischemia represents a profound detrimental factor, with no other resolution than reperfusion, the rapid recovery of basal conditions paradoxically carries the potential to exacerbate damage itself in a process tightly correlated to the duration and severity of the ischemic insult[58]. Thus, despite being ischemia the most detrimental factor, the cell response to reperfusion directly impacts on the extent of damage[47] In the context of ischemia and I/R, Akt has a number of positives effects as is the case of the inhibition of apoptosis and the promotion of angiogenesis[36–38]. Several reports have shown an activation of Akt during early reperfusion after ischemia[59]. Furthermore, the extent of this activation inversely correlates with damage, and thus Akt have been proposed as a central element of the I/R injury and the so called reperfusion injury salvage kinase (RISK) pathway[60,61]. Interestingly, while the extracellular ATP generation through the ecATP-S has been reported to activate Akt signalling via purinoreceptors[20], no activation of Akt was seen during ischemia in any condition. However, the ischemic exposure to DJ-1 in either form resulted in a more pronounced Akt activation at reperfusion, which may reflect a physiologic adaptation or an enhanced viability of ECs at reperfusion, rather than a consequence of purinergic signalling. A faster formation of capillary-like structures during reperfusion was seen for the treated cultures in the tube-formation assay, suggesting an enhanced angiogenic potential. Moreover, these reported effects were seen to be independent of cell death, as proven by the Casp-3 cleavage analysis, where no induction of apoptosis was found in any tested condition. Previous reports have proven a role for the ecATP-S in angiogenesis, as the treatment with targeted antibodies show an inhibitory effect[62,63]. Whether this effect is indeed dependent on the extracellular ATP generation, a secondary effect of cell pH regulation, or another feature conditional to the ecATP-S, remain to be clarified. Altogether, the data here reported supports a role for the ecATP-S and DJ-1 in the preservation of endothelial homeostasis in ischemia and I/R.

Methods

Cell culture. Human umbilical vein endothelial cells (HUVEC) were cultured in gelatin (G1890; Sigma, Saint Louis, MI, USA) coated flasks with M-199 Hank's medium (22350-029; Biological Industries, Beit-Haemek, Israel) containing 20% (v/v) FBS (04-007-1A; Thermo Fisher Scientific, Waltham, MA, USA), Endothelial Cells Growth Supplement (02-102; Millipore, Burlington, MA, USA), heparin (H3149; Sigma, Saint Louis, MI, USA), HEPES (15330-056; Thermo Fisher Scientific, Waltham, MA, USA), penicillin–streptomycin (15140-122; Thermo Fisher Scientific, Waltham, MA, USA), L-glutamine (25030-024; Thermo Fisher Scientific, Waltham, MA, USA), and pyruvate (11360-039; Thermo Fisher Scientific, Waltham, MA, USA), at 37 °C in 5% CO_2 atmosphere. All experiments were performed between passage 4 and 8.

For knock-down studies cells were transfected with a *park7* siRNA (s22305; Thermo Fisher Scientific, Waltham, MA, USA) using the Amaxa Cell Line Nucleofector Kit V (VCA-1003; Lonza, Basilea, Switzerland) following manufacturer's instructions. Both DJ-1 protein content and *park7* gene expression were assayed 72 h after transfection. All experiments were performed at 72 h post-transfection.

Cultures were treated with human recombinant full-length DJ-1 (MBS143125; MyBioSource, San Diego, CA, USA) or DJ-1ΔC (made upon request; GenScript, Piscataway, NJ, USA) at 100 nM, when indicated.

Ischemia and reperfusion model. Cell cultures were either subjected to in vitro ischemia or ischemia–reperfusion (I/R). In vitro ischemia was performed as previously described[64], incubating cells in acidic PBS (pH = 6.4) under hypoxic atmosphere (1% O_2). The culture of cells in growth medium and normoxic conditions, after a period of ischemia, is the modelling of reperfusion.

Angiotube-formation assay. Tube-formation assay was performed as previously described[65]. Briefly, HUVEC were seeded in growth factor reduced MatriGel (Corning, New York, NY, USA) coated 48-well plates at a density of 30.000 cells/cm^2, and let sit for 30 min. Then cultures were washed with PBS and subjected to 1 h in vitro ischemia in the presence and the absence of DJ-1 or DJ-1ΔC at 100 nM. Immediately after, ischemia buffer was replaced with basal growth media (without DJ-1 and DJ-1ΔC), and random acquisitions were taken every 2 h. Pictures were then analyzed in ImageJ (U.S. National Institute of Health, Bethesda, MD, USA).

Secretome analysis. In order to avoid protein interference, analysis of the secretome was performed in serum-free conditions. HUVEC cultures were washed twice with PBS, and subjected to 1 h in vitro ischemia or kept in normoxia (1 h control). Immediately after, supernatants were collected and replaced by fresh medium. Cells were allowed to recover for 2 h. After such time, reperfusion and 2 h control supernatants were collected, and cells counted with a cell counter (Beckmann Coulter, Brea, California). Supernatants were centrifuged to discard detached cells and debris. Then, supernatants were either mixed with 5× radio-immune precipitation assay (RIPA) buffer supplemented with a protease inhibitior cocktail (05056489001; Roche Diagnostics, Man-

nheim, Germany), freeze, thawed, and sonicated, in order to lysate any extracellular vesicles (EVs) present, or ultracentrifuged for 1 h at 100.000 × *g*, to remove EVs. Appropriate volumes to normalize samples through the number of cells were precipitated with acetone at −20 °C overnight, re-solubilized with 1% SDS, dialyzed against PBS, and analysed by western blot.

Western blot. Cell cultures were washed with PBS and lysed in ice-cold RIPA buffer supplemented with a protease inhibitior cocktail (05056489001; Roche Diagnostics, Mannheim, Germany). 10 µg of total protein were loaded and separated by SDS-PAGE with a Mini protean 3 system (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were then transferred to a nitrocellulose membrane (1620115; Bio-Rad Laboratories, Hercules, CA, USA), and immediately stained with Ponceau S total protein staining (P3504; Sigma, Saint Louis, MI, USA), to normalize differences in the loaded protein amounts between lanes. Blots were then blocked with 5 % bovine serum albumin (MB04603; NZYTech, Lisboa, Portugal) diluted in TBS-Tween20 and incubated overnight with either mouse anti-DJ-1 (MCA-4H4, EnCor Biotechnology, Gainesville, FL, USA) at 1:4000, rabbit anti-ATP5B (MA5-32589, Invitrogen, Carlsbad, CA, USA) at 1:1000, rabbit anti-phospho-Akt Ser473 (4060; Cell Signaling Technology, Danvers, MA, USA) at 1:1000, rabbit anti-Akt (9272; Cell Signaling Technology, Danvers, MA, USA) at 1:1000, or rabbit anti-Casp-3 (9662; Cell Signaling Technology, Danvers, MA, USA) at 1:1000. Horseradish peroxidase-coupled rabbit anti-mouse IgG secondary antibody or goat anti-rabbit IgG (P0260/P0448; Dako, Santa Clara, CA, USA) were used to detect primary antibodies together with SuperSignal reagent (34076; Thermo Fisher Scientific, Waltham, MA, USA). All images were acquired with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time qPCR. Cells were washed with PBS, and total RNA was isolated with the RNeasy Mini Kit (74106; Quiagen, Hilden, Germany) according to the manufacturer's instructions. Transcript levels were analysed by real-time quantitative polymerase chain reaction (qPCR) with on-demand TaqMan assays (DJ-1: Hs00994893_g1; Thermo Fisher Scientific, Waltham, MA, USA). rRNA-18S was used as an endogenous control (Hs99999901_s1; Thermo Fisher Scientific, Waltham, MA, USA). Taq-man real-time qPCR was performed as previously described[66].

Ectopic ATP-synthase activity. ecATP-S activity was analysed as previously described[19]. Briefly, cells were washed once in HEPES buffer (10 mM HEPES, 150 mM NaCl), and incubated for 5 min in HEPES buffer supplemented with 2 mM $MgCl_2$. Extracellular ATP generation was then initiated with the addition of HEPES buffer supplemented with 2 mM $MgCl_2$, 20 mM KH_2PO_4 and 200 µM ADP. After 20 s of reaction, supernatants were collected in EDTA containing tubes at 5 mM final concentration and centrifuged at 300x*g* 10 min at 4 °C to discard detached cells. ATP concentration was then assayed in supernatants with a luminescent ATP detection assay kit (ab113849; Abcam, Cambridge, United Kingdom) following manufacturer's instructions. After supernatants collection, cells were immediately lysed and protein amounts were quantified to normalize between wells.

Immunostaining. After indicated treatments, cells were washed with PBS and fixed for 15 min with 4% paraformaldehyde at room temperature. No permeabilization step was performed. Unspecific bindings were then blocked with 1% bovine serum albumin (MB04603; NZYTech, Lisbon, Portugal) diluted in PBS, for 15 min two times. Then cells were rinsed twice, incubated for 30 min with Image-IT FX signal enhancer (136,933; Invitrogen, Carlsbad, CA, USA), washed again and incubated with rabbit anti-ATP5B (MA5-32589, Invitrogen, Carlsbad, CA, USA) at 1:200, 1 h at room temperature. Cells were then thoroughly rinsed and incubated for 1 h with Alexa Fluor 488 coupled donkey anti-rabbit (A21206; Invitrogen, Carlsbad, CA, USA) at 1:100, counterstained with Hoechst 33342 (H3570; Thermo Fischer Scientific, Waltham, MA, USA) at 1 µg/mL, and mounted in Prolong Gold (P36931; Invitrogen, Carlsbad, CA, USA). Five random images per sample were then acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica microsystems, Wetzlar, Germany). Between 10 and 20 acquisitions per field were taken with a z-stack step size of 0.5 µm, in order to capture the whole sample volume. Maximum projections were then analysed with ImageJ (U.S. National Institute of Health, Bethesda, MD, USA).

Immunoprecipitation. Cell cultures were lysed in ice-cold (RIPA) buffer supplemented with a protease inhibitior cocktail (05056489001; Roche Diagnostics, Mannheim, Germany). Lysates were then incubated overnight with rabbit anti-ATP5B (MA5-32589, Invitrogen, Carlsbad, CA, USA) at 1:100, with gentle rocking at 4 °C. Protein G Sepharose beads (17-5280-04; GE Healthcare, Chicago, IL, USA) were used for precipitation according to the instructions of the manufacturer.

Statistical analysis. Normality was assessed with the Shapiro–Wilk method. When normality could be assumed, statistical differences between groups were analysed by two-tailed t-test (for comparisons between two groups), one-way ANOVA (for multiple groups) or two-way ANOVA (for multiple groups and two factors). Tukey's Honestly Significant Difference (HSD) *post hoc* test was performed to correct significance for multiple-comparisons. Kruskal–Wallis rank sum test was performed when normality could not be assumed. Data is presented as mean ± SEM. All the analyses were performed in RStudio (RStudio, Boston, MA, USA).

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.G.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing—original draft. L.B.: Conceptualization; Funding acquisition; Methodology; Resources; Supervision; Writing—review and editing.

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Competing interests

LB received institutional research Grants from AstraZeneca; consultancy fees from Sanofi, Pfizer and Novartis; speaker fees from Sanofi and Novartis. LB is shareholder of the academic spin-off companies GlyCardial Diagnostics S.L. and Ivestatin Therapeutics S.L. All unrelated to the present work. LB is author of the patents EP3219326A1 and WO2017157958A1 regarding the use of DJ-1-derived polypeptides for the treatment of ischemia/reperfusion injury. AG declares no conflict of interest.

Additional information

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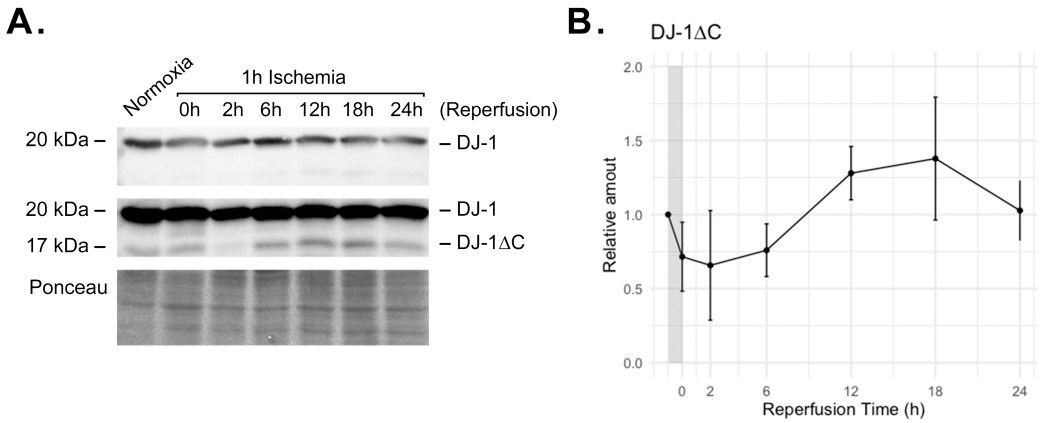
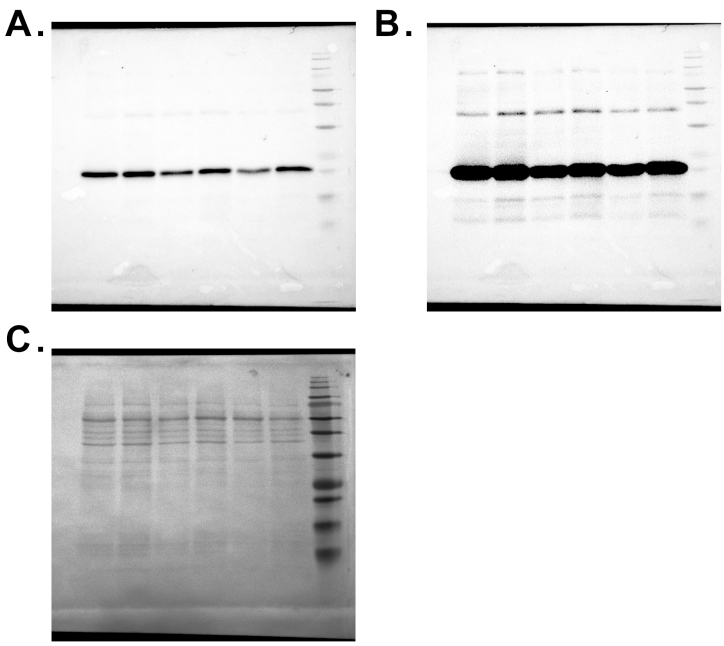
Supplemental Figure 1

Time course analysis of endothelial DJ-1ΔC at reperfusion. HUVEC cultures were subjected to 1h of *in vitro* ischemia followed by reperfusion, and relative DJ-1ΔC content was assayed at different time-points in cell lysates by western blot. **A.** Representative western blot of DJ-1 and DJ-1ΔC protein content over reperfusion. **B.** Quantification of intracellular relative DJ-1ΔC content over reperfusion. Shaded area indicates ischemia. HUVEC, Human umbilical-vein endothelial cells. Uncropped western blots acquisitions can be found in Supplemental Figure 8.



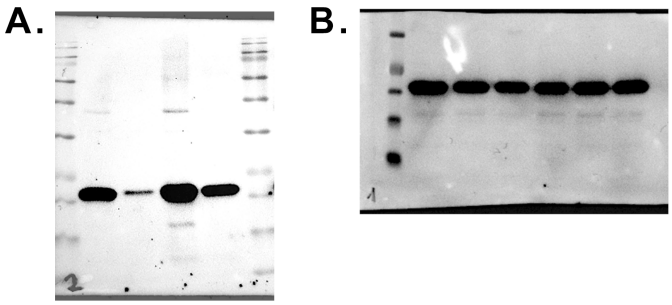
Supplemental Figure 2

Endothelial DJ-1 content declines during I/R injury - supporting gels. ECs cultures were subjected to either 30 min or 1 h of in vitro ischemia followed by 2 or 22 hours of reperfusion, and DJ-1 content was analysed by western blot. **A.** Uncropped western blot acquisition for DJ-1. **B.** Uncropped western blot overexposure for DJ-1ΔC. **C.** Corresponding uncropped Ponceau-S total protein staining acquisition. ECs, Endothelial cells; I/R, Ischemia-reperfusion.



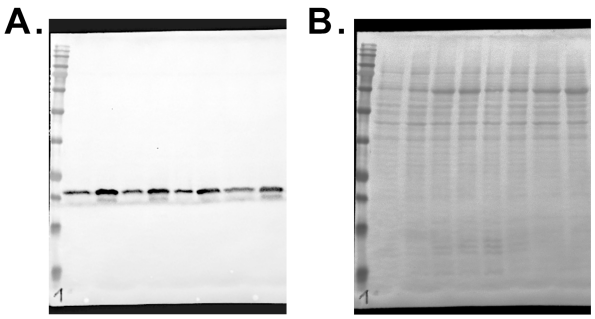
Supplemental Figure 3

In vitro I/R promotes DJ-1 and DJ-1ΔC endothelial secretion - supporting gels. ECs cultures were subjected to 1 h of in vitro ischemia followed by 2 h of reperfusion, and supernatants from ischemia, reperfusion, and control cultures, were proved for DJ-1. **A.** Uncropped western blot acquisition for secreted DJ-1. **B.** Uncropped western blot acquisition for DJ-1, before and after ultracentrifugation for 1h at 100.000*g* (alternate samples). ECs, Endothelial cells.



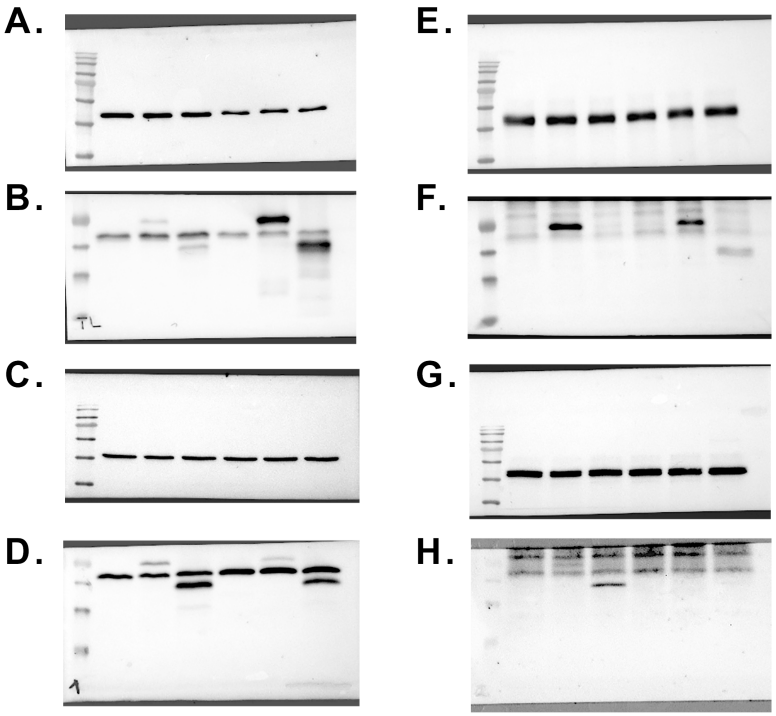
Supplemental Figure 4

Extracellular ATP generation after ischemia is dependent on DJ-1 - supporting gels. ECs cultures were transfected with a siRNA targeted to DJ-1, and subjected to 1 h of *in vitro* ischemia. Extracellular ATP generation was then evaluated. **A.** Uncropped western blot acquisition for DJ-1 for transfected and control cultures (alternate samples). **B.** Corresponding uncropped Ponceau-S total protein staining acquisition. ECs, Endothelial cells.



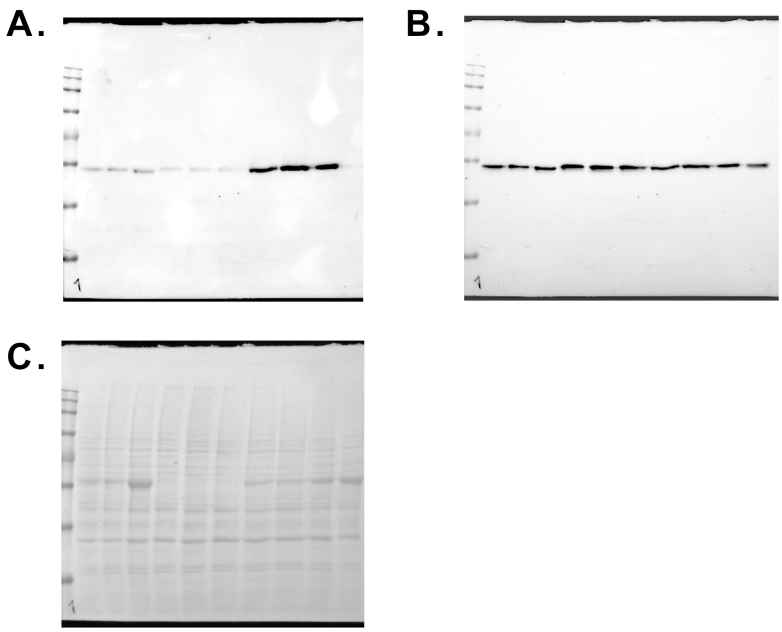
Supplemental Figure 5

ATP-synthase co-immunoprecipitation with exogenous DJ-1 and DJ-1ΔC in ischemia and I/R - supporting gels. ECs cultures were exposed to either 1h of ischemia or normoxia, in the presence and the absence of exogenously administrated with DJ-1 or DJ-1ΔC at 100 nM, with and without a reperfusion period of 2h and 24h in the absence of exogenous DJ-1/DJ-1ΔC. After indicated treatment, cells were thoroughly rinsed, lysed, and immunoprecipitated against ATP-synthase (ATP5B). Both total lysates (**A**-**D**) and IP-captures (**E**-**H**) were probed for ATP5B (**A**,**C**,**E**, and **G**) and DJ-1 (**B**,**D**,**F**, and **H**). Corresponding uncropped western blot acquisitions are presented. Membranes were split in two before incubation. ECs, Endothelial cells; IP, immunoprecipitate; I/R, ischemia/reperfusion.



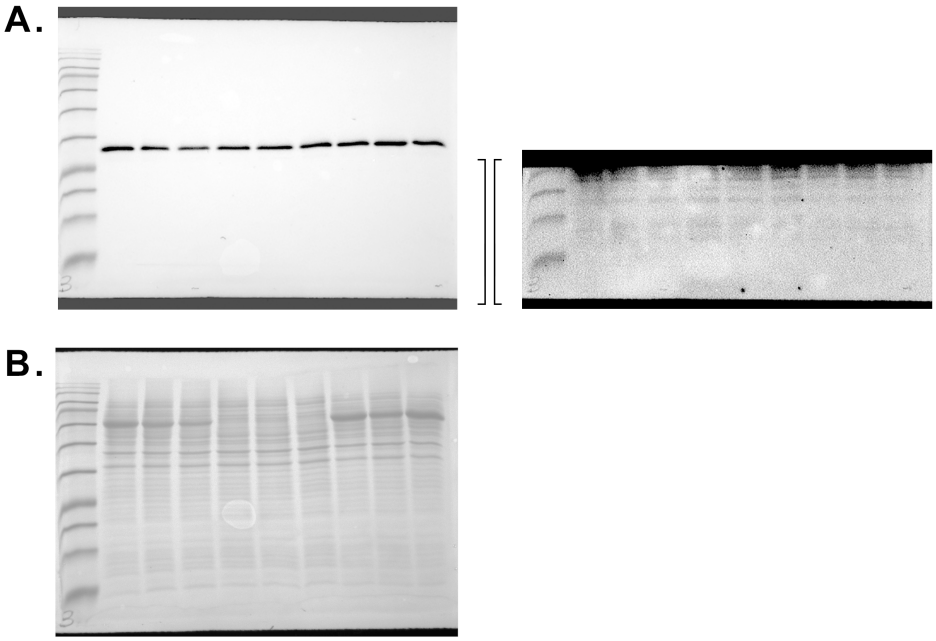
Supplemental Figure 6

Effect of DJ-1 and DJ-1ΔC ischemic exposure in Akt signalling - supporting gels. ECs cultures were exposed to either 1h of ischemia or normoxia, in the presence and the absence of exogenously administrated DJ-1 or DJ-1ΔC at 100 nM, with and without a reperfusion period of 2h, and the phosphorylation status of Akt was analysed upon cell lysates. **A.** Uncropped western blot acquisition for phospho-Akt (Ser473) analysis. **B.** Uncropped western blot acquisition for total Akt analysis. **C.** Corresponding uncropped Ponceau-S total protein staining acquisition. ECs, Endothelial cells; I/R, Ischemia/reperfusion.



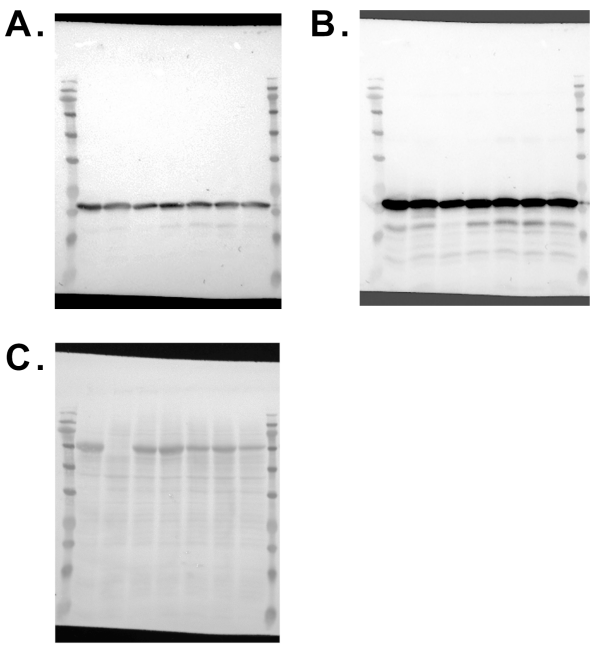
Supplemental Figure 7

Effect of DJ-1 and DJ-1ΔC ischemic exposure in Casp-3 cleavage - supporting gels. ECs cultures were exposed to either 1h of ischemia or normoxia, in the presence and the absence of exogenously administrated DJ-1 or DJ-1ΔC at 100 nM, with and without a reperfusion period of 2h, and the cleavage of Casp-3 was analysed upon cell lysates. **A.** Uncropped western blot acquisition for Casp-3 analysis, and overexposure of the low molecular weight section of the membrane. **B.** Corresponding uncropped Ponceau-S total protein staining acquisition. Casp-3, Caspase-3; ECs, Endothelial cells; I/R, Ischemia/reperfusion.



Supplemental Figure 8

Time course analysis of endothelial DJ-1ΔC at reperfusion - supporting gels. HUVEC cultures were subjected to 1h of *in vitro* ischemia followed by reperfusion, and relative DJ-1ΔC content was assayed at different time-points in cell lysates by western blot. **A.** Uncropped western blot acquisition for DJ-1 analysis. **B.** Uncropped western blot overexposure for DJ-1ΔC analysis. **C.** Corresponding uncropped Ponceau-S total protein staining acquisition. ECs, Endothelial cells; I/R, Ischemia-reperfusion.



5.3.- Article 3

DJ-1 administration exerts cardioprotection in a mouse model of acute myocardial infarction

Alex Gallinat, Guiomar Mendieta, Gemma Vilahur, Teresa Padró and Lina Badimon

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Objective 3.- To evaluate the therapeutic potential of recombinant protein DJ-1 for minimizing myocardium damage following acute myocardial infarction.

Highlights:

- A systemic administration of recombinant DJ-1 prior to acute myocardial infarction reduces infarct size and modifies the transcriptomic response of the myocardium to reperfusion.
- Gene sets corresponding to G-protein coupled receptors signaling and immune response are enriched in the myocardium transcriptome at reperfusion when animals were treated with a systemic administration of recombinant DJ-1.
- A systemic administration of recombinant DJ-1 prior to acute myocardial infarction reduces leukocyte infiltration, oxidative stress, and apoptosis, at reperfusion.

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DJ-1 administration exerts cardioprotection in a mouse model of acute myocardial infarction

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Cardiovascular diseases, and particularly acute myocardial infarction (MI), are the most common causes of death worldwide. Infarct size is the major predictor of clinical outcomes in MI. The Parkinson's disease associated protein, DJ-1 (also known as PARK7), is a multifunctional protein with chaperone, redox sensing and mitochondrial homeostasis activities. Previously, we provided the evidence for a central role of endogenous DJ-1 in the cardioprotection of post-conditioning. In the present study, we tested the hypothesis that systemic administration of recombinant DJ-1 exerts cardioprotective effects in a mouse model of MI and also explored the associated transcriptional response. We report a significant treatment-induced reduction in infarct size, leukocyte infiltration, apoptosis and oxidative stress. Effects potentially mediated by G-protein-coupled receptor signaling and modulation of the immune response. Collectively, our results indicate a protective role for the exogenously administrated DJ-1 upon MI, and provide the first line of evidence for an extracellular activity of DJ-1 regulating cardiac injury *in vivo*.

KEYWORDS

cardioprotection, DJ-1, PARK7, ischemia, reperfusion, myocardial infarction, ischemia/reperfusion injury

Introduction

Cardiovascular diseases are the leading cause of mortality worldwide. Among them, ischemic heart disease is the most common pathology (Benjamin et al., 2017). Myocardial infarction (MI) is defined as ischemia of the myocardial tissue, commonly caused by the occlusion of the coronary artery. Consequently, necrosis develops within the myocardium at risk (Reimer et al., 1979). Therefore, both the duration and the severity of the ischemic insult are major determinants of the final infarct size. Due to the limited regenerative capacity of the adult human heart, there is little replacement of lost functional tissue following MI. Rather, nearby fibroblasts are

activated to replace the damaged myocardial tissue with fibrous tissue (Frangogiannis, 2006; Aix et al., 2018). Since the scar lacks contractile function, it decreases cardiac output, eventually leading to heart failure and free wall rupture (Kutty et al., 2013), Therefore, infarct size is the major predictor of clinical outcomes following MI (Sobel et al., 1972; Kelle et al., 2009).

Considering the time-dependent transition from ischemia to necrosis of the myocardium at risk, early reperfusion remains the definitive treatment for the ischemic heart. However, the rapid transition from ischemia to normoxia during reperfusion carries the potential to exacerbate damage in a process known as ischemia/reperfusion (I/R) injury (Yellon and Hausenloy, 2007). This is in part related to the generation of reactive oxygen species (ROS) (Zweier, 1988) affecting, among others, the integrity of the sarcoplasmic reticulum which leads to calcium overload, hyper-contracture, and the opening of the mitochondrial permeability transition pore, eventually causing cell death. Also, the changes in intracellular pH and the triggered immune response compromise the viability of the myocardium upon reperfusion (Lemasters et al., 1996; Liu et al., 2016). The aforementioned detrimental effects of reperfusion occur within minutes of restoration of coronary flow and account for a significant part of the final infarct size (Yellon and Hausenloy, 2007; Garcia-Dorado et al., 2009). A large number of pharmacological agents have shown to reduce infarct size in pre-clinical studies, but they have failed to demonstrate significant clinical benefits (Hausenloy et al., 2013; Heusch, 2017). Thus, cardioprotection is currently an unmet clinical need.

The early-onset Parkinson's disease associated protein DJ-1 (also known as PARK7) is a multifunctional protein with cardioprotective effects against I/R injury (Dongworth et al., 2014; Shimizu et al., 2016; Shimizu et al., 2020) and oxidative stress (Billia et al., 2013). In a previous study, where the mitochondrial proteomic response to I/R and post-conditioning was analysed in a pre-clinical model of MI, we provided evidences for a central role of DJ-1 in the cardioprotection conferred by post-conditioning (Gallinat et al., 2022). The reported functions of DJ-1 include chaperone (Shendelman et al., 2004), protease (Chen et al., 2010), and deglycase (Richarme et al., 2015; Richarme and Dairou, 2017) activities, regulation of transcription (Takahashi-Niki et al., 2017), redox sensing (Wilson, 2011) and modulation of mitochondrial homeostasis (Hayashi et al., 2009; Heo et al., 2012). Also, some reports suggest extracellular activities for DJ-1. It is secreted under several pathological conditions including breast cancer (Le Naour et al., 2001), Parkinson's disease (Maita et al., 2008; Tsuboi et al., 2008) or stroke (Allard et al., 2005), and a protective role against ischemia (Kaneko et al., 2014) and I/R (Han et al., 2017). Likewise, we have reported endothelial DJ-1 secretion under ischemia and I/R, and evidenced its effects on endothelial cell function at reperfusion, thereby suggesting a role in regulating cardiac injury (Gallinat and Badimon, 2022). In this study, we tested the hypothesis that systemic administration of recombinant protein DJ-1 exerts a cardioprotective effect in a mouse model of acute MI.

Materials and methods

Murine model of myocardial infarction

The present study was performed in male CH3 mice of 8–10 weeks' old weighing 25–30 g ($n = 26$; Jackson Laboratory, Bar Harbor, ME, United States). Mice were randomly given an intraperitoneal injection of 50 μg of DJ-1 ($n = 10$; full length recombinant human DJ-1 > 95% purity, MBS143125, MyBioSource, San Diego, CA, United States); or equal PBS volume for vehicle/controls ($n = 16$), 60 min prior to the induction of MI by 45 min ligation of the left anterior descending coronary artery (LAD), as previously described (Cubedo et al., 2016; Mendieta et al., 2019). Briefly, animals were anesthetized with a mixture of O_2/isoflurane, intubated and mechanically ventilated (rate 90 breath/min, tidal volume 0.1 ml; TOPO dual mode ventilator, Kent Scientific Corporation; Torrington, CT, United States). Core temperature was continuously monitored throughout the surgery and maintained within 37–38°C using a heat pad and heat lamp. An anterior thoracotomy was performed; the heart was exposed and the LAD coronary artery was occluded with an intramural stitch (7–0 silk suture) for 45 min. The success of complete coronary ligation was verified by electrocardiographic visualization of the ST-elevation-MI pattern that was continuously monitored and the visualization of a pale and hypokinetic ventricular region distal to the site of occlusion. After 45 min of ischemia, animals were sacrificed (Ischemia group; $n = 11$ vehicle and $n = 5$ DJ-1) or reperfused for 2 h (I/R group; $n = 5$ vehicle and $n = 5$ DJ-1). A sham-operated group ($n = 4$), which underwent the same surgical procedure without ligation of the LAD was included. Afterwards, hearts were carefully excised and processed for the following procedures. The dose of DJ-1 was chosen based on previous studies (Arac et al., 2011; Cubedo et al., 2016). All analyses were performed blindly with regard to the treatment received by the animals.

Morphometric assessment of infarct size

Hearts ($n = 26$) were immersed in a fixative solution (4% paraformaldehyde), embedded in optimal cutting temperature compound (OCT) and sequentially cross-sectioned from the

apex to the base (10 µm thick sections 200 µm distanced). Sections were then stained with haematoxylin-eosin and infarct size analysis was morphometrically determined with the image analysis software ImageJ (Schindelin et al., 2012). Infarct size was calculated as the sum of myocardial infarct areas between total left ventricle wall surface, as previously reported (Takagawa et al., 2007; Cubedo et al., 2016). Three measurements per each histological section were determined.

Immunohistochemical analysis

OCT-embedded frozen specimens were cut into 5 µm thick serial sections, placed on poly-L-lysine coated slides, and processed for immunohistochemistry. The sections were incubated for 2 h with rabbit polyclonal antibodies against DJ-1 (AP01249PU-N; 1:50 dilution; Acris Antibodies GmbH, Herford, Germany), neutrophil elastase (ab68672; 1: 100 dilution; Abcam, Cambridge, United Kingdom), inducible nitrogen oxide synthase (iNOS; NB300-605; 1:20 dilution; Novus Biologicals; Littelton, CO, United States) and cleaved caspase-3 (Asp175; 9,661; 1:200 dilution; Cell Signaling; Danvers; MA, United States), mouse monoclonal antibody against monocyte/ macrophages (ab33451; 1:50 dilution; Abcam, Cambridge, United Kingdom), or goat polyclonal antibody against 8-hydroxy-deoxy-Guanine (8-OHdG; MBS536217; 1: 300 dilution; MyBioSource, San Diego, CA, United States). Thereafter, sections were rinsed and incubated with the appropriate biotinylated antibodies (1:200 dilution; Vector Laboratories, Burlingame, CA, United States). Endogenous peroxidase activity, as well as unspecific unions were blocked before incubation with primary antibodies. Finally, sections were incubated with avidin-biotin complex (Vector Laboratories, Burlingame, CA, United States), and 3,3′-diaminobenzidine was used as the substrate for peroxidase. Images were acquired with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan), digitized by a Retiga 1300i camera (Teledyne Photometrics, Tucson, AZ, United States), and imported to ImageJ (Schindelin et al., 2012). Positive signal was then quantified and expressed as the percentage of total area. For leukocyte infiltration and cleaved caspase-3 analyses, the number of positively labelled cells per field were counted. Six random fields per sample were analysed.

Apoptosis assessment

Apoptosis was histologically analysed by the dUTP neck-end labeling (TUNEL) assay according to manufacturer's specifications (Chemicon Inc.; Pittsburgh, PA, United States). The apoptosis rate was measured in a section below the occlusion and expressed as the percentage of TUNEL-positive cells per field (5 random fields per heart). All images were acquired in the same conditions.

RNA extraction

Frozen tissue was grinded using mortar and pestle, and total RNA was extracted using a combined organic extraction and silica-membrane columns method (RNesasy Mini Kit, Qiagen; Valencia, CA, United States). RNA was then quantified by spectrophotometry using the Nanodrop ND-1000 (Thermo Fisher Scientific). RNA quality was assessed with the Agilent 2100 Bioanalyzer technology (Agilent Technologies; Santa Clara, CA, United States) and the Agilent RNA 6000 Nano Kit (Agilent Technologies; Santa Clara, CA, United States). Only RNA samples with an RNA Integrity Number (RIN) > 7 were chosen for microarray experiments.

Transcriptomic and *in silico* analysis

Myocardial gene expression changes were analysed with a GeneChip Mouse 1.0ST array approach (Affymetrix, Santa Clara, CA, United States). Using the Ambion WT Expression Kit (Ambion, Life Tecnologies, Carlsbad, CA, United States) 100 ng of total RNA (mixed with poly-A controls; Affymetrix, Santa Clara, CA, United States) were retro-transcribed to double strand DNA, in two steps, in order to obtain cRNA. Single strand DNA was generated from 10 ug of cRNA. Then 5.5 µg of single strand DNA were fragmented and labelled with biotin using the WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, United States). Hybridization controls from the Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA, United States) were added to the sample. Thereafter, every sample was hybridized to a GeneChip Mouse Gene 1.0 ST array for 16 h at 45°C and 60 rpm, according to manufacturer's instructions. Hybridization, washing, staining, and scanning of microarrays were performed according to Affymetrix instructions using the Affymetrix GeneChip 3000 7G System (645 Hybridization Oven, 450 Fluidic Station and GeneChip 3,000 7G Scanner). Raw data were pre-processed (background correction, normalization and median polish summarization of the probes) with Robust Multiarray Average (RMA) method. Microarray quality control and statistical analyses were performed using Expression Console (Affymetrix, Santa Clara, CA, United States) and the Partek Genomics Suite software (Partek Inc., St Louis, MI, United States). *p*-values were adjusted for multiple testing with Benjamini and Hochberg method, and false discovery rates (FDR) were calculated. Bioinformatic analysis was performed with WebGestAlt (ZhangLab; http://www.webgestalt.org/)

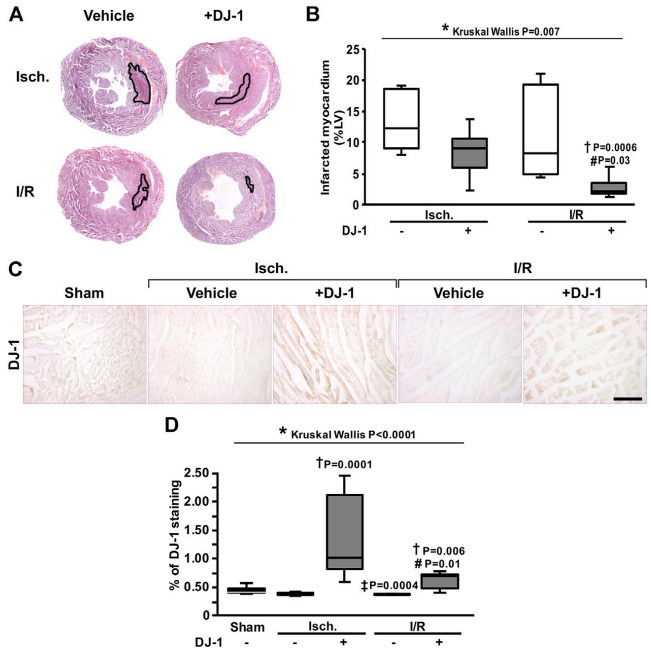


FIGURE 1
Effects of systemic DJ-1 administration on infarct size in a mouse model of MI. **(A)**. Representative images of infarcted heart sections. Infarct area is outlined in black. **(B)**. Infarct size quantification after ischemia and ischemia/reperfusion, in the presence and the absence of a systemic administration of DJ-1. Infarct measures are expressed as percentage of left ventricle area. **(C)**. Representative immunohistochemical acquisitions of DJ-1 in the myocardium after ischemia and ischemia/reperfusion, in the presence and the absence of a systemic administration of DJ-1. A sham-operated group was included as baseline. **(D)**. DJ-1 myocardium content quantification across groups. Scale bar: 100 µm; *$p < 0.05$, Kruskal-Wallis; †$p < 0.05$ vs. ischemia without DJ-1, ‡$p < 0.05$ vs. ischemia + DJ-1, #$p < 0.05$ vs. I/R without DJ-1, Dunn's test. LV, Left Ventricle; Isch., Ischemia; I/R, Ischemia/Reperfusion.

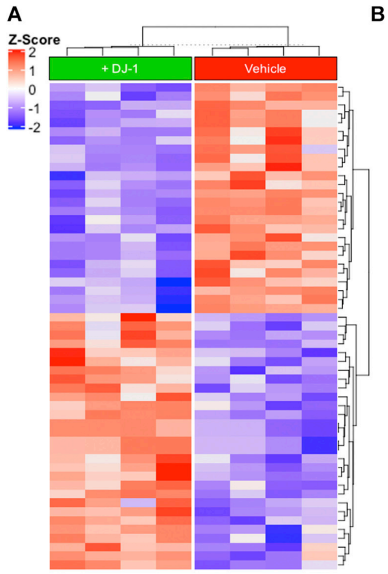
following a Gene Set Enrichment Analysis (GSEA) (Xin et al., 2019) approach and defining gene ranks by log-fold change (logFC). Gene sets available from Wikipathways (Martens et al., 2021), Molecular Signature Data Base (MSigDB) (Liberzon et al., 2015), and Panther (Thomas et al., 2003) pathway collections were considered for the analysis. Raw expression data have been deposited in the NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible under the GEO Series accession number GSE66307.

qPCR

Validation of myocardial gene expression changes was performed in tissue samples from the I/R group. *Gprc5a, Inos* and *Casp3* mRNA levels were analysed by real-time polymerase chain reaction, as previously described (Luquero et al., 2022). On-demand TaqMan RT-PCR assays for indicated genes were employed (Thermo Fisher Scientific Inc., Waltham, MA, United States).

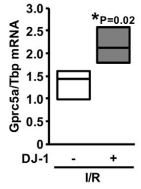
Statistical analysis

Because data were not normally distributed as assessed by the Shapiro-Wilk test, a non-parametric statistical analysis was employed. Non-parametric Kruskal-Wallis followed by Dunn's test for multiple comparisons and Mann-Whitney test were used to assess differences between groups. Results are reported as median with inter-quartile range unless otherwise stated. Correlations were determined with Spearman's rank correlation coefficient. All statistical analyses were performed with the statistical software package Statview 5.0.1 (SAS Institute Inc.; Cary, NC, United States).



B

Top Differentially Expressed Genes			
Up-regulted		Down-regulated	
Gene Symbol	**logFC**	**Gene Symbol**	**logFC**
Kir3dl2	0.9	Lphn2	-0.6
Olfr95	0.8	Aplnr	-0.6
Adamts9	0.8	Akr1c14	-0.6
Gprc5a	0.7	Klhl4	-0.6
Olfr362	0.6	Arhgap18	-0.6
Olfr591	0.6	Prlr	-0.6
Slc4a1	0.6	Hist1h4f	-0.6
Defb33	0.6	Fam198b	-0.6
Pfpl	0.6	Olfml1	-0.5
Ugdh	0.6	Prox1	-0.5



D

Collection source: Wikipathways			
Gene Set	**Description**	**Normalized Enrrichemnt Score**	**FDR**
WP3632	Lung fibrosis	2.0	0.014
WP234	Peptide GPCRs	1.9	0.009
WP441	Matrix Metalloproteinases	1.9	0.015
WP1397	Odorant GPCRs	1.8	0.029
WP522	Biogenic Amine Synthesis	1.8	0.027
WP460	Blood Clotting Cascade	1.8	0.028
WP189	GPCRs, Class A Rhodopsin-like	1.7	0.037
WP1396	Non-odorant GPCRs	1.7	0.045
Collection source: MSigDB			
Gene Set	**Description**	**Normalized Enrrichemnt Score**	**FDR**
M5890	TNFa Signaling via NFkB	2.9	0.017
M5897	IL6/JAK/STAT3 Siignaling	2.8	0.017
M5932	Inflamatory Response	2.6	0.017
M5950	Allograft Rejection	2.3	0.017
M5913	Interferon Gamma Response	2.3	0.017
M5921	Complement	2.3	0.017
M5956	KRAS Signaling DN	2.1	0.017
M5939	p53 Pathway	2.1	0.017
M5891	Hypoxia	2.0	0.017
M5902	Apoptosis	1.9	0.017
Collection source: Panther database			
Gene Set	**Description**	**Normalized Enrrichemnt Score**	**FDR**
P02727	Androgen/estrogene/progesterone biosynthesis	1.9	0.006
P00017	DNA replication	-2.2	0.006

FIGURE 2
Differential gene expression profile after ischemia/reperfusion, in the presence and the absence of a systemic administration of DJ-1. **(A)**. Heat-map representation of differentially expressed genes. **(B)**. Top ten up- and down-regulated genes after ischemia/reperfusion in the presence of DJ-1 systemic administration. **(C)**. *Gprc5a* transcript analysis by qPCR. **(D)**. Gene-set enrichment analysis result. Gene-set collections from Wikipathways, Molecular Signature, and Panther databases were considered. *$p < 0.05$, Mann-Whitney. I/R, Ischemia/Reperfusion; logFC, log-Fold Change.

Results

Systemic administration of recombinant DJ-1 protects the heart against I/R injury

In order to test whether the administration of DJ-1 exerts cardioprotection after MI, a group of mice were intraperitoneal treated with human recombinant DJ-1 (50 μg) 1 h before LAD coronary artery ligation, in a double-blind experimental design, and heart samples were processed for histological analysis. Computer-assisted morphometric assessment of infarct size revealed an infarct size reduction of about 75% for the animals treated with DJ-1 (Figure 1A,B). Interestingly, also a reduction in infarct size of about 20% was detected in the ischemia group, for the animals treated with DJ-1 (Figure 1A). Importantly, a 5.4-fold mean increase in the DJ-1 signal was found in the myocardium of the treated animals (Figure 1C,D), meaning the administrated DJ-1 reached the heart.

DJ-1 administration modifies the myocardial transcriptomic response to I/R

In order to outline possible mechanisms at play, we analysed the transcriptomic response of the myocardium upon MI in the presence and the absence of a systemic administration of DJ-1. As depicted in the heat-map, the administration of DJ-1 before I/R induced a multi-genic response in the myocardium different from that of the vehicle group (Figure 2A). Figure 2B shows the top 10 up- and down-regulated genes in the DJ-1-treated mice compared to the vehicle group.

Genome-wide RNA expression data was then analysed following a GSEA (Subramanian et al., 2005) approach considering all gene set collections available at Wikipathway (Martens et al., 2021), Molecular Signature (Liberzon et al., 2015), and Panther (Thomas et al., 2003) databases. As a result, different pathways were detected to be significantly enriched (FDR < 0.05) within each analysis (Figure 2D). From them all, G protein-coupled receptors (GPCR) mediated signaling and immune response-related gene sets were the most consistently detected. Interestingly, an apoptosis-related gene set was also significantly enriched (FDR = 0.017).

As a surrogate of the GPCR-mediated signaling-related gene set, the expression of *Gprc5a* gene was validated by qPCR (Figure 2C). Effects upon the immune response and apoptosis related gene sets were functionally validated as follows.

Systemic DJ-1 administration reduces myocardial leukocyte infiltration following I/R

In order to functionally validate the predicted effects of DJ-1 administration in modulating the immune response to I/R, we quantified the leukocyte infiltration by immunohistochemistry (Figure 3). As a result, animals treated with DJ-1 exhibited significantly lower infiltration of neutrophils and macrophages, both after ischemia and I/R.

Anti-apoptotic effects of DJ-1 administration

An effect of a systemic DJ-1 administration upon the induction of apoptosis following myocardial I/R was further investigated. Notably, animals treated with DJ-1 exhibited a diminished expression of *Casp3* gene after I/R, as assayed by qPCR (Figure 4A). Consistently, we could detect a significant reduction of the cleaved-Casp-3 myocardial content ($p = 0.04$), as well as the decrease of the TUNEL-positive cells ($p = 0.009$) following I/R for the DJ-1 treated group (Figure 4B,C).

Anti-oxidant effects of DJ-1 administration

Because ROS-induced damage has a central role in I/R injury, we evaluated whether a systemic administration of DJ-1 has an effect upon the oxidative damage to the myocardium following I/R. As a result, the administration of DJ-1 significantly reduced the expression level of *Inos* gene, as well as the staining of the oxidative damage marker 8-hydroxy-deoxy-Guanine (8-OHdG) (Figure 5). Additionally, we found a negative correlation between the staining signals of DJ-1 and iNOS (Figure 5E), and between DJ-1 and 8-OHdG (Figure 5F). A positive correlation between iNOS and 8-OHdG staining was also evidenced (Figure 5G).

Discussion

In the present study, we explored the effects of a systemic administration of recombinant DJ-1 upon ischemia and I/R injury in a mouse model of acute MI. We report a significant reduction in infarct size, leukocyte infiltration, apoptosis, and oxidative stress associated with the treatment. Also, we analysed the transcriptional response of the myocardium.

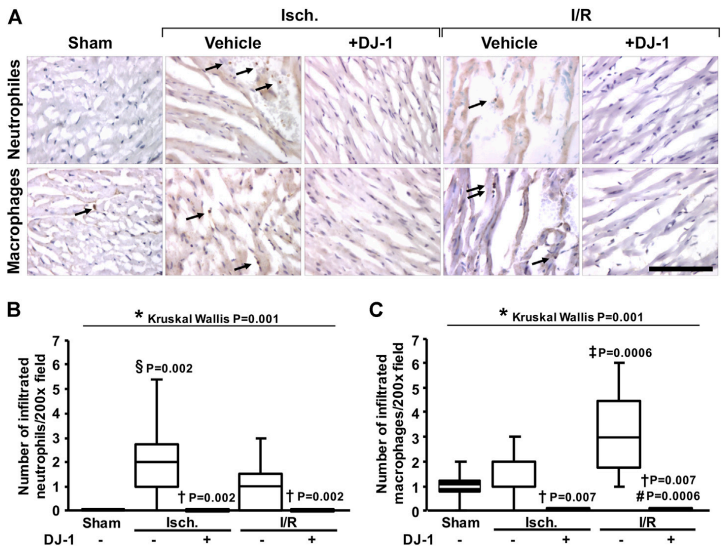


FIGURE 3
Effects of DJ-1 systemic administration on myocardial leukocyte infiltration after ischemia and ischemia/reperfusion. **(A)**. Representative immunohistochemical acquisitions of myocardial neutrophils (upper panel) and macrophages (lower panel) after ischemia and ischemia/reperfusion in the presence and the absence of a systemic administration of DJ-1. A sham-operated group was included as baseline. **(B)** and **(C)** quantification of infiltrated leukocytes. Scale bar: 100 μm; *$p < 0.05$, Kruskal-Wallis; §$p < 0.05$ vs. sham, †$p < 0.05$ vs. ischemia without DJ-1, ‡$p < 0.05$ vs. ischemia + DJ-1, #$p < 0.05$ vs. I/R without DJ-1, Dunn's test. Isch., Ischemia; I/R, Ischemia/Reperfusion.

A role for the endogenous DJ-1 in cardioprotection has already been proven by gain- and loss-of-function studies. Hence, while DJ-1 deficiency do not affect cardiac performance at baseline, animals lacking DJ-1 exhibited larger infarcts, increased mitochondrial fission, and worse left ventricular function upon LAD ligation, compared to wild-type littermates (Dongworth et al., 2014; Kaneko et al., 2014; Shimizu et al., 2016; Dong et al., 2018; Xin et al., 2019). Similar results were found in experimental models of stroke, where the lack of DJ-1 resulted in larger infarcts *in vivo*, and enhanced cell death *in vitro* (Aleyasin et al., 2007). In a previous study, we have described the up-regulation of DJ-1 following post-conditioning in a pre-clinical model of MI (Gallinat et al., 2022). In pressure overload animal models, mice lacking DJ-1 exhibited a higher oxidative stress level, exaggerated cardiac hypertrophy, and were more prone to develop heart failure (Billia et al., 2013). Collectively reinforcing a cardioprotective role for DJ-1.

Over the past decades, DJ-1 have been extensively studied, and many functions have been reported. These include chaperone (Shendelman et al., 2004), protease (Chen et al., 2010), and deglycase (Richarme et al., 2015; Richarme and Dairou, 2017) activities, regulation of transcription (Takahashi-Niki et al., 2017), redox sensing (Wilson, 2011) and the modulation of mitochondrial homeostasis (Hayashi et al., 2009; Heo et al., 2012). However, the exact molecular function of DJ-1, as well as its dynamics and regulation remain elusive. Interestingly, some reports suggest an extracellular activity, as it is secreted under some pathologic conditions such as breast cancer (Le Naour et al., 2001), Parkinson's disease (Maita et al., 2008; Tsuboi et al., 2008) and stroke (Allard et al., 2005). Also, a pro-survival role for the extracellular form, has been reported in ischemia (Kaneko et al., 2014) and I/R (Han et al., 2017). Likewise, we previously described the endothelial secretion of DJ-1 during ischemia and I/R, and evidenced a role in regulating endothelial cell function (Gallinat and Badimon, 2022). The data here reported support a cardioprotective role for DJ-1 in MI, presumably for the extracellular form.

ROS are a group of small molecules derived from the reduction of the oxygen molecule, that are continuously produced in small amounts as by-products of cell respiration and metabolism, and eliminated by the endogenous antioxidant systems. Within the heart, ROS play a role as second messengers for the excitation-contraction coupling, cell differentiation, and regulation of

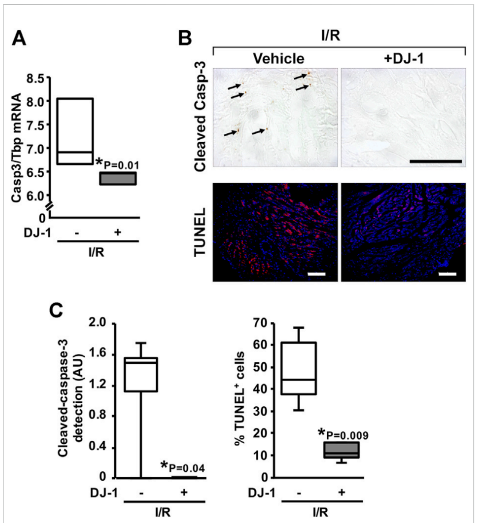


FIGURE 4
Effects of DJ-1 systemic administration on myocardial apoptosis after ischemia/reperfusion. **(A)** *Casp3* transcript analysis by qPCR. **(B)** Representative acquisitions of myocardial cleaved Casp-3 immunohistochemistry (upper panel) and TUNEL assay (lower panel) after ischemia/reperfusion. **(C)** myocardial cleaved Casp-3 immunohistochemistry and TUNEL positive cells quantification. Scale bar: 100 µm; *$p < 0.05$, Mann-Whitney. I/R, Ischemia/Reperfusion.

blood flow (Forman et al., 2004; Burgoyne et al., 2012). However, the accumulation of ROS or the unbalance between ROS production and the antioxidant mechanisms (termed, oxidative stress) is detrimental and cause several macromolecular modifications, such as, lipid peroxidation, protein misfolding, and DNA damage. Indeed, oxidative stress is involved in the aetiology of a number of pathologies and cellular insults. At the onset of reperfusion, the reintroduction of O_2 cause a burst of ROS within the mitochondria (Jassem et al., 2002), which challenges the antioxidant mechanisms and impairs the mitochondrial electron transport chain. This compromise the mitochondrial function, the ATP production and cell viability (Arduini et al., 1988). The significant decrease in the 8-OHdG staining signal following I/R for the treated animals, indicates that the administration of DJ-1 mitigates the oxidative damage to the myocardium. This observation was reinforced by the significant negative correlation found between DJ-1 and 8-OHdG staining signals.

In order to highlight possible mechanisms at play, we performed a whole-transcriptome analysis of the myocardium. Unexpectedly, we could not detect any of the previously described functions of DJ-1 enriched in the transcriptomic analysis, which suggests a distinct role for the extracellular and intracellular forms of DJ-1. GPCR-mediated signaling and immune response were rather the most consistently detected enrichments.

A body of evidences has linked GPCR signaling to cardioprotection. Endogenous GPCR ligands released during ischemia and reperfusion (i.e., autacoids) have been proposed as triggers of the cardioprotection conferred by ischemic pre- and post-conditioning (Heusch, 2015). This is the case for bradykinin (Goto et al., 1995; Oldenburg et al., 2004), opioids (Schultz et al., 1995; Zatta et al., 2008), acetylcholine (Yao and Gross, 1993), catecholamines (Tsuchida et al., 1994), angiotensin II (Liu et al., 1995), and endothelin-1 (Wang et al., 1996). Also, a number of GPCR agonists have proven to protect the heart against I/R injury.

GPCRs couple to a membrane-anchored heterotrimeric $G_{\alpha\beta\gamma}$ protein. Upon activation, GPCRs undergo a conformational change that allows the exchange of a GDP molecule, bound to the Gα subunit, for a GTP molecule, activating the G_α subunit. Thereafter, G_α dissociates from the remaining $G_{\beta\gamma}$ subunits and triggers downstream signaling. GPCRs can be classified depending on the downstream effects of the activated G_α subunit into: $G_{\alpha s}$, which stimulate the adenylate cyclase, $G_{\alpha i/o}$, which inhibit adenylate cyclase, $G_{\alpha q/11}$, which activate phospholipase C, and $G_{\alpha 12/13}$ which regulate Rho GTPase activity. Most of the GPCRs that play a role in cardioprotection couple to $G_{\alpha i/o}$ or $G_{\alpha q/11}$ proteins (Heusch, 2015). Furthermore, GPCR kinases, and β-arrestins, that are also involved in GPCR-mediated signaling (Gurevich and Gurevich, 2019), play a role in regulating cardiac injury upon MI (Brinks et al., 2010; Wang et al., 2017). The systemic administration of DJ-1 induced the up-regulation of several GPCRs following MI, some of them belonging to the odorant GPCRs family. Although still poorly understood, olfactory receptors have been reported to be expressed in the heart and to play a role in regulating cardiac function and angiogenesis (Kim et al., 2015; Jovancevic et al., 2017).

From the onset of ischemia to reperfusion and infarct healing, the immune response has a central role (Vilahur and Badimon, 2014; Ong et al., 2018). The delicate balance between pro-inflammatory and healing signals exerts a great impact on the outcome. As a matter of fact, several cardioprotective strategies have focused on the modulation of the immune response (Grilo et al., 2017; Andreadou et al., 2019; Zuurbier et al., 2019). Importantly, both pro- and anti-inflammatory signals are needed for the optimal resolution of MI (Yap et al., 2019; Sun et al., 2021). For the treated animals, we could detect several pathways related to the immune response enriched in the myocardium after MI. We further report, a diminished leukocyte infiltration following I/R for the treated animals. Amongst the immune response related

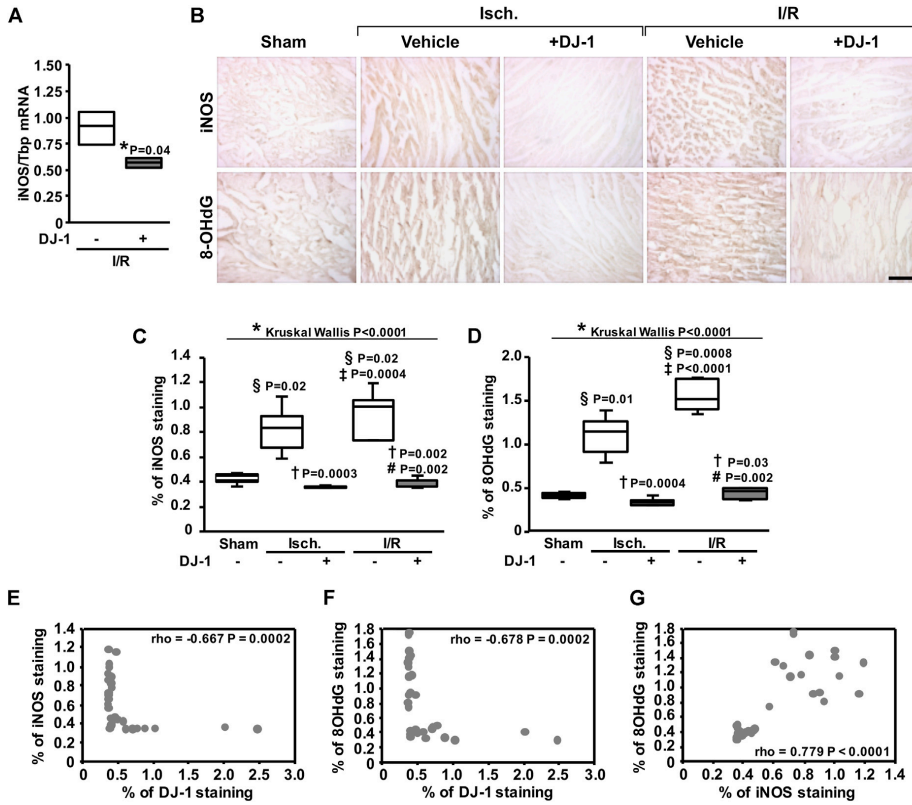


FIGURE 5
Systemic DJ-1 administration effects on oxidative stress after ischemia and ischemia/reperfusion. **(A)** *Inos* transcript analysis by qPCR. **(B)** Representative immunohistochemical acquisitions of myocardial iNOS (upper panel) and 8-OHdG (lower panel) after ischemia and ischemia/reperfusion in the presence and the absence of a systemic administration of DJ-1. A sham-operated group was included as baseline. **(C)** Myocardial iNOS content quantification. **(D)** Myocardial 8-OHdG content quantification. **(E–G)** Correlation analyses between iNOS and DJ-1 **(E)**, 8-OHdG and DJ-1 **(F)**, and iNOS and 8-OHdG **(G)** staining signals. Scale bar: 100 μm; *$p < 0.05$, Kruskal-Wallis; §$p < 0.05$ vs. sham, †$p < 0.05$ vs. ischemia without DJ-1, ‡$p < 0.05$ vs. ischemia + DJ-1, #$p < 0.05$ vs. I/R without DJ-1, Dunn's test; rho, Spearman rank correlation coefficient. Isch., Ischemia; I/R, Ischemia/Reperfusion.

pathways detected, the tumour necrosis factor α (TNFα) signaling pathway was the most enriched. Interestingly, TNFα signaling has been shown to display cardioprotection through NFκB-mediated cardiomyocyte expression of keratin-8 and keratin-18, which preserve the integrity and function of the intercalated discs and mitochondria upon stress (Papathanasiou et al., 2015).

Collectively our results illustrate a cardioprotective role for the exogenously administration of DJ-1 upon MI, potentially mediated by GPCRs signaling and the modulation of the immune response. The described extracellular activities of DJ-1 offer a therapeutic opportunity to limit I/R induced damage, which goes beyond MI. Whilst the present study provides the first line of evidence for an extracellular DJ-1 activity *in vivo*, the therapeutic potential of a systemic administration of recombinant DJ-1, requires further investigation regarding possible off-target activities of DJ-1. Also, long-term studies are needed to evaluate whether DJ-1 protective effects translate into the amelioration of adverse cardiac remodeling.

Limitations

The present study provides the first line of evidence on the infarct limiting effects of administering recombinant DJ-1 on a mouse model of acute myocardial infarction. This study needs further investigation to advance our understanding of DJ-1 target and off-target effects as well as the tentative side effects of recombinant DJ-1 preparations. A major concern for the clinical use of recombinant proteins is their potential to induce an unwanted immune response that may compromise safety. Furthermore, while infarct size is widely recognized as a major predictor of clinical outcomes, long-term effects have not yet been addressed. Further investigation, in appropriated models is warranted in order to assess whether the cardioprotective effects of DJ-1 administration are maintained in the long-term.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE66307.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committees (CEEA-IR Sant Pau) of ICCC-Hospital de la Santa Creu i Sant Pau.

Author contributions

Conceptualization, GV, TP, and LB; methodology, GV, TP, and LB; formal analysis and investigation, AG, and GM; animal experimentation, GM; *in silico* analysis, AG; writing—original draft preparation, AG; writing—review and editing, LB; funding acquisition, GV, TP, and LB; resources GV, TP, and LB; supervision, LB. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

LB received institutional research grants from AstraZeneca; consultancy fees from Sanofi, Pfizer and Novartis; speaker fees from Amarin, Lilly, Pfizer, and AstraZeneca. TP, GV and LB are shareholders of the academic spin-off companies GlyCardial Diagnostics SL and Ivestatin Therapeutics SL. All unrelated to the present work. LB, GV, and TP are authors of the patents EP3219326A1 and WO2017157958A1 regarding the use of DJ-1-derived polypeptides for the treatment of ischemia/reperfusion injury. AG and GM declare no conflict of interest.

Publisher's note

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5.4.- Article 4

DJ-1 regulates mitochondrial gene expression during ischemia and reperfusion

Alex Gallinat, Aleksandar Rakovic, Christine Klein and Lina Badimon

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Objective 4.- To characterize the functional implications of the RNA-binding activity of DJ-1 upon ischemia and reperfusion.

Highlights:

- DJ-1 preferably interacts with mitochondria-encoded RNA transcripts.
- The RNA-binding activity of DJ-1 notably increases during ischemia.
- DJ-1 deletion results in unstable hyper-fused mitochondria in normoxia that exhibit an increased fragmentation after ischemia/reperfusion.

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DJ-1 regulates mitochondrial gene expression during ischemia and reperfusion

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ABSTRACT

The early-onset Parkinson's disease protein DJ-1 is a multifunctional protein that plays a protective role against ischemia and reperfusion (I/R) injury and oxidative stress. Despite lacking a canonical RNA-binding domain DJ-1 exhibits RNA-binding activity and multiple transcripts have been identified. However, no functional characterization has been provided to date. Here, we have investigated the DJ-1-interacting transcripts, as well as the role of DJ-1 RNA-binding activity during ischemia and reperfusion. Among the identified DJ-1-interacting transcripts, we have distinguished a significant enrichment of mRNAs encoding mitochondrial proteins. The effects of DJ-1 depletion on mitochondrial protein expression and mitochondrial morphology were investigated using a CRISPR/Cas9 generated DJ-1 knockout (DJ-1^{KO}) cell model. DJ-1 depletion resulted in increased MTND2 protein expression in resting cells; however, after exposure to I/R, MTND2 levels were significantly reduced with respect to wild type cells. Increased mitochondrial fission was consistently found in DJ-1^{KO} cells after I/R exposure. *MTND2* transcript binding to DJ-1 was increased during ischemia. Our results indicate that the RNA-binding activity of DJ-1 shield mitochondrial transcripts from oxidative damage.

1. Introduction

Ischemic diseases, such as myocardial infarction (MI) and stroke, are the first cause of morbimortality worldwide. The early and successful recovery of blood supply (i.e., reperfusion) is virtually the only treatment for ischemia. However, at the time of reperfusion, there is a generation of reactive oxygen species (ROS) [1], which among other detrimental effects, promote the opening of the mitochondrial permeability transition pore and induce cell death [2,3]. The extracellular medium washout, pH recovery and the immune response also compromise viability upon reperfusion [2,3]. The reperfusion-associated injury significantly contributes to the extent of damage to the ischemic organ [4,5]. Given the limited regenerative capacity of the adult human brain and heart [6], controlling the ischemia-induced infarct size and reducing the reperfusion-induced organ damage is crucial to improve outcomes.

The early-onset Parkinson's disease protein DJ-1, encoded by the *PARK7* gene, is a multifunctional protein highly conserved from bacteria to humans [7]. Several functions have been proposed for DJ-1, including chaperone [8], protease [9], transcriptional regulator [10], redox sensor [11], mitochondrial homeostasis regulator [12,13] and cell signaling [14]. Over this plethora of functions, the exact role that DJ-1 plays in pathology remains elusive. Several studies have demonstrated a protective role against ischemia, ischemia-reperfusion (I/R), and oxidative stress [15]. Animals lacking DJ-1 exhibit larger infarcts, increased mitochondrial fission, and reduced heart function after MI compared to wild-type (WT) littermates [16–20]. Similar results were found for stroke models [21]. Conversely, DJ-1 overexpression is protective [17,19] and we have recently reported the reduction of myocardial infarct size upon systemic administration of recombinant DJ-1 [22].

Despite lacking a canonical RNA-binding domain, DJ-1 displays RNA-binding activity, as demonstrated by UV-cross-linked immunoprecipitation studies [23–25], and multiple transcripts have been identified [24]. In addition, DJ-1 was described as a regulatory sub-unit of a large RNA-binding complex [23]. Interestingly, the interaction between RNA and DJ-1 seems to be oxidation-dependent. So, DJ-1 may dissociate from RNA following oxidative stress, allowing translation, and

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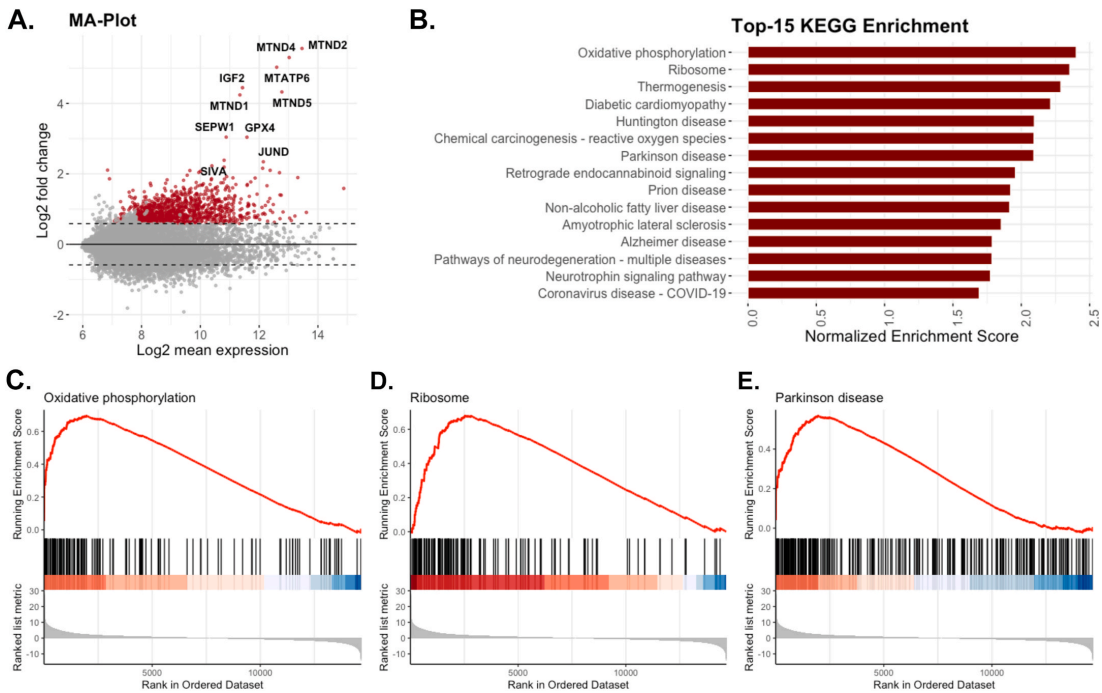

Fig. 1. Gene set enrichment analysis (GSEA) of DJ-1 interacting transcripts. **A.** MA-plot of normalized DJ-1 interacting transcripts. Coloring represents significant interaction. Top-10 DJ-1-interacting transcripts are labeled. **B.** Top-15 enriched gene sets detected. Normalized enrichment score is plotted. **C.-E.** GSEA plot for oxidative phosphorylation (**C.**), ribosome (**D.**), and Parkinson's disease (**E.**) gene sets. All gene sets available in the Kyoto encyclopedia of genes and genomes (KEGG) [31] were considered.

providing an additional mechanism to cope with stress [24,25]. In a different study, DJ-1 was found to interact with stress granules, reinforcing its possible role in regulating translation [26].

Here we have investigated DJ-1-interacting transcripts, as well as the biological implication of the DJ-1 RNA-binding activity in cells exposed to I/R.

2. Materials and methods

Cell culture – SH-SY5Y human neuroblastoma cells (ECACC, Public Health England, England, UK) were cultured in DMEM/F-12 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% (v/v) FBS (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in 5% CO_2 atmosphere.

CRISPR/Cas9 DJ-1 knockout cell line – DJ-1 knockout (DJ-1^{KO}) cells were kindly provided by Dr. Aleksandar Rakovic and Prof. Christine Klein [27]. Briefly, SH-SY5Y cultures were co-transfected with a spCas9 plasmid (Addgene, Watertown, MA, USA) alongside a modified pLKO.1 vector (pLKO1 Puro; Addgene, Watertown, MA, USA) expressing a gRNA targeting the sequence 5′-GTACAGTGTAGCCGTGATG-3′ from *PARK7* exon 3. Amaxa Cell Line Nucleofector Kit V (Lonza, Basilea, Switzerland) was employed for transfection following the manufacturer's instructions. After 24 h, effectively transfected cells were selected with puromycin (Thermo Fisher Scientific, Waltham, MA, USA) for 48 h and plated at a density of 1 cell/cm^2 for colony formation. Successful editing was assayed by *PARK7* exon 3 gene sequencing.

In vitro ischemia and reperfusion model – *In vitro* ischemia was performed as previously described [28,29]. Cells were incubated in acidic PBS (pH = 6.4) under hypoxic atmosphere (1% O_2) for the indicated times. Acidic PBS was supplemented with Ca^{2+} and Mg^{2+} to prevent cell detachment while under ischemic conditions. Restoring the cell culture to complete growth medium and normoxia was the *in vitro* modeling of reperfusion after ischemia.

RNA-binding in silico analysis – A publicly available dataset corresponding to the identification of DJ-1/RNA-immunoprecipitation captures in M17 human neuroblastoma cell line was employed [24] (Genomic Expression Omnibus reference code: GSE8632). Data were pre-processed using REA algorithm [30], which applies a Gaussian mixture distribution modeling to data, followed by a principal component analysis (PCA) to reduce the bias introduced by the immunoprecipitation performance. The first component of PCA was employed to rank genes for the gene set enrichment analysis (GSEA). All gene sets available in the Kyoto encyclopedia of genes and genomes (KEGG) [31] were considered. GSEA was performed with the R package 'ClusterProfiler' [32].

Western blot – Cell cultures were harvested by trypsinization and lysed in ice-cold radioimmunoprecipitation assay buffer supplemented with a protease inhibition cocktail (Roche Diagnostics, Mannheim, Germany). 25 μg of total protein were loaded and separated by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis in a Mini protean 3 system (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Ponceau S total protein staining (Sigma, Saint Louis, MI, USA) was employed to correct differences across lanes. Membranes were then blocked with 5% bovine serum albumin (NZYTech, Lisboa, Portugal) and incubated at 4 °C overnight with mouse anti-DJ-1 (MCA-

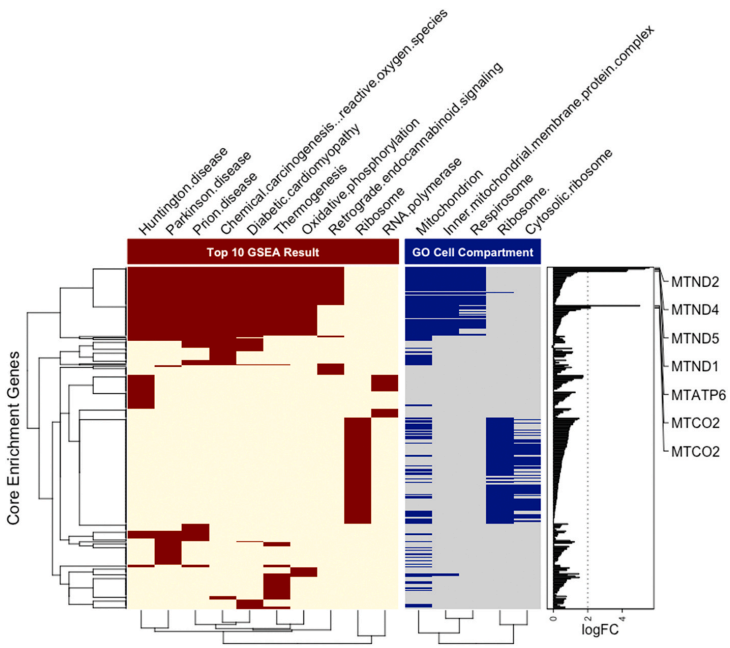

Fig. 2. Core enrichment analysis for the top-10 enriched gene sets. Overlap between gene subsets contributing the most to the enrichment signal of the top-10 enriched gene-sets is depicted as a binary heatmap and displayed alongside their associated Gene Ontology (GO) cell compartment terms, and log fold-change (red and blue coloring represents belonging to gene-set and GO terms, respectively). Fold-change is defined as the relative signal between immunoprecipitation captures and IgG control. Labeled transcripts exhibit a log fold-change greater than 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4H4; EnCor Biotechnology, Gainesville, FL, USA) at 1:4000 or rabbit anti-MTND2 (19704-1-AP; ProteinTech, Rosemont, IL, USA) at 1:1000. Horseradish peroxidase-coupled rabbit anti-mouse IgG or goat anti-rabbit IgG (P0260/P0448; Dako, Santa Clara, CA, USA) together with SuperSignal reagent (34076; Thermo Fisher Scientific, Waltham, MA, USA) were used to detect primary antibodies. Images were acquired with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA).

qPCR – Cells were harvested by trypsinization, and total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Real-time quantitative polymerase chain reaction (qPCR) with TaqMan assays (*PARK7*: Hs00994893_g1; *MTND2*: Hs02596874_g1; *rRNA-18S*: Hs99999901_s1; Thermo Fisher Scientific, Waltham, MA, USA) was performed as previously described [28].

RNA-immunoprecipitation – Cells were scraped and snap-frozen in lysis buffer (150 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM DTT. Samples were then thawed on ice, supplemented with Triton X-100 to a final concentration of 0.1%, and lysed with a 20G syringe. Lysates were incubated with 1:200 rabbit anti-DJ-1 (AP01249PU-N; OriGene, Rockville, MD, USA), with gentile rocking overnight at 4 °C, and precipitated with Protein G Sepharose beads (17-5280-04; GE Healthcare, Chicago, IL, USA) following manufacturer's instructions. Both free and DJ-1 bound RNA was then purified from immunoprecipitation captures and supernatants with QIAzol reagent (Qiagen, Hilden, Germany) and assayed by qPCR.

Mitochondrial morphology analysis – Cells were stained with Mito-Traker (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Four random acquisitions per sample were taken with a Leica SP-5 confocal system (Leica, Wetzlar, Germany) at maximum magnification and 0.5 μm z-stack step size [33]. Maximum projections were analysed in ImageJ [34]. ImageJ macro is provided as supplemental material.

Statistical analysis – As normality could be assumed (assessed by Shapiro-Wilk), statistical differences were analysed by Student's t-test, one-way ANOVA, or two-way ANOVA. Tukey's *post hoc* test was performed to correct significance for multiple comparisons. All analyses were performed in RStudio (RStudio, Boston, MA, USA).

3. Results

3.1. DJ-1 interacts with mitochondrial transcripts

The dataset generated by microarray hybridization of UV-cross-linked DJ-1/RNA-immunoprecipitation captures from M17 human neuroblastoma cell line (GSE8632) [24], was analysed following a GSEA approach [35]. Strong enrichments were found for oxidative phosphorylation and ribosomes, alongside Parkinson's disease (Fig. 1 and Supplemental Fig. 1 for a complete list of the enriched gene sets). Noteworthy, mitochondria-encoded genes were found among the top DJ-1-interacting transcripts. More specifically the mitochondria-encoded NADH:ubiquinone oxidoreductase core subunits *MTND2, MTND4, MTND5,* and *MTND1* exhibited the greatest fold-change, defined as the relative intensity between DJ-1 and IgG-control immunocapture signals (Fig. 1 A). The performed core enrichment analysis evidenced that a relatively small gene subset led the enrichment for most gene sets (Fig. 2). When combining the core enrichment analysis with Gene Ontology cell compartment terms for each gene, mitochondria, and particularly, inner mitochondrial membrane protein complexes, clearly defined the shared subset (Fig. 2).

3.2. MTND2 protein expression following I/R depends on DJ-1

To analyse the effect of DJ-1 on mitochondria-encoded protein expression, we used the SH-SY5Y human neuroblastoma DJ-1^{KO} model. Depletion of the *PARK7* gene completely abrogated DJ-1 protein expression (Fig. 3A–C). MTND2 protein expression was then analysed

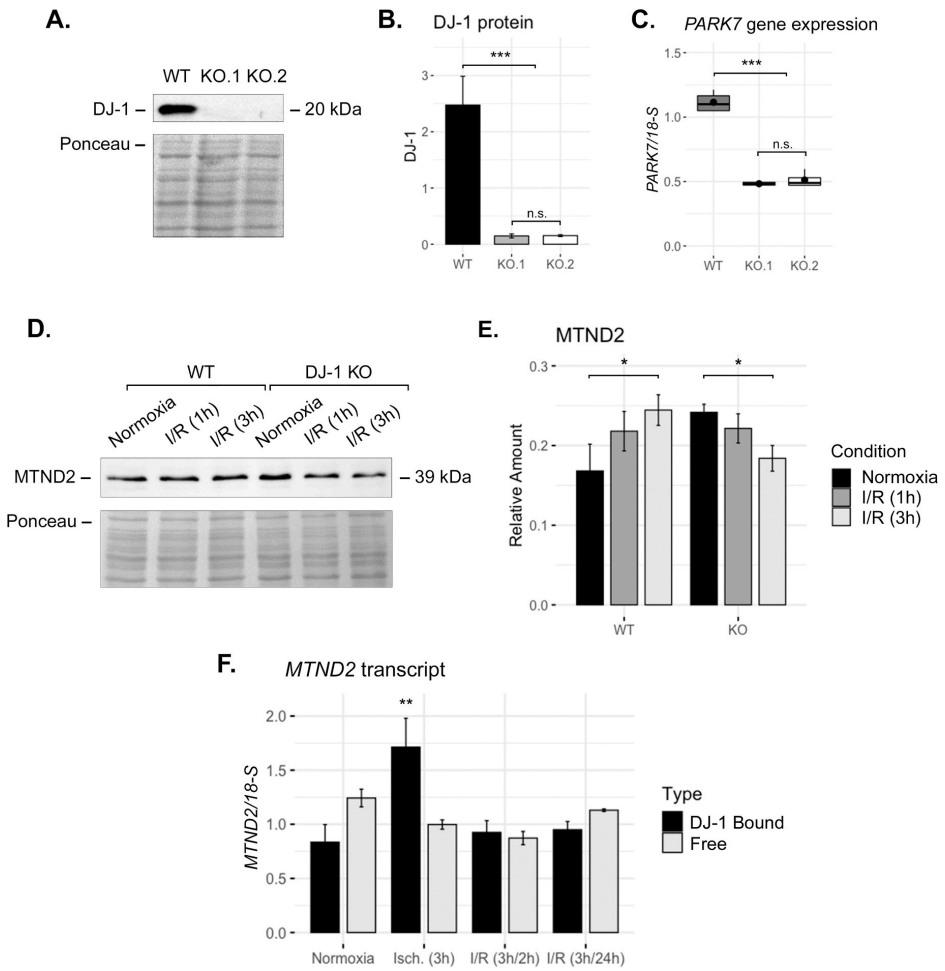

Fig. 3. MTND2 protein expression dynamics upon ischemia and reperfusion (I/R) in wild-type (WT) and DJ-1 knockout (KO) SH-SY5Y human neuroblastoma cell cultures. **A.-B.** DJ-1 protein expression of WT and KO cultures. Representative western blot (**A.**) and quantification (**B.**) are displayed. **C.** *PARK7* transcript expression analysis of WT and KO cultures. **D.-E.** MTND2 protein expression analysis upon I/R. Representative western blot (**D.**) and quantification (**E.**) are displayed. **F.** *MTND2* transcript analysis in WT cultures after DJ-1 immunoprecipitation. DJ-1 bound signal corresponds to immunoprecipitation captures, and free *MTND2* to DJ-1 depleted samples (immunoprecipitation supernatants). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Data presented as mean ± SEM.

when cells were exposed to I/R conditions. Two different times of ischemia were tested (1 h and 3 h), followed by 24 h reperfusion. MTND2 levels in resting cells were significantly higher in DJ-1^{KO} than in WT (Fig. 3 D and E); however, following exposure to I/R conditions, MTND2 levels increased in WT cells but decreased in DJ-1^{KO} cells (Fig. 3 D and E). The immunoprecipitation of DJ-1/RNA complexes showed that while the amount of *MTND2* free transcript remained constant, the DJ-1-bound fraction nearly doubled in ischemia in the WT cells (Fig. 3 F).

3.3. DJ-1 depletion results in unstable hyper-fused mitochondria

Mitochondrial morphology was also analysed. DJ-1^{KO} cells showed hyper-fused mitochondria, depicted by increased mitochondrial length and number of junctions in normoxia (Fig. 4). Furthermore, while no differences were found in WT cells after exposure to I/R conditions, both mitochondrial length and junction number were significantly reduced in DJ-1^{KO} cells.

4. Discussion

The early and successful recovery of blood supply to ischemic organs is virtually the only treatment to reduce organ damage. However, the needed reperfusion compromises cell function. The biochemical mechanisms at play during this reperfusion period are not fully understood. DJ-1 could be a key molecule in this process because DJ-1 mitigates the injury induced by myocardial infarction [22], a process in which oxidative stress has a central role [1]. DJ-1 was reported to interact with mRNA and promote translation upon oxidative stress [24,26]. Thus, in normal conditions, DJ-1 could withhold interactive transcripts from

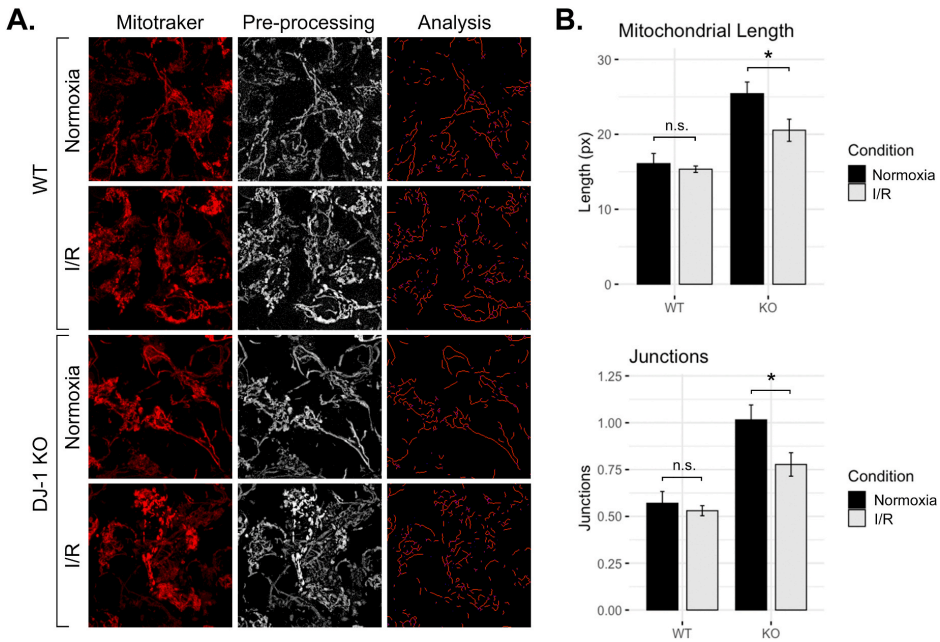

Fig. 4. Mitochondrial morphology analysis upon ischemia and reperfusion (I/R) in wild-type (WT) and DJ-1 knockout (KO) SH-SY5Y human neuroblastoma cell cultures. **A.** Representative acquisitions of raw Mitotracker staining signal, pre-processing result, and analysis input. **B.** Average mitochondrial length (upper panel) and junctions (lower panel) upon I/R in WT and KO cultures. $^{*}p < 0.05$; n.s., not significant. Data presented as mean ± SEM.

translation, releasing them upon increased ROS levels. The contribution of DJ-1 RNA-binding activity in the regulation of the cell responses to ischemia and reperfusion conditions is however unknown.

While several transcripts were identified to interact with DJ-1 [24], no functional characterization was defined. We analysed the DJ-1-interacting transcripts and found a significant enrichment of mitochondria-encoded genes, of which *MTND2* exhibited the highest affinity. Interaction with nuclear-encoded transcripts was also found, but again nuclear-encoded mitochondrial genes showed the highest enrichments. These results match with functions adjudicated to DJ-1, such as the regulation of mitochondrial function [36], the mitochondrial import in stress [37], the boosting of both mitochondrial [38] and ectopic ATP-synthase activity [28], and the protection of the electron transport chain (ETC) complex-I integrity [12,13]. The RNA-binding activity of DJ-1 may explain some of these functions.

Mitochondria are the primary source of ROS through electron leakage and reverse electron transport (RET). While ischemia completely disrupts the ETC, a non-canonical Krebs cycle leading to succinate accumulation remains active. Reperfusion restores ETC activity, and the accumulated succinate is oxidized, reducing the ubiquinone pool and increasing the mitochondrial potential [39]. As ADP availability is minimal following ischemia, the ETC collapses, and RET through the complex-I is promoted, generating ROS [40,41]. MTND2 subunit encompasses the main superoxide production site at complex-I, thus being particularly susceptible to oxidative damage [42]. The mitochondrial genome encodes for 13 proteins, of which 7 belong to the ETC complex-I. Therefore, DJ-1 detachment from RNA could speed up the translation of transcripts counteracting ROS effects. Indeed RNA-binding proteins play a pivotal role in regulating mitochondrial RNA translation, stability, and degradation [43].

DJ-1 depletion, using the CRISPR/Cas9-generated DJ-1^{KO} model, has effects on the expression of interacting transcripts when these cells are exposed to I/R conditions. While MTND2 protein expression tends to increase after I/R in WT cells, MTND2 protein expression decreased significantly in DJ-1^{KO} cells, supporting the initial hypotheses. Interestingly, MTND2 levels in resting normoxic cells were higher in DJ-1^{KO} cells than in WT cells, which is consistent with a putative translational inhibitory effect of DJ-1 in the absence of stress. However, the RNA-immunoprecipitation analysis revealed that rather than dissociating from RNA, *MTND2* transcript binding to DJ-1 significantly increased during ischemia. DJ-1 has three cysteine residues, conferring the ability to quench ROS [44]. Thus, our results suggest that while DJ-1 binding to RNA may slow down translation, as shown by the increased MTND2 levels in DJ-1^{KO} cells, increased RNA binding during ischemia may protect the RNA from oxidative damage.

Additionally, effects of DJ-1 depletion were noticed in the mitochondrial morphology upon exposure of cells to I/R conditions. Similarly, to MTND2 dynamics, DJ-1 depletion resulted in unstable hyperfused mitochondria. While DJ-1^{KO} cells exhibited elongated mitochondria in resting normoxic conditions, they fragmented after exposure to I/R. Instead, WT cells exhibited a stable mitochondrial reticulum not affected by exposure to I/R conditions.

5. Conclusions

The data here presented supports a role for DJ-1 in regulating mitochondrial gene expression and stability during the exposure to ischemia and reperfusion.

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Declaration of competing interest

LB received institutional research Grants from AstraZeneca; consultancy fees from Sanofi, Pfizer, and Novartis; speaker fees from Sanofi and Novartis. LB is a shareholder of the academic spin-off companies GlyCardial Diagnostics S.L. and Ivestatin Therapeutics S.L. All unrelated to the present work. LB is the author of the patents EP3219326A1 and WO2017157958A1 regarding the use of DJ-1-derived polypeptides for treating ischemia/reperfusion injury. AG declares no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.10.315.

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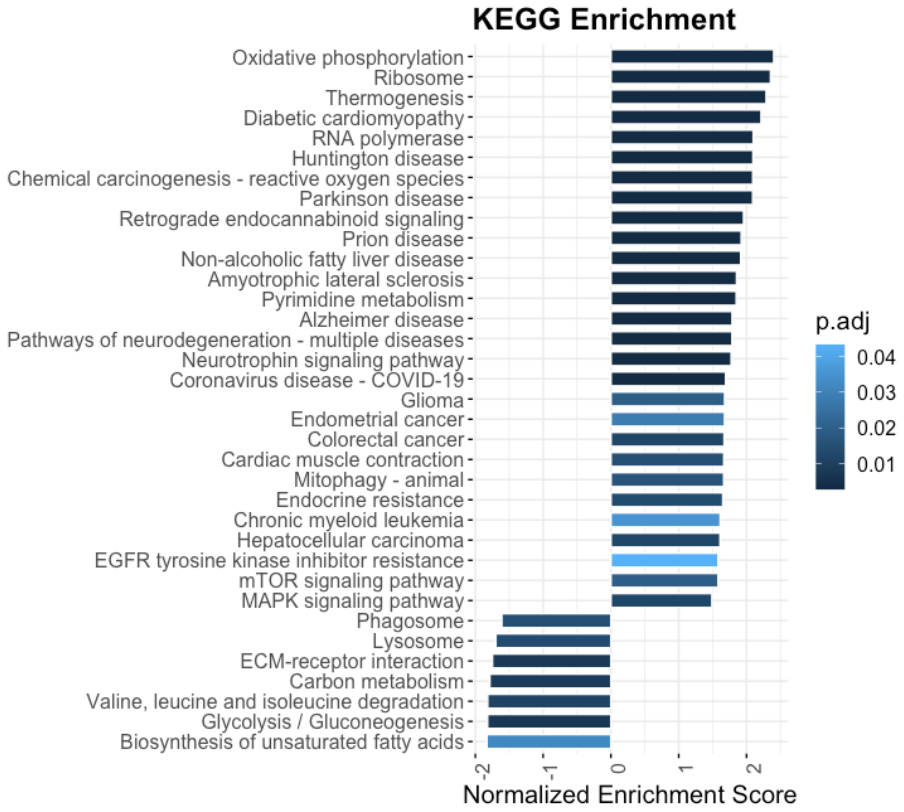
Supplemental Figure 1 – Complete gene set enrichment analysis result for DJ-1-interacting transcripts.



Supplemental File 1 – ImageJ macro employed for the analysis of mitochondrial morphology.

```
// This ImageJ macro has been generated by Alex Gallinat at
Prof. Lina Badimon's Lab //

macro "Mitochondrial Morphology Analysis" {

run("Set Scale...", "distance=0 known=0 pixel=1
unit=pixel");
run("Set Measurements...", "area shape redirect=None
decimal=2");
setOption("BlackBackground", true);
run("Clear Results");

setBatchMode(true);

rename("MAX_1");
run("8-bit");
selectWindow("MAX_1");

run("Subtract Background...", "rolling=5");
run("Apply LUT");
run("Enhance Contrast", "saturated=0.5 normalize");
run("Enhance Local Contrast (CLAHE)", "blocksize=127
histogram=256 maximum=3 mask=*None*
fast_(less_accurate)");
run("Tubeness", "sigma=1.0000 use");
run("8-bit");
run("Subtract...", "value=25");
run("Multiply...", "value=2");
run("Gaussian Blur...", "sigma=0.5");
run("Duplicate...", "Mask");
run("Bandpass Filter...", "filter_large=100 filter_small=7
suppress=None tolerance=5 autoscale saturate");
setAutoThreshold("MinError dark"); // Threshold to create
a cells broad selection.
run("Convert to Mask");
run("Invert");
run("Create Selection");
selectWindow("tubeness of MAX_1");
run("Restore Selection");
setForegroundColor(255, 255, 255);
setBackgroundColor(0, 0, 0);
run("Clear", "slice");
run("Select None");
setAutoThreshold("Li dark"); // Threshold to create
mitochondrial selection.
```

```
run("Convert to Mask");
run("Analyze Particles...", "size=0-9 display clear
include add");
setForegroundColor(0, 0, 0);
setBackgroundColor(255, 255, 255);
roiManager("Fill");
run("Skeletonize (2D/3D)");
run("Analyze Skeleton (2D/3D)", "prune=none");

for (i = 0; i < nResults(); i++) {
    v = getResult("# Branches", i)*getResult("Average
Branch Length", i);
    setResult("Length", i, v);
}

updateResults();

close("*");

run("Read and Write Excel", "stack_results");

setBatchMode(false);

}
```

6. Discussion

6.- Discussion

Cardiovascular diseases (CVDs) are a major cause of mortality and morbidity worldwide, being acute myocardial infarction (AMI) one of the main contributors to morbi-mortality [1–3]. Despite AMI mortality has dropped within the past decades, as a result of the improvement of reperfusion therapies [9,10], the consequent adverse remodeling of the heart and scar formation entail a high risk of developing heart failure for AMI survivors [11,12]. Given the minimal regenerative capacity of the adult human heart, infarct size becomes the major predictor of AMI clinical outcomes [6–8]. Currently, the only way of limiting infarct size is the early and complete restoration of coronary blood flow (i.e. reperfusion). However, the sudden re-introduction of oxygen at reperfusion, along with the extracellular medium washout and the triggered immune response paradoxically exacerbate damage in a process that is known as ischemia and reperfusion (I/R) injury [31,32]. Although the severity and duration of ischemia are the major contributors to infarct size, the damage associated with reperfusion significantly contributes to the final infarct size, therefore limiting the beneficial effects of reperfusion. The discovery of the conditioning phenomena, referring to the acquired cardioprotection arising from the application of transient cycles of ischemia and reperfusion, evidenced the existence of endogenous cardioprotective programs worth exploring. However, although broadly proven in animal models, clinical trials on ischemic conditioning techniques have brought poor, even futile, results [83–85]. Differences in the employed conditioning protocols, patient medications, comorbidities, and inclusion criteria are believed the main sources of disparity [86–88].

Over the past decades, the deciphering of the operating mechanisms behind cardioprotection has been a matter of intensive research, and a number of molecules and signaling pathways have been described to participate. Some of the identified players are susceptible to modulation with drugs, introducing pharmacologic conditioning as a new approach for cardioprotection. Pharmacologic conditioning offers the possibility to elude variation arising from conditioning protocols and may be a powerful approach to limit infarct size. However, whilst a huge number of compounds have proven cardioprotective in animal models, most failed at

demonstrating clinical benefits [14,15]. Reasons for this failure may arise from intrinsic limitations of the experimental models. As a matter of fact, pre-clinical research is usually carried out in healthy animals, lacking an underlying pathology, risk factors, and medications. Furthermore, knowledge gaps in the understanding of endogenous cardioprotective programs confound translation.

Although several receptors, pathways, and end-effectors have been so far identified as players of the endogenous cardioprotective program, a linear conception of cardioprotection may be too simplistic [90]. Indeed, cell signaling results from the interaction of input cues with dynamic and context-specific networks composed of a number of signaling molecules that collectively coordinate a cell response [270]. Despite reductionist models have helped in the understanding of the molecular basis of the process, interactions among signaling molecules and how they are affected by an underlying pathology seem to have been overlooked. Therefore, we firstly investigated whether a network biology approach would provide additional insights into the comprehension of the endogenous cardioprotective program. To this end, we have focused on the characterization of the mitochondrial proteomic response to ischemic post-conditioning (IPostC).

Mitochondria are central organelles in ischemia, reperfusion, and cardioprotection. As metabolic hubs, mitochondria provide the major source of ATP by oxidative phosphorylation (OXPHOS), which is tightly dependent on the O_2 availability and nutrient supply. Therefore, blood flow interruption during ischemia greatly impacts mitochondrial function, compromising the correct coupling of all metabolic reactions and ATP production. From the ionic unbalance to necrosis, most of the detrimental events following the onset of ischemia arise from the depletion of ATP. Whilst reperfusion is the only possible solution to this situation, the reintroduction of O_2, and the restoration of the electron transport chain (ETC), yield a reactive oxygen species (ROS) burst, that again compromises the mitochondrial compartment. Rather than just the 'powerhouse of the cell', mitochondria further play significant roles in signaling, cell cycle regulation, and apoptosis [271]. On the whole, the mitochondrial response and adaptation to ischemia and reperfusion determines cell fate after I/R, and consequently the damage extent. Not surprisingly,

nearly all identified signaling pathways involved in cardioprotection eventually converge in the mitochondria [16,272]. Hence, changes in the mitochondrial proteome following cardioprotective interventions portray the end-effectors of cardioprotection.

Within the performed network analysis, a tight correlation between network topology and cell function was evidenced. Strong enrichments were found for the OXPHOS system and the tricarboxylic acid (TCA) cycle, which is consistent with the known drivers of I/R injury [28].

Whilst members of the ETC complexes I and III were found down-regulated following I/R, they were strongly up-regulated when IPostC was applied. The ETC complexes I and III comprise the main ROS generation sites at the ETC, through reverse electron transport (RET) and electron leakage [273–275]. Therefore, their up-regulation following IPostC could reflect either a better handling of ROS at reperfusion or *de novo* protein synthesis. Such up-regulation was maintained when compared to the proteome of sham-operated animals, which suggests *de novo* synthesis. Consistently, the mitochondrial inner membrane protein (OXA1L) which is required for the correct insertion and assembly of inner mitochondrial membrane complexes [276,277], was found concomitantly up-regulated.

Further metabolic effects of IPostC were evidenced by a wide up-regulation of proteins belonging to the TCA cycle, and the coupling of the fatty acids oxidation (FAO) to the ETC; more specifically, the electron transfer flavoprotein subunits ETFB and ETFDH. While the proteomic signature of the myocardium at-risk following I/R may be explained both by the metabolic adaptation to ischemia and oxidative damage, the results found for IPostC suggest a metabolic re-programming. Altogether highlighting cardiac metabolism as an important driver for cardioprotection.

The analysis of the mitochondrial proteomic response to IPostC also evidenced a subset of proteins not related to cell metabolism that were differentially expressed following IPostC. From them, the voltage-dependent anion-selective channel 2 (VDAC2) and DJ-1 exhibited the highest fold-change both compared to the I/R and

sham groups, and have been previously related to cardioprotection [259,278]. Some of the functions previously described for DJ-1 are in line with the IPostC detected effects. This is the case of the regulation of the antioxidant response, the preservation of the ETC complex I under stress, and the maintenance of mitochondrial homeostasis. Noteworthy, the mitochondrial stress-70 protein (HSPA9; also known as Grp75), which is necessary for the mitochondrial import of DJ-1 during stress [214], was also up-regulated following IPostC.

Despite a protective role for DJ-1 over ischemia and I/R being widely recognized [259], the mechanism by which DJ-1 confers protection is still unclear, and many molecular activities have been proposed. Then, our next step was to characterize the dynamics of protein DJ-1 across ischemia and I/R. Although still in debate, the cleavage of the 15 carboxyl-terminal amino acids of DJ-1 (referred to as DJ-1ΔC) has been proposed as an activating mechanism [177,193,216]. Therefore, we analyzed both the full-length and the cleaved forms of DJ-1. For this part, we chose an endothelial *in vitro* model of ischemia and I/R. Endothelial cells (ECs) line the entire circulatory system and play important roles in the maintenance of heart homeostasis. Rather than just a barrier, ECs provide multiple autocrine and paracrine signals to support organ function [67,68,279]. Also, their specific location, between the bloodstream and the organ tissues, makes ECs key sensors and responders to hemodynamic alterations and ischemia, acting as an important source of autacoids in ischemic conditioning [67,111].

After evaluating the impact of ischemia and I/R upon the intracellular DJ-1 content, we described a significant and ischemia-dependent decline of the full-length form of DJ-1, further associated with secretion. Both forms of DJ-1 were detected within the analysis of the secretome. Whilst the full-length form of DJ-1 was detectable in the secretome of all tested conditions, its secretion was significantly promoted during ischemia and reperfusion. For the cleaved form, it was only detected in the secretomes associated with I/R, but never in the controls. Although lacking a conventional secretory signal peptide, DJ-1 has been described to be secreted under several pathologic conditions such as breast cancer [264], Parkinson's disease [265,280], and stroke [266]. However, as for the intracellular forms, the

extracellular activities of DJ-1 have not yet been elucidated. Previous *in vitro* experimentation using autophagy inhibitors and genetic models has proposed the autophagy-based unconventional secretory pathway as the mechanism for secretion [281]. Given that ischemia is a known autophagy-promoting stimulus, this could be a feasible mechanism to explain our observations.

To date, there are no known extracellular receptors for DJ-1, and most of the DJ-1 interacting proteins identified by proteomic techniques are soluble proteins, except for the F_0F_1 ATP-synthase [215,282]. Despite being mainly expressed in the mitochondria as an essential member of the OXPHOS system, the F_0F_1 ATP-synthase has been recognized to be ectopically expressed in the cell surface of many cell types. These include vascular ECs [283], hepatocytes [284], adipocytes [285], lymphocytes [286], keratinocytes [287], neurons [288], and muscular cells [289]. The ectopic ATP-synthase has proven active, thus allowing the extracellular generation or degradation of ATP [290]. It is primarily located in the lipid rafts and caveolae, which suggests a role in cell signaling [283,285,291]. It has been proposed to be functionally connected with purinoreceptors through the modification of local ATP/ADP concentrations [291]. Also, it has been identified as a receptor for angiotensin in ECs [290], and for apolipoprotein A-I in hepatocytes [284]. Experiments with specific antibodies targeted to the ectopic ATP-synthase have depicted a role in angiogenesis, proliferation, and the regulation of intracellular pH [292–294].

The ATP-synthase of either location mechanistically operates as a H^+ channel, therefore, the activity within the plasma membrane may provide an additional mechanism to regulate intracellular pH [291]. Given that ischemia rapidly leads to acidosis, and that acidosis is an important driver of the ionic unbalance of ischemic cells, the ectopic ATP-synthase activity has been proposed as a mechanism of ischemia tolerance for ECs [295]. Indeed, the exposure of cells to an acidic and hypoxic environment (i.e. ischemia-like conditions) promotes ectopic ATP-synthase activity [296].

Intracellular DJ-1 is known to directly interact with the mitochondrial F_0F_1 ATP-synthase, and to optimize its performance [215]. Hence, DJ-1 secretion in ischemia

may be functionally related to the ectopic ATP-synthase, acting as an autocrine/paracrine modulation factor, and promoting H^+ extrusion and/or downstream signaling. In order to test this hypothesis, we have studied the effect of both DJ-1 gene silencing, and the exposure to recombinant DJ-1/DJ-1ΔC, upon the extracellular ATP generation in ischemic ECs. As a result, while ischemia led to a significant increase in the ectopic ATP-synthase activity, the silencing of the DJ-1 gene abrogated the effect. Consistently, the exposure of ECs to recombinant DJ-1/DJ-1ΔC during ischemia greatly increased the extracellular ATP generation, with no regard to the employed DJ-1 form. Immunoprecipitation studies proved physical interaction between the administrated DJ-1 forms and ATP-synthase. The functional implications of the reported association were further investigated, and a role in preserving endothelial function upon reperfusion was evidenced by a higher induction of Akt signaling and a faster formation of capillary-like structures after ischemia. Importantly, all the reported effects were found independent of cell death, as proven by the analysis of caspase-3 cleavage, where no induction of apoptosis was detected at any condition.

In summary, our results collectively suggest the use of recombinant DJ-1 as a possible treatment for ischemia and I/R. Therefore, the next part of this study was the evaluation of the therapeutic potential of recombinant DJ-1 for myocardium salvage following AMI. To this end, we employed a mice model of AMI and explored the effects of an intraperitoneal administration of DJ-1 upon infarct size and the myocardial transcriptional response to reperfusion.

With this model, we have evidenced a significant amelioration of infarct size associated with the treatment. Interestingly, we also observed a reduction of infarct size in the treated animals that were subjected to ischemia alone, suggesting that the extracellular DJ-1 effects may span beyond reperfusion. A major driver of necrosis during ischemia is the ionic unbalance [28]. The metabolic reprogramming associated with ischemia implies the over-production of H^+, due to anaerobic glycolysis. These H^+ are then extruded by the Na^+/H^+ passive exchangers, coupled with the entry of Na^+. When ATP pools are critical, the Na^+/K^+ pump inactivates, and Na^+ accumulates in the cytoplasm. In the absence of a functional Na^+/K^+ pump, the

Na^+/Ca^{2+} exchangers allow the extrusion of Na^+ coupled to the entry of Ca^{2+}. In a normal situation, cytosolic Ca^{2+} is sequestrated within the sarcoplasmic reticulum in an ATP-dependent process. The lack of ATP during ischemia thus impedes Ca^{2+} sequestration, and both Ca^{2+} and Na^+ accumulate in the cytoplasm, progressively leading to intracellular edema and necrosis [28,29]. Against this background, the ectopic ATP-synthase may provide an additional mechanism for H^+ extrusion not disturbing the ionic balance. Therefore, the DJ-1-associated reduction of infarct size during ischemia may arise from the modulation of the ectopic ATP-synthase activity. However, further research is needed in order to evaluate the effects of ectopic ATP-synthase regulation *in vivo* within the context of ischemia and I/R.

The transcriptomic analysis revealed that a systemic administration of DJ-1 modified the myocardial response to reperfusion. Interestingly, we detected additional pathways to those previously associated with DJ-1, reinforcing a different role for the intracellular and extracellular forms. Importantly, G-protein coupled receptors (GPCRs) mediated signaling and immune response-related gene sets encompassed the highest enrichments.

GPCRs signaling is closely connected to cardioprotection, playing a central role in triggering the cardioprotective response following ischemic pre-conditioning, and IPostC. Local conditioning techniques induce the release of autacoids which then activate GPCRs, downstream triggering an endogenous cardioprotective program [89,90]. Several genes belonging to the GPCRs family were found up-regulated following reperfusion in the treated hearts. However, the mechanism by which extracellular DJ-1 promotes GPCRs signaling requires further investigation.

The immune response is central to infarct healing [5,47,48]. From the onset of reperfusion, the released necrotic products trigger a profound inflammatory response causing the early recruitment and activation of neutrophils and mononuclear cells. Whilst their main purpose is the removal of dead cells and matrix debris, the activation of immune cells (especially neutrophils) couples to the production and release of further ROS and proteases, which paradoxically aggravate damage [297]. Beyond the acute phase of AMI, the immune response is a major contributor to scar formation, thus being a major determinant of adverse cardiac

remodeling [49]. The existing balance between pro-inflammatory and healing signals determines the extent of activation and the nature of this immune response, greatly affecting the outcome [298]. Several immune response-related gene sets were detected significantly enriched within the transcriptomic analysis of the reperfused myocardium of the treated animals. The effects on the immune response were then functionally validated. We report diminished leukocyte infiltration and oxidative stress, associated with DJ-1 administration. Consistently, a reduction in the extent of myocardial apoptosis following AMI was also detected in the treated animals.

The potential for recombinant proteins to trigger an undesired immune response that may compromise safety is a serious concern for their clinical application. Thus, although this part of the study provides the first line of evidence for the therapeutic potential of recombinant DJ-1 to treat AMI, further research is needed to evaluate safety. Also, long-term effects upon adverse cardiac remodeling and angiogenesis are yet to be characterized.

Beyond the extracellular activities of DJ-1 here discussed, a body of evidence has linked the intracellular form of DJ-1 with oxidative stress, I/R, and cell protection [216,257,260–263]. Over the past decades, the deciphering of the intracellular activities of DJ-1 has been a matter of intense research, and multiple functions have been reported [176–182]. However, lacking a model conceiving the links and relationships across all reported functions, the comprehension of the role that DJ-1 plays in health and disease is still difficult to recognize. It is a possibility that some of the molecular activities attributed to DJ-1 result from a yet-to-be-understood upstream function. In such a way, we hypothesized that the reported RNA-binding activity of DJ-1 may provide some integrative view.

Despite lacking a conventional RNA-binding domain, DJ-1 has been reported to bind RNA, and multiple targets have been identified by cross-linked immunoprecipitation (CLIP) studies [225–227]. Such interaction has been described to exert an inhibitory effect upon translation and to be dependent on the oxidation status of DJ-1. Hence, DJ-1 would retain a pool of RNAs away from translation, and release them under oxidative stress, providing an additional

mechanism to cope with stress [226,227]. However, no functional characterization of DJ-1 interacting transcripts has been provided so far.

We have analyzed a publically available dataset resulting from the microarray identification of DJ-1 RNA-immunoprecipitation captures, and found great enrichments for OXPHOS and ribosome associated transcripts. A closer look at the genes encompassing these enrichments revealed that mitochondrial proteins, especially the mitochondria-encoded ones, were the most representative. Being the mitochondria-encoded NADH:ubiquinone oxidoreductase core subunit 2 (MTND2) the one exhibiting the highest affinity for DJ-1. In order to analyze the effect of DJ-1 upon mitochondria-encoded protein expression, we employed a CRISPR/Cas9-generated DJ-1 knockout (KO) *in vitro* model of ischemia and I/R. Because the original dataset was generated in human neuroblastoma cells [226], a similar cell line was chosen for the validation experiments. More specifically, we employed the SH-SY5Y human neuroblastoma cell line. And found that whilst MTND2 protein levels were basally higher for the DJ-1 KO cultures, they significantly fall after reperfusion. Instead, for the wild-type (WT) cultures, I/R induced the expression of MTND2 protein. Consistently, within the first study of this thesis, both MTND2 protein and DJ-1 were found up-regulated in the myocardium at risk of post-conditioned pigs.

These results seemed to match the original model, in which DJ-1 would retain a pool of RNAs away from translation until there is oxidative stress [226]. However, the RNA-immunoprecipitation study revealed that the amount of *MTND2* transcript bound to DJ-1 significantly increased during ischemia. Our results, then, suggest that while RNA binding to DJ-1 may prevent translation, it also may protect the RNA from oxidative damage and degradation.

This pattern of function resembles the dynamics of stress granules (SGs), with which DJ-1 reportedly interacts [228]. Those are cytoplasmatic granules composed of messenger RNAs stalled from translation and proteins, that rapidly assemble during stress [299,300]. Although the function of SGs is largely unknown, they are involved in cell survival, and a tentative functional role in protecting RNA from degradation upon stress has been hypothesized. SGs are enriched in RNA-binding proteins which

are involved in the assembly [299,300]. In such a way, DJ-1 could act as a factor targeting mitochondrial transcripts to SGs.

Moreover, we have explored the effect of DJ-1 depletion and I/R on mitochondrial morphology and found that the depletion of DJ-1 resulted in unstable hyper-fused mitochondria, that fragmented after the exposure to I/R. Instead, no effects on mitochondrial morphology were detected for WT cultures after I/R.

Amongst all the molecular functions previously associated with DJ-1, the major consensus entails redox sensing and the maintenance of mitochondrial homeostasis upon stress. Nevertheless, the participation of DJ-1 in mitochondrial stability could be conceived as the combination of several other molecular activities that DJ-1 plays in the mitochondria. This is the case of the interaction with the mitochondrial F_1F_0 ATP-synthase and the mitochondrial complex I, the regulation of the antioxidant response, and ROS quenching. Our results on the functional perspective of the RNA-binding activity of DJ-1, add another aspect for DJ-1 participation in the maintenance of mitochondrial homeostasis.

Overall, our studies support a protective role for DJ-1 both in ischemia and reperfusion. So far, we identified the involvement of DJ-1 in the cardioprotection triggered by IPostC, characterized both an extracellular activity of DJ-1, and the functional identity of DJ-1-binding transcripts, and provided a tentative therapeutic application to limit infarct size after AMI. Although many questions regarding the function and regulation of DJ-1 remain to be elucidated, these results advance our understanding of the endogenous cardioprotective programs and propose new tentative therapeutic approaches to protect the heart from ischemia and reperfusion damage.

7.

Conclusions

7.- Conclusions

The conclusions of this thesis are the following:

1. Network biology provide a powerful approach for the understanding of complex biological processes such as ischemia and reperfusion (I/R), and the endogenous cardioprotective programs.
2. Whilst the myocardium retains an ischemia-adapted metabolism after I/R, ischemic post-conditioning induce a metabolic reprogramming together with the up-regulation of the cardioprotective proteins DJ-1 and VDAC2.
3. Both DJ-1 and DJ-1ΔC are secreted during ischemia by endothelial cells (ECs), and act as autocrine and/or paracrine factors to regulate the activity of the ectopic ATP-synthase in ischemia.
4. The axis DJ-1/ectopic ATP-synthase plays a role in maintaining ECs function during reperfusion.
5. A systemic administration of recombinant DJ-1 prior the induction of acute myocardial infarction ameliorates infarct size, leukocyte infiltration, and oxidative stress following ischemia and I/R in a mouse model.
6. Intracellular DJ-1 displays an increased RNA-binding activity during ischemia, preferentially binding mitochondria-encoded RNA transcripts.

Collectively, our findings highlight the implication of DJ-1 in the molecular basis of ischemic post-conditioning and cardioprotection, and offer new therapeutic opportunities to limit infarct size.

8. References

8.- References

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