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Mitochondrial stress signaling during ischemia reperfusion injury: The roles of Mitochondrial Ca2+ uptake, dynamics, and cell death

Introduction:

Mitochondria are intracellular organelles that consist of two membranes, the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM), and the intermembrane space between them. Mitochondria are responsible for producing ~90% of cellular energy in the form of ATP, therefore it is known as the "powerhouse" of the cell. Beyond ATP production, mitochondria maintain ion homeostasis, produce precursors for macromolecules, such as lipids, proteins, and DNA, and generate and sequester potentially damaging metabolic byproducts such as ammonia and reactive oxygen species (ROS). The heart is the high energy consuming organ that utilizes ATP to maintain myofibrillar contractility through the actin-myosin ATPase as well as cellular ion homeostasis and different anabolic processes[20]. Hence, the heart exhibits a rapid response to oxygen deprivation and mitochondrial dysfunction induced by oxidative and energy stresses.

The heart contains up to 5,000 mitochondria per cell, making it one of the most mitochondria abundant tissues in the body[47]. There are three different types of mitochondria based on their localization in subcellular compartments: the interfibrillar, subsarcolemmal, and perinuclear mitochondria[26]. These organelles are strategically located to meet the energetic demands of high ATP-consuming tissues, particularly, the heart. The mitochondria produce energy through five protein complexes located in the IMM. The electron transport chain (ETC), composed of complexes I-IV, is responsible for transporting electrons from NADH (complex I) or FADH2 (complex II) to oxygen at complex IV. Transfer of electrons through the respiratory chain is accompanied by pumping H+ by complexes I, III, and IV from the mitochondrial matrix to the intermembrane space generating an electrochemical gradient or the proton motive force

across the IMM. The proton motive force is used to produce ATP by FOF1-ATP synthase (or complex V), the process known as oxidative phosphorylation (OXPHOS). Adenine nucleotide translocase is the ADP/ATP transporter which exchanges the matrix ADP for ATP in the intermembrane space. The inorganic phosphate carrier is responsible for the import of Pi and H+ to support the OXPHOS.

Under physiological conditions, mitochondrial function works to maintain the energetic demands of cardiac activity. However, after an ischemia/reperfusion (IR) event, mitochondrial function becomes compromised, thus no longer capable of supporting the heart. Cardiac IR injury is divided into two phases: ischemic phase, which is when any reduction in blood flow resulting in decreased oxygen and nutrient supplies to a tissue and reperfusion phase, which is the restoration of oxygen and nutrient supplies to a tissue. During ischemia, cardiac cells attempt to survive by producing energy via anaerobic respiration, however this is at the cost of creating a more acidic environment. In addition, the lack of O2 damages the mitochondria's ability to produce ATP, therefore reducing the ionic transport capacity that inevitably leads to an imbalance of ionic homeostasis (specifically Ca2+). During reperfusion, mitochondria begin to produce ROS due to damage induced on the ETC. Moreover, the mitochondria try to correct the excess of Ca2+ in the cytosol, which leads to a pathological swelling effect that alters mitochondrial dynamics (fusion and fission) and the initiation of cell death signaling. Over the years, Ca2+ uptake, mitochondrial dynamics, and cell death signaling have been demonstrated to play a vital role in mediating cardiac recovery after IR injury. In this review, we will evaluate how mitochondrial Ca2+ uptake, dynamics, and cell death signaling are influenced during IR injury.

Objective:

We will explore the roles of mitochondrial Ca2+ uptake, dynamics, and cell death signaling during IR injury.

Development:

Role of mitochondrial calcium uptake via the MCU

Intracellular Ca2+ are some of the most versatile signaling molecules, their functions range from promoting muscle contraction to participating cell migration. However, in excess, Ca2+ may function as a mediator of cell death mechanisms, specifically apoptosis, thus maintaining regulating intracellular Ca2+ levels is vital. The mitochondria serve a critical role in maintaining intracellular Ca2+ levels by buffering excess Ca2+ via the mitochondrial Ca2+ uniporter (MCU). The mitochondrial calcium uniporter (MCU) is a ~700 kD multi-subunit channel located in the inner mitochondrial membrane[27]. The MCU is an essential one directional channel that regulates Ca2+ into the mitochondrial matrix, this process is driven by the mitochondrial membrane potential which is generated from the proton motive force[25].

Regulation of the MCU is key to conserving calcium homeostasis in the mitochondria, heightening the importance and demand for MCU regulators. The MCU is regulated by two central regulators called MICU1 and MICU2. The mitochondrial calcium uptake 1 (MICU1) contains two calcium-binding EF-hands and is reported to be a channel gatekeeper by maintaining closure of the MCU at low cytosolic calcium levels[35]. In addition, there are chemical regulators of the MCU such as ruthenium red/Ru360 which have been used to prevent Ca2+

entry[27]. The regulation of Ca2+ uptake by the MCU has been found to have a beneficial role against a myriad of pathologies, in this review we will be focusing on its role in cardiac IR.

The MCU has been demonstrated to play a physiological role in mitochondrial Ca2+ loading to promote ATP production during high cardiac workload conditions. Alternatively, after IR, this Ca2+ loading effect tends to be the main driver of mitochondrial dysfunction[19]. The effect of pathological Ca2+ overload in the mitochondria include the production of ROS, the opening of the mitochondrial permeability pore, inhibition of mitochondrial fusion and the release of pro-apoptotic factor cytochrome C (cyt C)[33,19]. Studies performed with MCU KO mice have demonstrated that inhibition of the MCU has led to a resistance in the activation of the mitochondrial permeability pore, however little to no protection was provided in an in-vivo cardiac IR injury model[36]. Nevertheless, this study didn't consider the effects of Ca2+ regulation in mitochondrial fusion. This opens the question as to whether the effects of calcium uptake on mitochondrial fusion play a role in the recovery from cardiac IR injury.

Mitochondrial fusion mechanisms

The mitochondrion is a dynamic organelle which changes shape and place to maintain an adequate distribution of the organelle and the energy demand of the cells, this depends on the physiological need in which the mitochondrion is exposed. Mitochondrial dynamics, fusion and fission, operate in equilibrium in order to maintain mitochondrial health. Mitochondrial fusion is very important for the repair of damaged mitochondria structures, triggering the exchange of material between damaged and undamaged mitochondria[42]. Due to the dynamic process of mitochondrial fusion, the maintenance of important cellular procedures such as mitochondrial respiratory activity, the distribution of mitochondrial DNA, apoptosis, cell survival or calcium signaling occurs. Mitochondrial fusion is regulated by the interactions between the proteins

GTPases Mitofusin (MFN) 1 and 2 at the outer mitochondrial membrane and optic atrophy protein 1 (OPA-1) at the inner mitochondrial membrane[42].

MFN 1 and MFN 2 are responsible for the fusion of the outer mitochondrial membrane. MFN1 proteins can interact with other mitochondrial membranes, joining two opposing mitochondria[18]. As for MFN2, these proteins interact with each other, but also heterooligomerize with MFN1 to promote mitochondrial fusion[12]. MFN2 also participates in the physical interaction between the endoplasmic reticulum and mitochondria, to promote the Ca2+ signaling[6]. While Mitofusins mediate outer mitochondrial membrane fusion, inner mitochondrial membrane fusion is produced by OPA-1. Regardless of its role in mitochondrial fusion, OPA-1 regulates the remodeling and maintenance of apoptotic cristae[16]. In addition, OPA-1 maintains the morphology of mitochondrial cristae, which has a direct metabolic effect by stabilizing the mitochondrial ETC supercomplexes; a super structure of the ETC that allows for more ATP production. Disruption of OPA-1 under pathological conditions would lead to increased mitochondrial fission, fragmentation, and even cell death; therefore, this demonstrates that OPA-1 is the main regulator of mitochondrial fusion[38].

One of the first experimental studies to investigate the role of OPA-1 in the heart found that myocardial levels of OPA-1 were reduced in ischemic heart failure patients and in a rat model of ischemic heart failure[7]. Mitochondrial swelling, which occurs due to IR injury, has been demonstrated to fragment OPA-1 from it's long to its short form[21]. Attempts to link the degradation of OPA-1 to intramitochondrial enzyme OMA-1 remain obscure[41]. Loss of OPA-activity after IR injury has been associated with an increase in mitochondrial fission mediated by DRP-1[19]. Mitochondrial fusion is typically balanced with fission, however after ischemia

reperfusion mitochondrial fission is higher than the fusion process, thus taking over the mitochondrial quality control during heart failure.

Mitochondrial fission mechanisms

The role of mitochondrial fission consists of dividing functional portions of the mitochondrial network to relocate them within the cell for maintaining energy production[1]. These processes that are accompanied by fusion manage the mitochondrial distribution inside the cell and maintain quality control by regulating many cellular activities including the activation of apoptosis. A lack of balance in mitochondrial dynamics may lead to organelle fragmentation and cell death, this is mainly driven by changes in the FIS-1 and DRP-1 proteins. Dynamin-related protein-1 (DRP-1) plays an important role in mitochondrial homeostasis[30]. Mainly, it is a dynamin related protein that regulates the fission process through the outer mitochondrial membrane. DRP-1 works by translocating to the outer mitochondrial membrane to eliminate mitochondria damaged through mitophagy, a mitochondrial degradation process[15].

DRP-1 is regulated through post-translational modifications via an inhibitory phosphorylation by PKA[40]. DRP-1 actively targets the outer mitochondrial membrane through non-GTPase receptor proteins, such as mitochondrial fission protein 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial elongation factor. The assembly of the fission apparatus is assisted by the endoplasmic reticulum, which contacts the mitochondria, creating a microdomain for the assembly of DRP-1, MFF and pro-apoptotic proteins. DRP-1 activity is rapidly regulated by opposite effects of phosphorylation on two serine keys. Phosphorylation of serine 616 increases the activity of DRP-1, while phosphorylation of serine 637 reduces it. Each serine is the target of different kinases and phosphatases, thus linking mitochondrial fission to

crucial cellular processes. The ratio of phosphorylation of serine 616 to serine 637 determines DRP-1 activity and reflects the aggregated effects of many kinases and phosphatases[11].

Mitochondrial fission plays an important role in ischemic reperfusion injury. During ischemic reperfusion, if any injury occurs, the protein dynamin (DRP-1) is activated, which causes mitochondrial fission and the generation of reactive oxygen species (ROS)[24]. This protein has been shown to contribute to the prevention of long-term heart failure as seen in the KO model[46]. Another KO model in mice found that DRP-1 plays a protective role against cardiac pressure overload during heart failure by preventing mitochondrial dysfunction[44]. The DRP-1 protein inhibition will help preserve mitochondrial function by eliminating the dysfunctional parts of the mitochondria and maintaining morphological stability in healthy mitochondrial fractions[34]. Compromising this quality control mechanism plays a crucial role in inducing cell death, understanding the relationship between the two may help facilitate the production of mitochondrial directed interventions to prevent IR injury.

Mitochondrial apoptosis signaling in the heart

Apoptosis (programmed cell death) is a cellular self-destruction mechanism that is essential for a variety of biological events, such as developmental sculpting, tissue homeostasis, and the removal of unwanted cells. Mitochondria play a crucial role in regulating cell death[22]. Apoptosis is characterised by morphological changes in the structure of the cell, together with enzyme-dependent biochemical processes[10]. The common characteristics of apoptosis include cellular shrinking, condensation and margination of the chromatin, protein cleavage, DNA breakdown and phagocytosis[37]. The mechanisms that are responsible for the morphological characteristics and changes observed during apoptosis are: the receptor ligand mediated

mechanism, a mitochondrial pathway, and a mechanism in which the endoplasmic reticulum plays an important role[9].

There are many factors involved in the activation, regulation, and execution of apoptosis, these are mainly proteins. The most important are the Bcl-2 family of proteins, the caspases, the p53 gene, the amyloid-B peptide and the heat shock proteins[37]. Heat shock proteins are known as protectors from cell death. The role of the Bcl-2 protein family is to regulate cell death through complex stoichiometric equilibrium[23]. Similarly, caspases co-ordinates the demise of the cell, this process is mediated by members of a family of cysteine proteases and these substrates include many proteins[43]. The mechanisms that are responsible for the morphological characteristics and changes observed during apoptosis are also related to the activation of caspases[9]. The p53 protein also plays a key role in growth arrest and apoptosis after cell stress, by regulating the transcription of select downstream target genes in the cell[39]. In addition, amyloid beta peptide leads to cell death through apoptosis after the cell passes through oxidative stress/damage[32].

The Bcl-2 Proteins family are the ones responsible for the programmed cellular death. Thus, proteins are mediators either for pro or anti-apoptotic activities[17]. One of the distinguishing pro-apoptotic protein are Bak/Bax proteins which create a permeable stable pore on the mitochondrial outer membrane (MOM).These pores give exit to mitochondrial apoptotic factors such as cyt C[5]. Their activity is regulated by two other factions of the Bcl-2 family: proapoptotic BH3-only proteins, which play an activating role, and anti-apoptotic Bcl-2 type proteins, including Bcl-2, which inhibit proapoptotic partners. One of the best-known pathways of apoptosis occurs when, after an intercellular stress, cyt C is released from the mitochondria. Apoptotic protease-activating factor 1 (Apaf1) interacts with cyt C creating a scaffold that

functions as a signal to begin apoptosis. The scaffold interacts with caspase9 and caspase3 which give rise to apoptosis[8]. Meaning the localization and control of Bcl-2 proteins on mitochondria is essential for the intrinsic pathway of apoptosis. Anti-apoptotic Bcl-2 proteins reside on the outer mitochondrial membrane (OMM) and prevent apoptosis by inhibiting the activation of the pro-apoptotic family members Bax and Bak. The Bcl-2 subfamily of BH3-only proteins can either inhibit the anti-apoptotic proteins or directly activate Bax or Bak[29].

Therefore, the mitochondria are a key regulator of the cell's fate, controlling cell survival via the production of ATP that fuels cellular processes and inducing cell death via apoptosis through release of pro-apoptotic factors such as cyt C[3]. But other than apoptosis there are various forms of cell death these being necrosis, oncosis, pyroptosis and autophagy. Apoptosis results in the clearance of cells from the body, with minimal damage to the surrounding tissues, so if apoptosis fails damaged cells will accumulate in the body which can cause various forms of cancer. While necrosis is characterized by the uncontrolled death of the cell expanding into and damaging surrounding tissues[10]. Prolonged periods of myocardial ischemia are related to an increase in the rate of necrosis, whereas, paradoxically, reperfusion leads to an enhancement in apoptosis. Because reperfusion restores oxygen and glucose supply to the cell, giving it the required energy for the completion and acceleration of the apoptosis process. The pathways involved in preconditioning, growth factor induced myocardial protection, apoptosis, and hypertrophy are crucial for reperfusion-induced cell death[13].

In rats, ischemia-induced apoptosis going into necrosis has been demonstrated. Of the cell death 86% was based on apoptosis versus 14% on necrosis. Although the onset of cell death is different, transitions from apoptosis to necrosis were observed. There is more supportive evidence for ischemia-related apoptosis as mitochondrial dysfunction has been demonstrated

with leakage of cyt C, and caspase activation following global ischemia in the isolated rat heart showed that caspase activation was dependent on the time of ischemia. The release of cyt C following mitochondrial-permeability transition (MPT) activation seems to be the apoptosis inducing mechanism. The level of apoptosis was shown to depend on the duration of reperfusion. These studies confirm that ischemia by itself can trigger apoptosis. Reperfusion accelerates the process. The results by Gottlieb et al. were different. They suggest that apoptosis is initiated by reperfusion only. In a more recent study in dogs, apoptosis was detected in myocardium subjected to a brief period of ischemia followed by reperfusion, and not in ischemic tissue without reperfusion[13]. Experiments also showed the importance of the duration of reperfusion for the level of apoptosis. There is a possibility to reduce apoptosis during reperfusion by blocking caspase activation and through blocking of the sodium hydrogen exchanger. Cell death mechanisms such as apoptosis are important targets for developing therapeutics for cardiac IR injury.

Discussion:

Many of the studies analyzed in this review contained remarkable findings and provided new insight for future research. However, they all have certain limitations that need to be looked into to create a complete picture of the role of Ca2+ uptake via the MCU in cardiac IR. For example, a limitation that most of the studies had was that they focused solely on the mitochondria and ignored the endoplasmic reticulum (ER), which is known to play a prominent role in calcium buffering. Therefore, resulting in a narrow understanding of the dynamics regarding Ca2+ homeostasis. Further research is needed to explain the relationship between mitochondrial and ER during Ca2+ buffering after cardiac IR injury. Another notable limitation is that the contact site or active target of where Ca2+ induces permeability transition of the inner

mitochondrial membrane remains to be elucidated, given that the molecular identity of the pore is unknown[4]. For this reason, identifying the permeability transition pore may help gain an understanding of how to develop therapeutics to prevent that process, thereby diminishing cell death signaling.

Lastly, an MCU KO study on mice acknowledged that the lack of MCU expression did not affect mice viability. They demonstrated that the absence of MCU expression, in the context of the whole animal, has little to no effect on basal energetics, implying that there could be a slow and low capacitive mechanism that controls intracellular Ca2+[36]. Therefore, further research should look into non-cardiac organs to explore possible mechanisms that deal with Ca2+ homeostasis and buffering in their context and see if there could be any similarities that can be applied to further our understanding of cardiac IR. Overall, all of the research covered provides new insight into the relationship of the MCU with the uptake of Ca2+. Notably, from the current research, we conclude that the MCU is an essential tool that facilitates high capacity and rapid uptake of Ca2+. However, we can infer that the MCU is not the only Ca2+ pathway and that other mechanisms could control Ca2+ levels in the cytosol. Creating a multi-layered understanding of Ca2+ uptake could help us find ways to relate those findings to the MCU, which in the end, would help promote mitochondrial fusion and prevent cell death by inhibiting pro-apoptotic factors.

In recent studies, it has been seen that long, membrane-bound forms of OPA1 are required for mitochondrial fusion, but their processing to short, soluble forms limits fusion and can facilitate mitochondrial fission. It is unclear what role the short isoform of OPA-1 has within mitochondrial stress signaling pathways, however it has been proposed to expedite cristae organization[28]. Additionally, it remains unclear how OMA1, the enzyme responsible for cleaving

the long OPA1 isoform, is regulated under IR injury. A study using agents against oxidative stress and mitochondrial swelling were unable to prevent OMA1 activity nor degradation of OPA1[41]. This implies that an alternative mechanism must be taken into account when considering OPA1 cleavage.

During the research it was understood that mitochondrial fusion gives responses to multiple stimulations and diseases consequent to aging specifically in heart failure. The mitophagy process was slowed down by the mitochondrial fission generated by the overexpression of the DRP-1 protein. It was found that there is a DRP-1 dominant negative mutant, DRPK38A, which blocks mitochondrial division, affecting the phenotype in expressed cells. The fact that this mutation inhibits apoptosis associated activation is counteracted resulting in a limitation in its natural physiological process. However, more work needs to be done in understanding the regulatory role of post-translational modifications in DRP-1 and its relationship to mitochondrial fission. There are still assertions that indicate the cause of the changes in the mitochondrial phenotype based on homologous proteins related to dynamin (DRP-1). DRP-1 has been a proposed target for preventing IR injury. Mdivi-1, a selective inhibitor of DRP-1 has shown to improve cardiac recovery in left coronary artery ligation IR models[31]. This demonstrates the importance of mitochondrial fission activity in cardiac recovery and that DRP-1 is an effective therapeutic target for IR injury.

A setback that stands out in our research is that the permeabilization mechanism of MOM is not known with certainty. Although the participation of Bak / Bax proteins are widely suspected as responsible, it is unknown if they are responsible for properly establishing the permeabilization pore on the mitochondria. As mentioned before in this paper, the mechanism most supported by researchers gives the responsibility of creating the pores in the MOM to the

Bak / Bax proteins, these being the ones that give way out to apoptotic factors. However, other researchers consider that the true role of Bak / Bax proteins is to facilitate other existing transition pores in the MOM such as the voltage-dependent anion channel (VDCA)[8]. Recently, it has been argued that oligomerization of the Bax protein may form either a proteinaceous channel or induce lipidic pores on the outer mitochondrial membrane, further studies are required to confirm this notion[45]. This uncertainty of the true permeabilization mechanism exerted by Bak / Bax proteins represents an imminent need for future research.

There are different mechanisms of cell death that could be related to the time of ischemia and reperfusion but the studies mainly focus on apoptosis and necrosis. The activation of apoptosis during ischemia reperfusion has been demonstrated in vivo with rats, dogs and rabbits. The rodent models showed that ischemia by itself can trigger apoptosis and that reperfusion accelerates the process. While other studies of rabbits suggest that apoptosis is initiated by reperfusion only. In a more recent study in dogs apoptosis was detected in myocardium subjected to ischemia followed by reperfusion, but not in ischemic tissue without reperfusion[13]. Whether these findings are related to each specific species remains unclear, since the findings from the studies with rabbits and dogs suggests apoptosis is initiated by reperfusion while the studies with rats showed that both ischemia and reperfusion cause apoptosis. In addition, recent studies have found the participation of alternative cell death mechanisms which play a role in the heart such as ferroptosis. Liproxstatin-1, an anti-ferroptotic agent, has demonstrated a protective effect on mouse myocardium against ischemia/reperfusion injury by decreasing VDAC1 levels and restoring GPX4 levels[14]. More research needs to be done to see how the different mechanisms of cell death are activated in respect to changes in the time of ischemia and reperfusion.

Conclusion:

In conclusion, mitochondria play a key regulatory role in cell survival during ischemia reperfusion injury. The mitochondria's role in regulating Ca2+ level was demonstrated to play a vital role in both cell death signaling and mitochondrial dynamics. Maintaining a balance between fusion and fission is essential for sustaining healthy cardiac activity and an imbalance among them could lead to cardiac dysfunction. Additionally, IR injury was demonstrated to be heavily regulated by mitochondrial cell death signaling via the release of cyt C. Understanding how these mechanisms work could help develop therapeutics or manufacture new drugs that help mediate cardiac recovery after IR injury.

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