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DEGLI STUDI
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Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Biologia

SCUOLA DI DOTTORATO DI RICERCA IN : BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO: BIOCHIMICA E BIOFISICA

CICLO XVIII

THE ROLE OF THE MITOCHONDRIAL CALCIUM UNIPORTER (MCU) IN THE CARDIAC INJURY INDUCED BY ISCHEMIA AND REPERFUSION

Direttore della Scuola : Ch.mo Prof. Paolo Bernardi

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1. ABBREVIATIONS

CNs cortical neurons

CsA cyclosporine A

DHE dihydroethidium

ETC electron transport chain

LDH lactate dehydrogenase

I/R ischemia reperfusion

IMM inner mitochondrial membrane

IPC ischemic preconditioning

MCU mitochondrial calcium uniporter

MICU1 mitochondrial calcium uptake 1

MCUR mitochondrial calcium unicomplex regulator

MTR mitotracker red

NRVMs neonatal rat ventricular myocytes

OMM outer mitochondrial membrane

PLA2 phospholipase A2

PTP permeability transition pore

ROS reactive oxygen species

TMRM tetramethylrhodamine methyl ester

2. SUMMARY

Studies on mechanisms underlying cardiac injury caused by ischemia and reperfusion (I/R) have provided an enormous contribution to our current understanding of the role of mitochondrial dysfunction in cardiac pathophysiology, and it appears clear that mitochondria are a central target of I/R injury.

The rise in Ca^{2+}, which occurs during ischemia and/or at the onset of reperfusion, represents a powerful mechanism of injury by acting simultaneously on contractile proteins, thus increasing ATP demand and mitochondria, thus potentially reduces ATP supply. The deleterious action of Ca^{2+} is exacerbated by the formation of reactive oxygen species (ROS) due to a readmission of oxygen occurring during the post ischemic period. Indeed, both Ca^{2+} and ROS are suggested to play a pivotal role in I/R-induced loss of cell viability by promoting the opening of the PTP. In the past the relevance of the mitochondrial Ca^{2+} uniporter in this pathological context has been evaluated using Ruthenium derivatives that unfortunately lack selectivity. However, the gap of the knowledge regarding the molecular identity of the protein responsible for mitochondrial Ca^{2+} uptake, has been filled by De Stefani and Co.(De Stefani, Raffaello et al. 2011). The discovery of the molecular identity of MCU allowed us and others laboratories to investigate the role of MCU in cardiac I/R injury by using genetic approaches. The experiments illustrated in this thesis have been carried out by studying the effects of MCU overexpression in both isolated cardiomyocytes and intact heart.

The isolated myocytes were subjected to classical protocol of simulated I/R adding 5mM KCN for 60 minutes (i.e., simulated ischemia) followed by KCN washout (reperfusion) for 60 minutes. Initially we investigated the effects of MCU deletion by means of shRNA transfection. Under this condition, we observed an increase of loss of cell viability induced by I/R injury in NRVMs devoid of MCU. This finding appears to confirm the results obtained in Finkel's laboratory showing a lack of protection in hearts lacking MCU but this conclusion was then argued by more recent report (Kwong, Lu et al. 2015). Since this discrepancy is far to be solved , we focus our attention in the opposite strategy that has not been covered yet in cardiac studies. To this aim the effect of MCU overexpression was validated by observing an increase in mitochondrial Ca^{2+} uptake and oxygen consumption rate. We observed an increase in the maximal mitochondrial capacity probably due to the activation of the dehydrogenases involved in the Krebs'cycle and calcium-dependent. Moreover, NRVMs overexpressing MCU subjected to I/R injury, showed a decrease in the loss of cell viability that was parallel with the reduction in ROS levels in the onset of reperfusion. We hypothesized that MCU induced cardioprotection is a preconditioning-like effect involving the activation of protective pathways. To validate this hypothesis we investigated the activation of Akt, a pro-

survival kinase linked to the IPC phenomenon. Indeed, we showed an activation of Akt when MCU is overexpressed in NRVMs. This correlates with a slight increase of ROS formation under normoxia. Actually, MCU induced Akt activation detected as an increase phosphorylation of Ser473 and Tre308 was abrogated by treatment with the antioxidant mercaptopropionylglycine. Since Akt is not activated directly by ROS, we investigated the upstream processes focusing on the Ser/Thr phosphatase PP2A. Since phosphorylation inhibits PP2A activity the likely consequence is the activation and phosphorylation of Akt. These results demonstrate that MCU overexpression determines an increase of oxidative stress, that triggers the Akt activation, and it explains a reduction of cell death observed in the post ischemic injury.

The findings obtained *in vitro* were extended to *in vivo* system. To this aim we generated a mouse model overexpressing MCU in the heart by means of adeno-associated virus type 9 at P8 in C57/BLJ6 mice. We did not observe any significant alterations in mice development or heart morphology, neither in survival probability of the mice. When we performed the I/R protocol in the Langendorff model MCU overexpressing hearts showed a decrease of the extent of cell death during post-ischemic reperfusion. The loss of cell death was associated with a reduction of oxidative stress. Similarly to what we have observed in vitro, MCU overexpression induced an activation of Akt and a dephosphorylation of PP2A. Therefore, the sequence of events linking MCU overexpression with cardioprotection applies to both isolated myocytes and isolated heart. These findings appear to be in contrast with the common concept by which I/R injury is caused by an increase in mitochondrial Ca^{2+}. We hypothesized that this concept is all valid upon condition of severe elevation of the mitochondrial [Ca^{2+}]. To test this hypothesis, MICU1 was co-expressed with MCU to further increase mitochondrial Ca^{2+} accumulation. The co-expression abrogated the protective efficacy afforded by the overexpression of MCU only, this was detected as both an occurrence of cell death and ROS formation that was not different from wild type cells. In addition, the co-expression abolished the effects of MCU overexpression on Akt and PP2A that are likely crucial in determining the protective phenotype. Overall, the present data for the first time provide evidence of protective efficacy of an increase in mitochondrial calcium uptake driving by MCU overexpression. The link between the increase of Ca^{2+} uptake and reduction of cell death appears to involved a sequence of events where by a mild increase in ROS formation causes a decrease in protein phosphatase activity that eventually stimulates a pro-survival kinase. Nevertheless, the cardioprotective efficacy of a slight increase in mitochondrial Ca^{2+} uptake is lost when mitochondrial Ca^{2+} accumulation is further increase by the co-expression of MCU and MICU1. This supports the notion that mitochondrial Ca^{2+} overload plays a pivotal role in cardiac injury. Further studies are necessary to define the threshold between protective and deleterious levels

of mitochondrial calcium and ROS as well as clarifying the signaling pathways involved in both injury and protection of the ischemic heart.

2. SOMMARIO

Lo studio dei meccanismi coinvolti nel danno da ischemia-riperfusione hanno evidenziato il ruolo centrale delle disfunzioni mitocondriali, facendo emergere quest'ultimi come target centrale nel danno da ischemia/riperfusione (I/R). L'aumento di Ca^{2+}, che avviene in fase ischemica e nella fase iniziale della riperfusione rappresentano un meccanismo d'elezione per l'induzione del danno cellulare andando ad influire sia sulle proteine contrattili sia sull'incremento metabolico di ATP. L'azione deleteria del Ca^{2+} viene esacerbata dall'aumento della formazione delle specie reattive dell'ossigeno (ROS), dovuto alla re-introduzione dell'ossigeno nella fase di riperfusione. Inoltre, entrambi questi fattori, Ca^{2+} e ROS sono coinvolti nell'apertura del poro della permeabilità mitocondriale determinando così un aumento della morte cellulare. Nel passato il ruolo dell uptake di Ca^{2+} mitocondriale è stato studiato valutando l'effetto dei derivati del Rutenio, i quali tuttavia mancavano di selettività. Questo avveniva poichè la struttura della proteina responsabile dell uptake del Ca^{2+}mitocondriale era ignota. La scoperta dell'identità molecolare di tale proteina definita MCU, ha permesso di utilizzare un approccio genetico. Gli esperimenti riportati in questa tesi si occupano dello studio della sovraespressione di MCU sia nei cardiomiociti neonatali isolati che nel cuore intero.

I cardiomiociti isolati, in cui MCU è stato silenziato tramite transfezione con shRNA, sono stati sottoposti ad un protocollo di I/R utilizzando il KCN. Il dato ottenuto mostrava un'aumentata mortalità associata ad un aumento di ROS nella fase post ischemica. Questi dati erano in accordo con quanto pubblicato da Finkel, tuttavia in seguito alla pubblicazione di altri due paper con dati contrastanti al riguardo l'argomento risulta ancora molto dibattuto. In questo studio si è deciso di utilizzare una strategia alternativa valutando l'effetto della sovraespressione di MCU nel danno indotto da I/R. L'overespressione di MCU induceva un aumento dell'uptake di Ca^{2+} mitocondriale ed un aumento del consumo di ossigeno in condizioni di stress (con l'aggiunta di FCCP), probabilmente causata dall'accumulo di Ca^{2+} che attiva le deidrogenasi Ca^{2+}-dipendenti coinvolte nel ciclo di Krebs. Inoltre, abbiamo evidenziato una ridotta mortalità cellulare in seguito al danno da I/R in parallelo con una riduzione della formazione di ROS nelle prime fasi della riperfusione. Per spiegare l'aumentata protezione indotta da MCU si è supposto che esso inducesse l'attivazione di un meccanismo simile al preconditioning. Per confermare questa ipotesi abbiamo valutato l'attivazione di Akt, chinasi coinvolta nei meccanismi di preconditioning. Akt risultava più attiva quando MCU è sovraespresso, e questo correlava con un aumento di ROS in condizioni di basali. L'attivazione di Akt era abrogata dal trattamento con mercaptopropilglicina, un antiossidante. Tuttavia Akt non viene modulata direttamente dai ROS, perciò abbiamo focalizzato la nostra attenzione

su un meccanismo che ne potesse spiegare la maggiore fosforilazione. Focalizzandoci su PP2A, una fosfatasi che regola negativamente Akt e la quale risulta essere inattiva quando MCU è sovraespresso. Questi risultati dimostrano che la sovraespressione di MCU determina un aumento dello stress ossidativo che porta all'attivazione di Akt. Questo potrebbe spiegare la riduzione del danno post ischemico evidenziato.

I dati ottenuti in vitro sono stati poi ripetuti in un modello in vivo, ottenuto tramite l'iniezione di un virus adeno-associato di tipo 9 codificante per MCU-flag in topi C57/BLJ6, iniettati all'ottavo giorno dopo la nascita. I topi non mostravano alcuna alterazione nello sviluppo né nella morfologia cardiaca né nella sopravvivenza. Il cuore isolato sovraesprimente MCU sottoposto ad un esperimento di I/R in Langendorff mostrava una riduzione del danno cellulare, correlato ad una riduzione dello stress ossidativo. Similarmente a quanto osservato in vitro, abbiamo osservato un'attivazione di Akt che correlava con una maggiore fosforilazione di PP2A. I dati fin qui presentati sembrano apparentemente in contrasto con la nota nozione che il danno indotto da I/R è legato ad un aumento del Ca^{2+} mitocondriale. Per confermare questa nozione abbiamo co-espresso MCU e MICU1 per incrementare ulteriormente l'accumulo di Ca^{2+} mitocondriale. La co-espressione determinava una perdita dell'effetto protettivo visto nella sola espressione di MCU, causando una maggiore mortalità cellulare in seguito ad I/R e un aumento dello stress ossidativo. La mancata protezione correlava con una minore fosforilazione di Akt e conseguentemente una maggiore attivazione di PP2A. Questi dati, mostrano per la prima volta un fenotipo protettivo apportato da un aumento di Ca^{2+} dato dalla sovraespressione di MCU. La sequenza di eventi che da un aumento di calcio porta ad un aumentata cardioprotezione comprende la produzione di ROS che determinano l'inattivazione di alcune fosfatasi, che portano alla successiva attivazione di chinasi che aumentano la vitalità cellulare. Tuttavia un eccessivo incremento di calcio indotto dalla co-espressione di MCU e MICU1 ha un effetto deleterio sulla cellula. In ogni caso, altri studi saranno necessari per comprendere e definire qual è il livello di demarcazione tra un effetto benefico o meno di Ca^{2+} e ROS nel danno ischemico, e per specificare quale sia l'esatto meccanismo molecolare d'attivazione.

3. INTRODUCTION

3.1. The Ischemia/Reperfusion Injury

Ischemia and post ischemic reperfusion cause a wide array of mitochondria dysfunction. The interruption of oxygen and substrate supply during cardiac ischemia induces a block of the mitochondrial ATP synthesis. The first consequence of the inhibition of the electron transport chain is the loss of energy conservation and oxidative metabolism.

Impairment of energy metabolism

The energy is released from the flux of electrons from reducing to oxidizing components of the ETC, this energy is transformed into the extrusion of protons from the mitochondrial matrix into the intermembrane space. This movement of protons generates a protonmotive force that is composed of membrane potential and pH gradient. This protonmotive force is necessary to maintain the ions homeostasis and the protein import for the mitochondrial bioenergetics. During hypoxia, the inhibition of the ETC due to the lack of oxygen, causes the block of ATP synthesis at the level of F1FoATP synthase, for that reason mitochondria are not anymore the main source of ATP, however they become a powerful system for hydrolyzing glycolytically-produced ATP.

Impairment of oxidative metabolism

The main pathway for the reduction of oxidized pyridine and flavin nucleotides is the ETC. These coenzymes are critical for the cellular metabolism, they are not abundant in the cells, and they could not be replenished by extracellular sources. During hypoxia, their oxidation is mostly catalyzed by lactate dehydrogenase. This permits the prosecution of ATP synthesis under anoxic condition through anaerobic glycolysis in the cytosol.(Allen and Orchard 1987).

Impairment of calcium homeostatsis

Membrane potential and proton gradient are the crucial factors for the mitochondrial calcium uptake and release (Bernardi 1999). During ischemia, the inhibition of the ETC causes a profound alteration of the Ca^{2+} homeostasis. In ischemia, due to ETC inhibition the proton gradient is abrogated, and the mitochondrial calcium passively follows the rise in the cytoplasmic calcium. During the ischemia the sarcolemmal Na+/H+ exchanger is activated due to intracellular acidosis. Moreover , due to inhibition of the sarcolemmal Na/K ATPase as a consequence of a decreased of ATP/ADP ratio, cytosolic sodium increases, following the rise in cytosolic calcium via Na+/Ca2+ exchanger, that induces the rise in the cytoplasmic calcium. In the reperfusion the recovery of the protonmotive

force, determines an increase in mitochondrial Ca^{2+} uptake, that it is a crucial player involved in the triggering opening of the PTP.

Increased of ROS formation

ROS modulate a wide variety of process, such as the regulation of growth factors signalling, the hypoxic response, inflammation and the immune response. In mammalian cells ROS are produced in different cellular compartments such as the plasma membrane, cytoplasm, mitochondria and endoplasmic reticulum (ER), lysosomes and peroxisomes. However, mitochondria are well known to be major ROS producers. ETC is one of the main source of ROS, due to electrons lacking from complex I and III. Also, monoamine oxidases (MAO) play a relevant role in ROS generation. These flavoproteins located in the OMM catalyze the deamination of amines such as catecholamines, producing large amounts of ROS (Cadenas and Davies 2000). MAO are likely to be involved in the amplification of ROS in the cardiac I/R injury, accordingly MAO inhibition might have a therapeutic value for treating cardiac diseases (Kaludercic, Carpi et al. 2011). An additional source of ROS producers is p66Shc, that transfers electrons from cytochrome c to oxygen (Giorgio, Migliaccio et al. 2005). The cardioprotective efficacy associated with p66(Shc) has been shown to be afforded by other antioxidant interventions (Carpi, Menabò et al. 2009) .

3.2. The role of PTP in the I/R injury

The permeability transition pore (PTP) defines an increased permeability of the inner mitochondrial membrane to ions and solutes activated by matrix Ca2+ in the presence of specific inducers, such as Pi and thiol oxidants (Bernardi, Krauskopf et al. 2006). The phenomenon is caused by a large channel (i.e. PTP) located in the inner mitochondrial membrane (IMM). The PTP opening causes an increase of the inner membrane permeability to solutes with a molecular weights up to 1500 Daltons. PTP opening is favoured by various factors the most important of them are the rise in matrix calcium concentration, an increase in ROS levels and a decrease in mitochondrial membrane potential. Thus, PTP opening is involved in cell death, caused by many diseases, including heart ischemia (Griffiths and Halestrap 1993). The loss of cell viability is induced by prolonged PTP opening, causing the collapse of the membrane potential resulting in ATP and NAD+ depletion, matrix swelling, and rupture of the outer membrane space. On the contrary, very short open times are not deleterious for the cells. These different PTP opening states have been described in isolated mitochondria and intact cells (Hüser, Rechenmacher et al. 1998, Petronilli, Penzo et al. 2001).

The molecular identity of this protein has been elucidated recently(Giorgio, von Stockum et al. 2013).

Accordingly, the PTP is formed by a dimers of the FoF1 ATP synthase. FoF1 ATP synthase (complex V) is the rotary enzyme that synthesizes the vast majority of ATP in respiring cells (Giorgio, von Stockum et al. 2013). This complex is formed by the catalytic F1 portion, the membrane-bound proton-translocating Fo portion, and a lateral stalk linking F1 and Fo. CyPD, a well-characterized protein regulator of the PTP, binds the lateral stalk, which acts as a stator to counter the tendency of the F1-catalytic domain to rotate with the rotor containing the F1 subunits and a ring of Fo subunits (Rees, Leslie et al. 2009). This observation allows the identification of the elusive PTP as a dimer of the FoF1 ATP synthase. This finding solves a long-lasting issue in cell biology and readily accommodates the pathophysiological effectors of the pore described in the past. Indeed, Ca2+, Mg2+, adenine nucleotides, and Pi bind the catalytic core at F1, and the membrane potential and matrix pH, which are key PTP modulators (Giorgio, von Stockum et al. 2013).

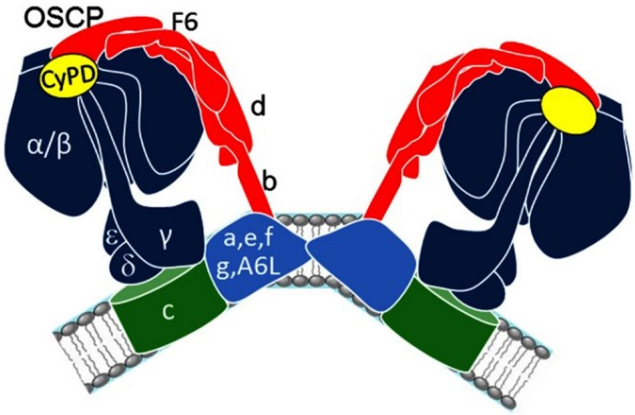


Figure 3.2.1. Schematic representation of F0F1ATP synthase dimers. F1(dark blue), F0(green and light blue), and stalk subunits (red) are illustrated based on recent structural.

3.2.1 *PTP effectors*

The most important effectors involved in the PTP opening are:

- Divalent cations: the opening of the PTP is strongly favoured by accumulation of Ca^{2+} in the matrix; at the opposite it is counteracted by Me^{2+} ions like Mg^{2+}, Sr^{2+}, Mn^{2+}
- ΔΨm : at physiological membrane potential the pore is in the closed state, while a membrane depolarization favours the opening of the PTP. Many factors are able to modify the threshold voltage, in this case PTP opening can be obtained by

either depolarization, or by changing the threshold potential. On the other hand, mitochondrial depolarization may prevent PTP opening reducing the Ca^{2+} uptake.

- Inorganic phosphate favours PTP opening
- Protons: the probability of PTP opening is sharply increased below and above pH 7.4. The inhibitory effect of H+ is exerted from the matrix side of the inner membrane, and is linked to reversible protonation of histidyl residues.
- Adenine nucleotides: the probability of pore opening is decreased by adenine nucleotides, ADP being more potent than ATP.
- ROS are well characterized factors increasing the opening probability of PTP. The link between PTP and ROS is bidirectionals, since PTP opening causes an increase in ROS levels. This link is also called ROS-induced ROS release and it promotes the amplification of the oxidative stress inducing cell injured.

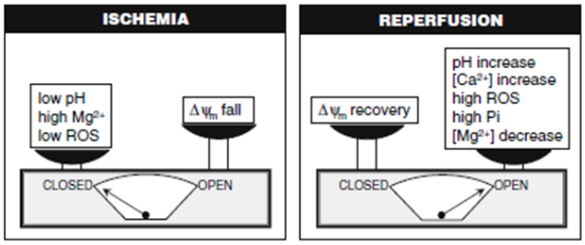


Figure 3.2. PTP effectors involved in PTP opening in I/R injury.

Findings obtained in many laboratories suggest that PTP opening occurs during post ischemic-reperfusion. Indeed, during ischemia, intracellular acidosis along with high levels of Mg^{2+} and ADP overrides the PTP promoting conditions established by ΔΨm decrease and increase in Ca^{2+} and Pi levels. On the contrary, upon reperfusion the recovery of pH and the burst of ROS, along with a sudden rise in matrix Ca^{2+} concentration and Pi represent the ideal scenario for PTP opening.

3.3. Mechanisms of Death: Role of Necrosis, Apoptosis, and Autophagy

Cell death following ischemia-reperfusion has been reported to have features of apoptosis, autophagy, and necrosis. The precise proportion of each form of death may depend on the model (adult versus neonatal, cultured cells versus in vivo). These three mechanisms are briefly described.

Apoptosis

Apoptosis is a physiological process that occurs in multicellular organisms, also called programmed cell death. It is a crucial player in the maintenance of tissue homeostasis, organogenesis and tissue remodeling during the embryogenesis in the vertebrates (Brill, Torchinsky et al. 1999). This process is also essential for the elimination of potentially

dangerous cells, such as autoreactive lymphocytes T, virus infected or cancer cells. The aim of apoptosis is to eliminate unnecessary cells from the organism, through a series of coordinated and programmed events (Steller 1995). Apoptosis contributes to homeostasis which is obtained when the rate of cell proliferation in the tissue is balanced by cell death, the loss of this equilibrium determines the development of numerous pathologies (Lowe and Lin 2000). However, some diseases are associated with higher rates of apoptosis and excessive cell death. For example, increase in apoptosis contributes to loss of cardiomyocytes following ischemic injury during myocardial infarction. Apoptosis was originally characterized by chromatin condensation and fragmentation, cell shrinkage and plasma membrane budding with release of apoptotic bodies that are phagocytised. In this way apoptosis limits the inflammatory response.

Apoptosis has been characterized by the deletion of proapoptotic proteins or increased expression of anti-apoptotic proteins have been evaluated. Activation of caspases is thought to be a major mechanism of apoptotic cell death, especially in I/R injury. (Czerski and Nunez 2004). Inhibition of caspases results in only a modest reduction in infarct size, that is less than that observed with overexpression of antiapoptotic proteins(Gottlieb 2005). However, a large number of studies have reported that addition of caspase inhibitors reduces infarct size, suggesting an important role for caspase activation in ischemia-reperfusion injury (Mocanu, Baxter et al. 2000). Caspase 9 has been reported to be activated during ischemia, whereas caspase 8 and 9 are activated during reperfusion (Stephanou, Brar et al. 2001). Caspases cleave a large number of targets that trigger cell death, such as many mitochondrial proteins accelerating the occurrence of cell death. Cytoskeleton or plasma membrane constituents are also possible targets through which caspase activation, could lead also to rupture of the plasma membrane.

Authphagy

Autophagy is another form of death which could play a role in ischemia reperfusion injury. Autophagy is a physiological mechanism that removes damaged organelles, such as mitochondria or endoplasmic reticulum. Autophagy is also initiated by starvation. However, prolonged autophagy can cause cell death. The role of autophagy in the I/R injury is still matter of debate. Some studies showed that inhibition of autophagy during ischemia or anoxia is detrimental, suggesting a beneficial role for stimulation of autophagy during ischemia (Dosenko, Nagibin et al. 2006). However, other papers demonstrate that a decrease in beclin1 (a protein that stimulates autophagy) reduces ischemia-reperfusion mediated autophagy and myocyte death(Takagi, Matsui et al. 2007). The issue is further complicate by connections between autophagy and other forms of cell death. For example, an increase in Ca^{2+}, as occurs during ischemia, has

been shown to increase autophagy (Hoyer-Hansen, Bastholm et al. 2007). Activation of calpain, a calcium activated protease has been reported to cleave Atg5, a protein involved in autophagy; cleaved Atg5 translocates to the mitochondria where it is reported to bind Bcl-2 and thereby stimulate apoptosis (Hamacher-Brady, Brady et al. 2006). Taken together these data suggest that an increase in calcium induce autophagy activation and at the same time could transform some autophagy events to apoptosis.

Necrosis

Necrosis is an accidental form of cell death, induced by exogenous stimuli that cause an irreversible injury. Necrotic cell death is characterized by cell swelling leading to irreversible rupture of the plasma membrane with release of cytosolic components. The release of cytosolic components triggers an inflammatory response. Necrosis is associated with a large variety of conditions or diseases, such prolonged exposure to injury, infection, cancer, infarction, poisoning and inflammation. In addition, also genetic mutations or damage to cell genome can induce necrosis. This form of cell death initiates with cell swelling, chromatin digestion, and disruption of both plasma and organelle and membranes. Late necrosis is characterized by extensive DNA hydrolysis, vacuolization of the endoplasmic reticulum, organelle breakdown, and cell lysis. Until recently, necrosis was described as a gene- and ATP independent process. However, recent studies have demonstrated that necrosis can be regulated and the interventions can reduce necrotic cell death (Golstein and Kroemer 2007).

Death following ischemia-reperfusion injury appears to be a mixture of apoptotic, autophagy, and necrotic cell death and it can have features of all three mechanisms, and all the three forms of cell death can be regulated and inter-related.

3.4. Role of Calcium

Ca^{2+} during ischemia

Each systole is initiated by Ca^{2+} entry via the L-type Ca^{2+} channel resulting in Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum, and the combined increase in Ca^{2+} leads to contraction. The Ca^{2+} that enters via L-type Ca channel is removed from the cell primarily by Na+-Ca^{2+} exchange (NCX) with a small contribution from the sarcolemmal Ca2+ ATPase (Bers 2002). The Ca2+ released from the sarcoplasmic reticulum is re-accumulated into the sarcoplasmic reticulum (SR) via SR/ER Ca2+, pumps that are ATP dependent. Studies showed that the rise in cytosolic Ca^{2+} during ischemia is primarily due to Ca^{2+} entry by reverse mode NCX, secondary to the rise in [Na+]i during ischemia (Imahashi, Pott et al. 2005). This occurs because of increased generation of protons during ischemia, which are extruded from the cell via Na-H exchange (NHE), resulting in

an increase in intracellular Na+ (Murphy, Perlman et al. 1991). Na+ has also been shown to enter the cell during ischemia on non-inactivating Na+ channels (Murphy, Cross et al. 1999). This rise in intracellular Na+, coupled with the depolarized plasma membrane results in a reversal of NCX to bring Ca^{2+} into the myocyte.

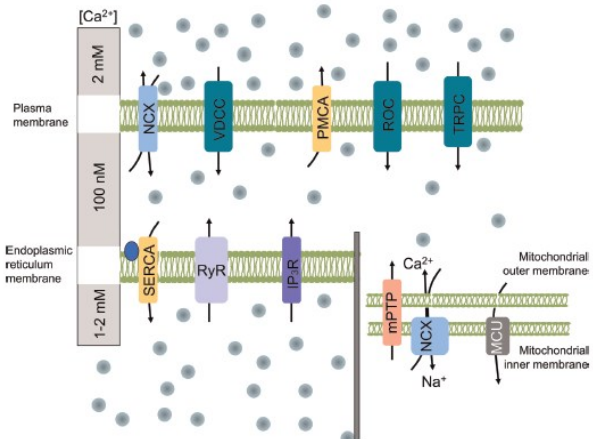


Figure 3.4. **Calcium transporters in the cell**. Calcium channels transport Ca^{2+} into the cytoplasm upon changes in membrane potential or ligand binding. Calcium pumps transport Ca^{2+} from the cytoplasm or into the endoplasmic reticulum (ER) and are energy dependent. Sodium calcium exchanger belong to antiporters transporting Ca^{2+} against sodium ions. Two receptors that are localized in the ER release Ca^{2+} from the ER store. In mitochondria Ca^{2+} transport is realized through the sodium Ca^{2+} exchanger, the mitochondrial Ca^{2+} uniporter and the PTP.

Ca2+ during reperfusion

During reperfusion extracellular pH rapidly returns to physiological values. However, initially the intracellular pH is still acidic and this pH gradient facilitates extrusion of H+ from the cell in exchange for Na+ on NHE. The increased cytosolic Na+ can be extruded by Na-KATPase or NCX in exchange for Ca2+, thereby raising (at least transiently) Ca^{2+}. This process could induce the generation of arrhythmias, that are triggered by Ca^{2+} dyshomeostasis and are one of the principal cause of cell death.

If arrhythmias are not generated, Ca2+ returns quickly to near normal levels in myocytes that survive. However, there can be Ca2+, oscillations (Piper, Meuter et al. 2003), which can lead to hypercontracture which will contribute to loss of cell viability. In some other case Ca^{2+} remains high during reperfusion; this causes an irreversible injury to myocyte. The different behaviour of myocytes depends on ATP levels (which could depend on PTP opening), intracellular Na+ levels, and damage to Ca2+ handling proteins such as the ryanodine release channel in the SR. The Ca^{2+} released from the SR can also be taken up by the mitochondria leading to opening of the PTP. Indeed, improving SR Ca^{2+} handling

has been published to rescue the damage caused by ischemic injury (Piper, Abdallah et al. 2008). Moreover, adenoviral mediated overexpression of SERCA was shown to reduce infarct size and improve function following ischemia (del Monte, Lebeche et al. 2004).

3.5. Role of the mitochondrial calcium

Ca^{2+} uptake into mitochondria occurs via the mitochondrial Ca^{2+}uniporter, which depends on membrane potential and pH gradient. During simulated ischemia, most studies suggest that there is a small rise in mitochondrial Ca^{2+} (Griffiths, Ocampo et al. 1998). Interestingly, Griffiths et al observed that the rise in mitochondrial Ca^{2+} during ischemia was inhibited by clonazepam (an inhibitor of mitochondrial NCX), thus suggesting a role for mitochondrial NCX operating in the reverse mode to increase mitochondrial matrix Ca^{2+} .Moreover, Griffiths et al reported that 0.1µM cyclosporin A (CsA), a PTP inhibitor, protected cells subjected to simulated ischemia and reperfusion. (Griffiths, Ocampo et al. 2000). These data highlight the crucial role of the mitochondrial Ca^{2+} in the I/R injury. However till now the role of the mitochondrial calcium uniporter has been investigated using a pharmacological approach that lack selectivity. The discovery of the protein responsible for the mitochondrial Ca2+ uptake (the mitochondrial Ca2+ uniporter, MCU), has opened the way to genetic approach to elucidate the role of the mitochondrial calcium uniporter in the cardiac injury induced by ischemia and reperfusion.

3.6. Role of ROS

ROS are derived from molecular oxygen by electron transfer reactions resulting in the formation of superoxide anion radical (O_2), and subsequently hydrogen peroxide (H_2O_2), either spontaneously, or by the action of superoxide dismutases (SOD). In the presence of iron, superoxide and H_2O_2 can lead to the formation of highly reactive hydroxyl radicals which can harm cellular proteins, RNA, DNA and lipids. Interaction of ROS with nitric oxide or fatty acids can lead to the formation of peroxynitrite or peroxyl radicals, respectively, that are also highly reactive. Superoxide is not freely diffusible, but can cross membranes via ion channels. Extracellular superoxide has been shown to enter the cell via the anion blocker sensitive chloride channel-3 (Hawkins, Madesh et al. 2007), while voltage-dependent anion channels of the mitochondrial outer membrane can direct superoxide flux from mitochondria to the cytosol (Han, Antunes et al. 2003). On the other hand, hydrogen peroxide can diffuse through membranes and could act as a messengers in signaling pathways. Aquaporins can manage the transmembrane diffusion of hydrogen peroxide, regulating the levels of hydrogen peroxide in the cytoplasm, intracellular organelles, and the extracellular space (Bienert and Chaumont 2014). There is increasing evidence describing ROS as important signaling molecules

controlling cell proliferation, cellular migration, vascular tone, but also cell death. (Holmstrom and Finkel 2014).

ROS in the ischemia and reperfusion injury

ROS formation occurs at a low, if any, extent during ischemia. Controversially, during reperfusion, with the readmission of oxygen, a large burst of ROS has been consistently shown to occur (Zweier, Flaherty et al. 1987). The increase in ROS during reperfusion is probably due to alterations of electron transport chain components, that result in inefficient transfer of electrons, generating superoxide. ROS generated at the onset of reperfusion lead to extensive damage to the cellular components that hampers the maintenance of cell viability because ROS are the primary activators of the PTP opening (Kim, Jin et al. 2006). In addition cell death is contributed by PTP opening induced by ROS accumulation. It has already been published that the treatment of *in vivo* and *in vitro* hearts with antioxidants reduced ROS and stunning (Bolli 1998). However, in contrast to the general agreement regarding ROS as a crucial player triggering cell death in the I/R injury, there are some studies that find no reduction in infarct size upon antioxidants treatment. Indeed, ROS generation has been shown to be part of the protective signaling pathway causing the activation of the self-defence cellular machinery, and in this case the antioxidant treatment can inhibit cardioprotection (Chen, Gabel et al. 1995).

Mitochondria and ROS production

Mitochondria are thought to be both a major source of ROS as well as a major target for ROS damage. Mitochondrial electron transport is one of the primary sources of ROS in the cell. The electron transport chain (ETC) within mitochondria constitutes an important source of superoxide ($O_2{\cdot}-$) formation mainly due to leaking electrons from complex I (NADH-CoQ reductase) and complex III (cytochrome c reductase). Complex I produces $O_2{\cdot}-$ only within the matrix, while complex III can contribute to O_2 $-{\cdot}$ formation also in the intermembrane space. In addition to the ETC, there are other sources of ROS in the cell, such as the acetyl-CoA generating enzyme pyruvate dehydrogenase (PDH) and the Krebs cycle enzyme α-ketoglutarate dehydrogenase (KGDH). Moreover, recent finding suggests that also p66shc and MAO could contribute to ROS formation producing H_2O_2. It has been described that even though only low levels of ROS are generated during ischemia, this ROS can trigger the damage of the electron transport chain (Lesnefsky, Chen et al. 2004); this damage is then thought to lead to increased ROS production because of inefficient transfer of electrons. Inhibition of electron transport at complex I during ischemia reduces ROS generation. However, ROS generation has been shown to be part of the protective signaling pathway of preconditioning (Chen, Gabel et al. 1995), as for example diazoxide, an activator of the mitoKATP channel, has been

shown to increase ROS (Forbes, Steenbergen et al. 2001), and addition of antioxidants has been shown to block the protection afforded by diazoxide. This suggests that low levels of ROS generation are important in signaling of cardioprotection. However, high levels of ROS, as occurs in the post ischemic reperfusion, can lead to damage to the electron transport chain and activation of the PTP.

3.7. Calcium and ROS a fateful encounter

Ca^{2+} primarily supports ATP synthesis by stimulating enzymes of the Krebs'cycle and oxidative phosphorylation in the mitochondria, the resulting increase in oxygen consumption could favour respiratory chain electron leakage thus increasing ROS levels (Brookes, Yoon et al. 2004). Indeed, metabolic rate influences the mitochondrial ROS generation (Sohal and Allen 1985). There is further evidence that the metabolic state of the mitochondria determines the effects of Ca^{2+} on mitochondrial ROS levels. When the membrane potential is high (no ATP synthesis), Ca^{2+} uptake results in decreased ROS generation. When the membrane potential is set to a depolarized range (ATP synthesis), ROS generation is stimulated, or not influenced by Ca^{2+}, depending on the amount of the Ca^{2+} load (Adam-Vizi and Starkov 2010). When mitochondria are overloaded with Ca^{2+}, ROS production might increase independently of the metabolic state of mitochondria (Li, Fang et al. 2013). Finally, also the PTP is a voltage and Ca^{2+} dependent channel , whose prolonged opening determines a deleterious effect for the cell viability inducing a burst of ROS. There is reciprocal crosstalk between $Ca^{2+}$and ROS production. Indeed, as well as Ca^{2+} regulates ROS generation, also ROS have been shown to modulate the activity of a variety of Ca^{2+}channels, pumps and exchangers, such as several Ca^{2+} transporters localized in the plasma membrane, which are redox sensitive.

For example, the voltage dependent Ca^{2+}channels (VDCC) present a cysteine residues in the pore, that can be oxidized by ROS, inducing changes in activity, trafficking, expression and open-time probability. Also the L-type calcium channel can be regulated by ROS in smooth muscle cells, increasing the calcium entry. Finally oxidants modulate store-operated Ca^{2+}entry (SOCE). ROS can also regulate some intracellular Ca^{2+} channels, such as ryanodine receptors oxidizing (redox sensing) thiol groups and the sacro/endoplasmic reticulm Ca^{2+}ATPase (SERCA), their oxidation compromises pumping-activity (Gorlach, Bertram et al. 2015).

It appears clear that Ca^{2+} and ROS are mutually interconnected. Ca^{2+} can increase production of ROS, and at the same time ROS can significantly alter Ca^{2+} influx into the cell as well as intracellular homeostasis.

3.8. ROS as regulators of signaling pathways

The action of ROS in various signaling networks is connected to both physiological and pathological role. Various stimuli, such as nutrients like fatty acids, growth factors, hormones, coagulation factors, cytokines, and hypoxia were shown to act at least partially via regulated ROS generation.

Preconditioning

Preconditionig (PC) has been originally described by Murry et al. It consisted of 4 cycles of 5 minutes of ischemia and 5 minutes of reperfusion just prior to a sustained period of ischemia (Murry, Jennings et al. 1986). This protocol conferred resistance against a subsequent lethal episode of myocardial ischemia, reducing infarct size, the generation of lactate and the rate of fall in ATP. The reduced rate of ATP hydrolysis during ischemia could result from a decrease in anaerobic glycolysis. In this respect it can be proposed that glycogen is reduced in IPC protocol . An early hypothesis to account for the reduced ATP hydrolysis was that PC might inhibit ATP breakdown by reverse mode of the F1FoATPase. A second hypothesis was that IF1 binds F1FoATPase inhibiting its activity (Rouslin, Erickson et al. 1986) and other observations demonstrated that glycogen is significantly reduced during the PC protocol, so anaerobic glycolysis is limited and less lactate can be generated during the sustained period of ischemia in PC hearts (Steenbergen, Perlman et al. 1993). A decrease in glycolisis results a decrease in lactate formation and acidosis. Consequently, IPC reduces the activation of the Na-Ca exchanger so that intarcellular Na+ has not to be extruded from the Na-Ca exchanger. In this way IPC reduces the extent of intracellular Ca^{2+}(Fujio, Nguyen et al. 2000) overload. An additional mechanism to reduce ATP hydrolysis is through inhibition of Fo-F1 ATPase. Beside this metabolic action a large body of evidence support the notion that IPC efforts cardioprotection by activating pro-survival pathways. In this respect relevant role is played PI3K-Akt-GSK3β pathway.

Akt-PI3K-GSK3 β pathway

Initially Akt was described as a oncogenic protein. Akt is upregulated in many types of tumors, contributing to facilitating tumor growth through a reduction in apoptosis. In the heart Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K) in response to stimulation of receptor tyrosine kinases (Fujio, Nguyen et al. 2000), glycoprotein 130, and G-protein coupled receptors. (Tong, Chen et al. 2000).

PI3K is a lipid and protein kinase, that catalyzes the phosphorylation of the inositol ring of phosphoinositides at D3 position. Its activation was associated with cytoprotection, and it is involved in β-adrenergic receptor endocytosis. Its action is usually attributed to

generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) which facilitates PDK1 phosphorylation of substrates, such as Akt, p70S6K, PKC. Downstream of PI3K, Akt mediates protection by phosphorylating various targets, such as GSK-3β, endothelial nitric oxide synthase (eNOS) , the proapoptotic Bcl2-family member BAD, caspase 9.

Glycogen synthase kinase 3 was initially described in the process of glycogen synthesis. This kinase is involved in the regulation of many other cellular functions. Two isoforms exist but the β isoform is the most extensively studied in the cardiac pathophisiology. In the heart this kinase is involved in pro-apoptotic pathway and that is a suppressor of hypertrophic responses.

Under basal conditions GSK-3β is active. Akt inhibits GSK-3β activity through phosphorylation at Ser9. GSK-3β phosphorylation has been reported to be involved in the PC. One modality of protection appears to depend on its interaction with ANT, leading to a decrease in the association of ANT with Cyp-D that has been proposed to decrease the opening probability of PTP (Nishihara, Miura et al. 2007). A more recent study suggested that GSK-3β inhibition might decrease the mitochondrial membrane potential, reducing the driving force for the mitochondrial Ca^{2+}uptake and ROS formation in reperfusion. The resulting reduction in mitochondrial Ca^{2+} accumulation and ROS level decreases the loss of cell viability .(Nishihara, Miura et al. 2007).

3.9. MCU

Mitochondria are very essential components of intracellular Ca^{2+}signaling and cell metabolism. Importantly Ca^{2+}regulates key enzymes of the TCA cycle (Denton and McCormack 1990). Thus, by determining the rate of ATP production Ca^{2+} regulates the metabolic adaptations to nutrients. (Rizzuto, De et al. 2012) .

In particular, Ca^{2+} in the mitochondrial matrix regulates aerobic metabolism. Indeed, there are two dehydrogenases of Krebs'cycle (isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) are stimulated by Ca^{2+} as well as pyruvate dehydrogenase (Melendez-Hevia, Waddell et al. 1996). By activating these hydrogenases more electrons are made available for the electron transport chain. The increase flux of electrons results in an increase rate of ATP synthesis that matched the ATP demand detected by contractility activated by Ca^{2+} in the cytosol. The entry of Ca^{2+} depends on the mitochondrial membrane potential and according to Nerst equation and the intramitochondrial [Ca^{2+}] could be six orders of magnitude larger than cytosolic [Ca^{2+}]. This enormous Ca^{2+} accumulation is prevented by release pathways, the most important in the heart is the mitochondrial Na-Ca exchange. Although the key role of Ca^{2+} in mitochondrial function and aerobic metabolism has been recognized starting from the 60s (Carafoli, Rossi et al. 1965), the field has been limited by the lack of knowledge of

the molecular identity of the mitochondrial calcium uniporter. This issue was solved in 2011 by two independent laboratories (De Stefani, Raffaello et al. 2011) (Baughman, Perocchi et al. 2011). The investigation of the molecular identity of MCU benefited from the genome-wide approach based upon the availability of "MitoCarta". This mitochondria geneteque was obtained by performing mass spectrometry analyses on both highly purified and crude mitochondrial preparations from 14 different mouse tissues. Another helpful information was that the yeast is not devoid the mitochondrial calcium uptake. Finally, MCU has no orthologue in the yeast S. cerevisiae, which lacks a Ruthenium Red-sensitive mitochondrial Ca^{2+} uptake system (Carafoli, Balcavage et al. 1970). The first protein identified was "mitochondrial calcium uptake 1" (MICU1). This protein containing an EF domanis, was recognized as a putative regulator of the uniporter. The MitoCarta and MICU1 allowed the final identification of the protein CCDC109A, which was termed as MCU and fulfils all the requirements to represent the mitochondrial calcium uniporter. Indeed, this protein demonstrated a Ca^{2+} selectivity in the IMM, and its reconstitution in a planar lipid bilayer generated a Ca^{2+} current. Moreover, a single point mutation abolished the sensitivity of MCU to Ruthenium Red (Chaudhuri, Sancak et al. 2013). Notably, the overexpression of CCDC109A/MCU almost doubled the mitochondrial Ca^{2+} content in both intact and permeabilized HeLa cells, parallel to a significant decrease in the cytosolic Ca^{2+}content due to enhance the mitochondrial buffer activity (De Stefani, Raffaello et al. 2011). On the other hand, the down-regulation of MCU strongly inhibits mitochondrial Ca^{2+} uptake while the re-introduction of the MCU in knock-down cells fully rescues Ca^{2+} uptake.

The nuclear MCU gene, located on chromosome 10, encodes a 40-kDa protein that looses its cleavable target sequence during mitochondrial import, resulting in a 35-kDa mature form. Although the topology of MCU was initially a matter of debate , it is now clear that both its N- and C-terminal domains are located in the mitochondrial matrix , while its 9-aa linker domain faces the intermembrane space (Baughman, Perocchi et al. 2011). The two transmembrane α-helixes are highly conserved among different species.

The fact that MCU contains only two transmembrane domains has suggested that functional uniporter channel could be formed by MCU oligomers. The predicted quaternary structure is compatible with a tetramer, in which eight helices line the putative pore region, and the clustering of charged residues in proximity of the pore generates a negative electrostatic potential that promotes the passage of Ca^{2+}(Raffaello, De Stefani et al. 2013). Indeed, that MCU oligomerizes in the mitochondrial inner membrane as part of a larger complex, migrating at an apparent molecular weight of 480 kDa. Beside, strong evidences support that the uniporter complex includes different regulatory subunits and components, described below.

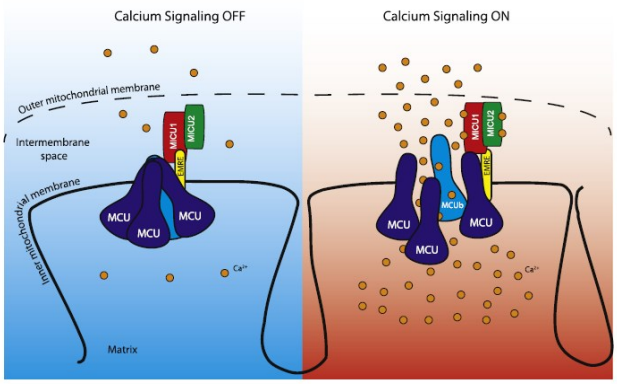


Figure 3.8. **Schematic representation of MCU complex**

MCU B

Based upon genomic analysis a gene closely related to MCU, has been discovered, originally reported as CCDC109B and now known as MCUb (Raffaello, De Stefani et al. 2013). The encoded protein sharing 50% similarity to MCU possesses two coiled-coil domains and two transmembrane domains separated by a short loop that slightly differs from MCU. MCUb is conserved in most of the vertebrates and in many plants, but it is absent in organisms devoid of MCU. MCU can self-oligomerize or hetero-oligomerize with MCUb. A crucial amino acid substitution in the loop region (E256V) removing one critical negative charge in MCUb causes an impairment in Ca^{2+} permeation both in HeLa and in planar lipid bilayers. In addition, the concomitant expression of MCU and MCUb greatly decreased the open probability in planar lipid bilayer, even when MCUb is present in low amount. Therefore, the notion that the insertion of a small fraction of MCUb within the oligomer can efficiently inhibit Ca^{2+} channeling activity. MCUb silencing in HeLa cells has been reported to increase the mitochondrial Ca^{2+} uptake. Therefore, MCUb has been suggested to be the dominant negative component of MCU complex. Notably, the MCU/MCUb ratio varies among different tissue and this is likely to explain differences in mitochondrial calcium uptake displayed in different cell types.

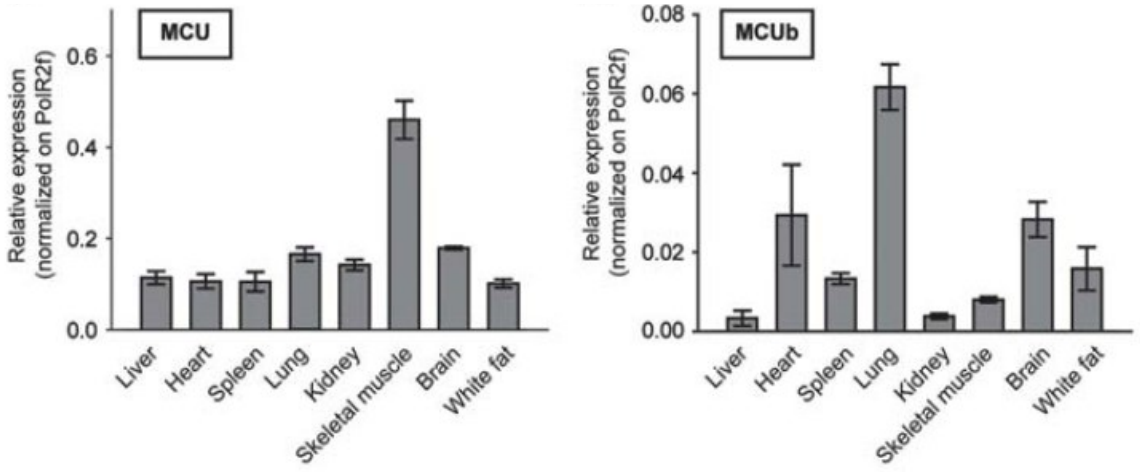


Figure 3.8.2. **MCU and MCUb relative expression in mouse tissue.** The different ratio of MCU/MCUb opens the possibility that this ratio set the overall mitochondrial Ca2+, carrying different capacity in different

EMRE

Although, in a planar lipid bilayer MCU is sufficient per se to form a Ca^{2+} channel, the situation in vivo is likely to be more complex. In particular the activity of MCU in situ, seems to be controlled by EMRE. This 10 kDa protein that spans the IMM and it is characterized by possesses a highly conserve C-terminus rich in aspartate residues (Sancak, Markhard et al. 2013). This protein has been suggested to be required for Ca^{2+} channeling activity keeping the MICU1/MICU2 dimer attached to the MCU complex. However, it has to be pointed out that species, such as plant where MCU and MICU1 are highly conserved EMRE or possible homologues are not present. In any case the relevance of EMRE is supported by the observations that in mammalian mitochondrial EMRE knockdown abrogated completely the mitochondrial Ca^{2+} uptake even when MCU is overexpressed. The interaction between EMRE and MCU could result in a reciprocal stabilization. In fact, EMRE stability is impaired in the absence of MCU, while in EMRE MCU complex became smaller. Therefore, EMRE could play a crucial for an efficient assembly of the MCU complex.

The MICU family

A group of MCU regulators termed as MICU family promotes the closest stay close in resting condition as opposed to more active stay at high [Ca^{2+}] concentration. These regulators located in the intermembrane space, they have an EF hand domain to increase Ca^{2+} sensitivity (Mallilankaraman, Doonan et al. 2012). The MICU family is responsible for the sigmoidal response of MCU to external Ca^{2+}.

MICU1, the first discovered member of MICU family, is described as a gatekeeper for the channel activity. It keeps MCU in the close state when the [Ca^{2+}] is low, yet its absence

decreases the mitochondrial Ca^{2+}uptake (Csordas, Golenar et al. 2013). Additional members have been discovered, such as MICU2 and MICU3 (Lam, Martell et al. 2015). Also these regulators are located in the intermembrane space, but their specific role is far from being elucidated conclusively. The stability of MICU2 is MICU1 dependent, and it appears to act as a gatekeeper at low Ca^{2+}.

Less is known about MICU3 that is predominantly expressed in the CNS (Plovanich, Bogorad et al. 2013).

MCUR1

The MCU complex includes also MCUR1. This 40kDa protein of the IMM displays one predicted transmembrane domain, and one coiled-coil region, with the N-terminal facing the intermembrane space and the major part of the protein exposed to the matrix. MCUR1 and MICU1 could co-immunoprecipitate with MCU, but MICUR1 does not interact directly with MICU1.

SLC25A23

SLC25A23 was recently identified as an additional member of MCU complex (Hoffman, Chandramoorthy et al. 2014). It appears to participate in mitochondrial Ca^{2+} uptake through interaction with MCU and MICU1. However the characterization of its role needs further investigation.

3.10. The role of MCU in I/R injury

Since the molecular identity of MCU has remain elusive until recently, the role of mitochondrial calcium uptake has been extensively investigated by means of pharmacological approaches the interest in this field is related to both the Ca^{2+} dependent control of substrate oxidation and the pathological associations with a wide variety of disease, especially cardiac I/R injury.

The discovery of the molecular identity of MCU is a crucial information to elucidate the role of the mitochondrial calcium uptake in myocardial ischemia and develop potential inhibitors. However data obtaining deleting MCU has generated a controversial set of information.

Initially, Pan and Colleagues generated a mice model lacking MCU in CD1 mice, that was subjected to I/R injury using the Langendorff model. This model revealed that MCU knockdown mice displayed the same degree of I/R injury of WT in addition and surprisingly MCU deletion abolished the protective efficacy of CsA. Therefore, these findings suggest that the occurrence of cardiomyocyte death during post ischemic reperfusion does not depend on both mitochondrial Ca^{2+}uptake and PTP opening.

However, the alternative mechanism causing the loss of viability was not at all elucidated. It is tempting to hypothesized that the extent of Ca^{2+} in the cytosol due to the lack of mitochondrial uptake might stimulate the activity of various of Ca^{2+} dependent enzymes, such as PLA2 , calpain, and CaMK that have been associated with the exacerbation of myocardial injury. The surprising results of initial report were argued by two more recently reports that attribute the expected protection to MCU deletion in the heart . Molkentin and Collogues generated a C57/BL6J mice with adult and cardiomyocyte-specific deletion of MCU. Their mitochondria were refractory to acute Ca^{2+} uptake, with impaired ATP production, and inhibited PTP opening upon acute Ca^{2+} challenge (Kwong, Lu et al. 2015). Mice lacking MCU in the adult heart was protected from acute ischemia-reperfusion injury. Even if resting/basal mitochondrial Ca^{2+} levels were normal in hearts of MCU-deleted mice, this mice was unable to immediately sprint on a treadmill. Thus, these data suggest that MCU is a dedicated regulator for the mitochondrial Ca^{2+} loading underlying a ‘‘fight-or-flight’’ response that acutely matches cardiac workload with ATP production. In addition, Anderson and Collogues using a dominant negative mouse heart (DN-MCU), demonstrated that hearts had preserved $\Delta\Psi m$ and reduced ROS during ischemia reperfusion but were not protected from myocardial death as compared to WT (Rasmussen, Wu et al. 2015). This paper appears to support the notion brought by the paper by Pan and all suggesting that chronic inhibition of myocardial MCU leads to previously unanticipated compensatory changes that prevent the protection against I/R injury that will be expected from MCU deletion. Further studies are necessary to clarify how those possible compensatory changes promote the loss of viability during post ischemic reperfusion.

The current debate on the role of MCU is only based upon studies using MCU deletion no information have been generated overexpressing MCU in the heart. We followed this approach to generate the information describing in this Thesis.

4. MATERIALS AND METHODS

4.1. Cells culture

4.1.1. Isolation of neonatal rat ventricular myocytes (NRVMs)

Ventricular myocytes were isolated from 1-3 days old Wistar rats. Hearts were excised from rats anaesthetized with diethyl ether, transferred to an ice cold, sterile solution (solution A) containing 140mM NaCl, 4.8mM KCl, 1.2mM MgSO4, 4mM NaHCO3, 1.2mM NaH2PO4, 12.5mM D-glucose, 10mM HEPES, pH 7.4, and washed twice. Hearts were then cut in small fragments. Tissue fragments were dissociated with an enzyme solution containing trypsin (Invitrogen) at 4 °C for overnight, under continuous stirring.

The day after the small fragments were further dissociated by incubating them with an enzyme solution containing Collagenase type I (Gibco), at 37°C for 2 minutes 4 times, under continuous stirring. Pooled cell suspensions were pelleted by centrifugation (1000 rpm, 7 min) . To purify them, cells were filtered and resuspended in growth medium consisting of MEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (100U/ml penicillin and 100ug/ml streptomycin), and are pre-plated for 90 min.

After this incubation, only fibroblasts are attached to the flask, while myocytes remain in the supernatant. Cells are counted, diluted to appropriate concentration with MEM supplemented with 10% FBS, antibiotics, non-essential amino acids (NEAA) , and 0.1mM BrdU, necessary to inhibit cell proliferation. Cells are plated in gelatin O.1% coating and cultured for 24 hours in 5% CO2 incubator at 37 °C. The day after the cultured media is changed with MEM supplemented with antibiotics, NEAA and 1x ITS.

4.1.2. Transfection

Twenty-four hours after plating, the cells were transfected by lipofectamine (Lipofectamine 3000, Life Technologies) following manufacturer's instructions. Briefly, 5 µg of DNA and 7.5 µL of lipofectamine were added to 100 µL of optiMEM (Gibco) and distributed upon each well of a 6-well plate. After 2 hours of incubation, the transfection mix was replaced with fresh complete medium.

The cells were transfected with mitochondrial HyPer; to measure the H2O2 formation; or mitochondria GCaMP to evaluate calcium content within mitochondria, or MICU1 to reconstitute the uniporter complex in the cells. Transfected cells were analyzed 24/48 hours after transfection.

4.2. Amplification and purification of plasmid DNA

4.2.1. Preparation of competent E. coli cells

DH10B E. coli cells were inoculated in a 10 mL overnight culture of Luria Bertani (LB) broth at 37°C. On the following morning, 1 L of LB broth was seeded with the 10 mL overnight culture. The culture was incubated at 37°C while shaking at 250 rpm until the optical density measured at 600 nm (OD600) reaches 0.3-0.4. From this point forward, the remainder of the preparation was done in the cold room. The 1 L culture was split into 50 mL sterile tubes and the cells were pelleted by chilled centrifugation at 2500 g for 15 min. The supernatant LB broth was discarded and the pellet resuspended by gentle swirling with 100 mL of CaCl2 100 mM. The resuspended cells were incubated on ice for 30 min and then pelleted by chilled centrifugation at 2500 g for 15 min. The resuspension and centrifugation steps were repeated another time. Finally, the cell pellet was gently resuspended in 5 mL of CaCl2 100 mM plus 20% glycerol and divided into 60 µL aliquots. The aliquots were flash freezed in liquid nitrogen and stored at −80°C.

4.2.2. Transformation of E. coli

100 ng of plasmid DNA were added to one aliquot of competent DH10B E. coli cells. Another aliquot of competent cells was used as negative control. The DNA-cell mixture was incubated on ice for 30 min and then heat shocked by keeping it at 42°C in a termoblock for 90 sec. The aliquots were immediately returned to ice for 2 min. The cells were recovered by adding 900 µL of LB broth and incubating at 37°C while shaking at 250 rpm for 1 h. The cells were pelleted by centrifugation at 4000 g for 5 min. The two pellets was resuspended in a small volume of LB broth and distributed into previously prepared LB agar plates containing ampicillin 100 µg/ml. The plates were incubated in a stationary 37°C incubator to grow the bacterial colonies.

4.2.3. Amplification and purification of plasmid DNA from E. coli

One colony from LB agar plate seeded with transformed DH10B E. coli was collected and inoculated in a 250 mL overnight culture of LB broth at 37°C. On the following morning, the bacteria culture was centrifugated at 4000 rpm for 10 min and plasmid DNA was extracted and purificated following the manufacturer's instructions of a commercial kit (PureLink HiPure Plasmid Maxiprep Kit, Life Technologies).

4.3. Fluorescence Microscopy

4.3.1. Measurement of reactive oxygen species (ROS) production in situ

ROS formation in intact cell was measured using two different probes: MitoTracker Red (MTR, Molecular Probes, ʎ ec=554nm , ʎ em=576nm) and mitochondrial Hyper. The MTR is a rosamine derivative that accumulates selectively in the mitochondria due to the difference in the mitochondrial membrane potential. The MTR presents a chloromethyl moiety that can bind to the thiol groups present at the level of mitochondrial proteins and be retained in these organelles. The increase in the fluorescence intensity is due to the oxidation of MTR that occurred detected in presence of ROS production. At the moment of the experiments, cells were incubated for 25 minutes at 37°C with 25nM MTR in HBSS. Following the incubation , cells were washed twice with HBSS.

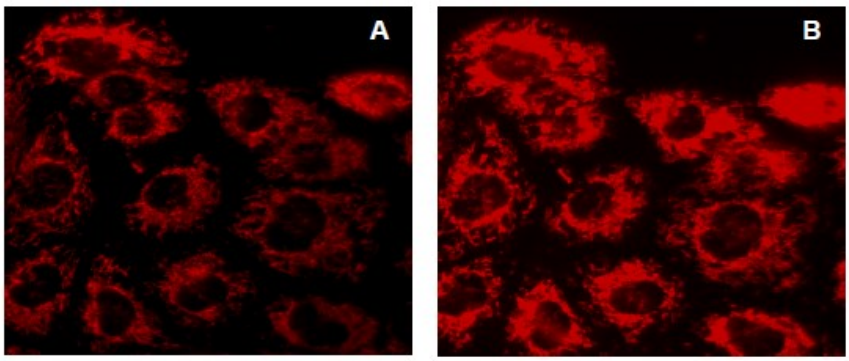


Fig.4.3.1 **HL-1 cardiomyocytes loaded with 25nM MTR and visualized by fluorescence microscopy**. Images were acquired at the beginning of the experiment (panel A) and after hydrogen peroxide administration, to appreciate an increase of fluorescence intensity due to ROS formation (panel B).

The second probe that has been used is Mitochondrial HyPer (HyPermit, Evrogen, λec=420-480nm, λem=520nm). It is fully genetically encoded fluorescent sensor capable of detecting intracellular peroxide (H2O2). It has been developed on the basis of yellow fluorescent protein inserted into the regulatory domain of E. coli protein OxyR. Hyper demonstrates submicromolar affinity to hydrogen peroxide and it is insensitive to other oxidants tested, and it does not cause artifactual ROS generation due to light exposure. The oxidation of OxyR determines a shift in protein conformation that causes an increase of fluorescence intensity.

Fig.4.3. 2. **NRVMs transfected with mitochondria-targeted HyPer mito, stained with mitochondria dye MTR and their overlap.**

4.3.2. Measurement of mitochondrial calcium oscillation

To detect the mitochondrial calcium oscillation in vivo, cell were transfected with mito GCaMP (λ ec=410-480nm, λem=520nm), an encoded genetically protein targeted to mitochondria. This probe has been developed on the basis of a GFP.

4.3.3. Determination of oxidative stress by DHE fluorescence staining

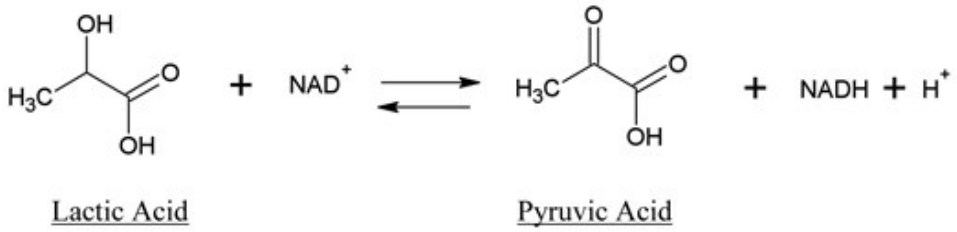
LV tissue was cryosectioned to 10 micron thick slices using a LEICA CM 1580 microtome, termostated at -24°C. Sections were attached to slides. ROS production was determined measuring variations in fluorescence intensity of the probe dihydroethidium (DHE). Once inside the cell, this probe can be oxidized by ROS to the fluorescent compound ethidium and irreversibly binds to nuclear DNA, that determines a further increase in fluorescence intensity. DHE (sigma) was prepared as 1mM stock solution in DMSO and then diluted to a final concentration of 5µM in degassed PBS. Because of the solution is photosensible, all the operations were carried out in the dark. The slides were incubated with DHE solution for 30 minutes at 37°C in a humid atmosphere, and the they were washed three times with degassed PBS to remove the excess of DHE. Slides were mounted with Prolong solution (Invitrogen) and covered with a cover slide. Slides were analysed using a fluorescence microscope and 20x air objective at 568nm and 585nm excitation and emission wavelengths respectively.

4.4. Determination of cell viability

4.4.1. LDH measurement

The release of lactate dehydrogenase (LDH) from NRVMs was measured to evaluate cell death occurring during post ischemic reperfusion . The same method was used to determine the amount of LDH released from the hearts exposed to I/R. In this latter

case, coronary effluent was collected at 1 min intervals during the 30 min of reperfusion. At the end of reperfusion hearts were collected and homogenized for assessing the residual activity of LDH in the whole tissue. Since all values were normalized to heart weight, the amount of LDH released was expressed as % of total (i.e., effluent + homogenate) to rule out possible changes due to variations in heart size. LDH activity was determined by spectrophotometrically measuring the absorbance of NADH at 340nm(reduction of pyruvate to lactate) accordingly to the following reaction.



Lactic Acid Pyruvic Acid

4.4.2. Tunel assay

The rate of apoptosis was measured in NRVMs using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method. NRVMs were seeded onto 12 well plate pretreated with gelatin and grown in MEM supplemented with antibiotics, NEAA and 1x ITS . Cells were then incubated in a solution of 10µM propidium iodide for 15 minutes at 37°C and visualization of all nuclei was performed by staining with Hoechst 33258. Cells were washed twice with PBS to remove the excess fluorescence background. The number of total and TUNEL-positive nuclei was determined in randomly selected fields by using a Zeiss (Oberkochen, Germany) Axioplan microscope (×40 magnification) equipped with a digital camera.

4.5. Oxygen consumption rate measurement

NRVMs (60000/well) were seeded onto Seahorse 24-well microplates 24 hours prior to the analysis on the Seahorse XF24 extracellular flux analyzer (Biosciences) following the manufacturer's instructions. All experiments were performed at 37°C. Oxygen consumption rate data consist of mean rates during each measurement cycles consisting of a mixing time of 30 sec and a waiting time of 3 min followed by a data acquisition period of 3 min. Rates displayed are basal respiration and rates following addition of 1 µg/ml oligomycin, 1µM FCCP, 1 µM rotenone and 1 µM antimycin A. The FCCP concentration was previously determined as that optimal concentration to maximize the stimulation of mitochondrial respiration of cells.

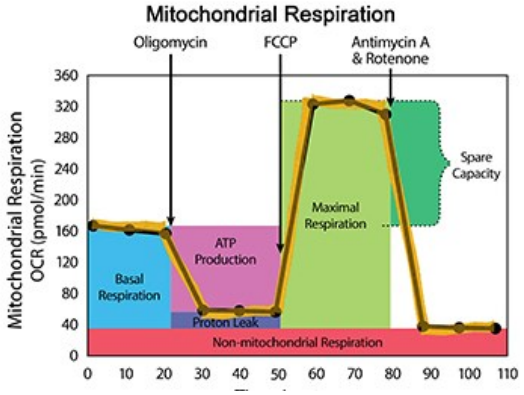


Figure 4.5.1. The fundamental parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, maximal respiration and spare respiratory capacity.

4.6. Ischemia Reperfusion Injury

4.6.1. Treatment protocols for I/R injury in mice

Adult (aged 4 months) C57/BLJ6 WT mice (Charles River, Milan, Italy) were anaesthetized with an intraperitoneal injection of Zoletil 100 (30 mg/kg). Hearts were perfused with bicarbonate buffer gassed with 95% O_2–5% CO_2 at 37 °C (pH 7.4) at a constant flux of 5 ml/min. Perfusion was performed in the nonrecirculating Langendorff model. The perfusion buffer contained (in mM) 118.5 NaCl, 3.1 KCl, 1.18 KH_2PO_4, 25.0 $NaHCO_3$, 1.2 $MgCl_2$, 1.4 $CaCl_2$ and 5.6 glucose. Hearts were treated as follows (n ≥ 3/group): after 10 min of stabilization, hearts were subjected to 40 min of global ischemia (I-40) by stopping the coronary flow and 30 min of reperfusion (R-30). After reperfusion hearts were quickly immersed into PBS containing 0.5% Triton X100 and homogenized for measurement of lactate dehydrogenase (LDH).

4.6.2. I/R protocol in NRVMs

Neonatal ventricular cardiac myocytes were incubated with a balanced salt solution. The composition was as followed: NaCl 115 mM, KCl 5 mM, KH_2PO_4 1 mM, $MgSO_4$ 1.2 mM; $CaCl_2$ 2 mM and HEPES 25 mM. Simulated ischemia was induced adding 5mM potassium cyanide (KCN) and 20mM 2-deoxy-D-glucose, and maintained for 60 minutes in a 5% CO2 at 37°C. simulated reperfusion was induced by removing cyanide and rinsing cells twice with PBS, after that cell were kept for additional 60 min in a solution composed of NaCl 150 mM, $MgSO_4$ 2mM, $CaCl_2$ 2mM, HEPES 20mM, Glucose 10mM for 60 minutes during the reperfusion period.

4.6.3. Hypoxia/Reperfusion protocol in NRVMs

The microfluidic chip used for H/R experiment is composed by three indipendent culture chambers and a climatic chamber in an overall surface of 17x15 mm. The chip is divided into three layers: the glass coverslip makes the culture surface, a thin layer of highly gas permeable PDMS confines the culture chamber separating the liquid phase from the upperlayer in which the gas flows. Fast transitions from normoxic to anoxic conditions can be achieved using this chamber. The cells were subjected to 3 hours of hypoxia (95% N_2 and 5% O_2) followed by 3 hrs of reperfusion (95% air and 5% CO_2).

4.7. Protein analysis by gel electrophoresis (Western Blot)

4.7.1. Samples preparation

Samples were prepared by frozen LV tissue stored at -80°C. 50mg was homogenized in 1ml lysis buffer. The composition of lysis buffer was:

50mM Tris

150mM NaCl

10mM $MgCl_2$

1mM EDTA

2% SDS

1% Triton

10% Glicerol

1X protease inhibitor mix (Roche)

1X phosphatase inhibitor (Roche)

Homogenized samples were centrifuged at 13000xg for 10 minutes at 4°C and the pellet was discarded. Protein concentration was determined by Bradford assay. To denature and solubilize the protein the sample buffer was added , and samples are heated at 70°C for 10 minutes at 1250rpm. Then they were loaded in a gel or aliquoted and stored at -80°C.

Other samples were obtained from NRVMs. Cells were washed twice with PBS and then 200µl of RIPA buffer was added in each 6 well plate. Ripa Buffer was composed of:

25ml PBS

1% NP-40 (100%)

0.25% $C_{24}H_{39}NaO_4$

2mM EDTA

1X anti protease inhibitor (Roche)

2X anti phosphatase inhibitor (Roche).

Cells were scraped and centrifuged at 12000xg for 10 minutes at 4°C. Then the pellet was discarded and the protein concentration was determined by Bradford assay. To denature and solubilize the protein the sample buffer was added , and samples are heated at 70°C for 10 minutes at 1250rpm. Then they were loaded in a gel or aliquoted and stored at -80°C.

4.7.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

Electrophoresis was performed on polyacrylamide gel prepared in glass slabs 1mm thick with 12% acrylamide in the separating gel and 4% in the stacking gel. The following solutions were used for the preparation of the gel and the electrophoresis run:

Acrylamide/bisacrylamide: 30% acrylamide and 0.8% bisacrylamide

Lower Tris-HCl (4x): 1.5M Tris-HCl and 0.4% SDS, pH 8.8

Upper Tris-HCl (4x): 0.5M Tris-HCl and 0.4% SDS, pH 6.8

Running buffer (4x): 0.1M Tris-HCl, 0.77M glycine and 0.4% SDS, pH 8.3

The polymerization of the gel was obtained by the addition of TEMED (Invitrogen) and ammonium persulfate 0.1 mg/ml (invitrogen). Samples were run on the gel at room temperature using an Electrophoresis Power Supply (Apelex) that provided a constant voltage of 70V in the stacking gel and 120V in the separating gel. In order to make the proteins accessible to antibody detection, they were moved from within the gel onto a nitrocellulose membrane.

Once the samples finished the run, the gel was washed from the excess SDS with Transfer Buffer (Tris 25mM, glycine 192mM, methanol 20%, pH 8.0). A 0.45µm nitrocellulose membrane (BioRad Laboratories) was placed on top of the gel, avoiding creating bubbles, and a stack of tissue papers placed on the top. This stack was then inserted into a transfer box filled with Transfer Buffer, so that the gel is oriented towards the cathode and the membrane towards the anode. When a current is applied to the electrodes, this causes the protein migration from the negatively charged cathode

to the positively charged anode towards the membrane. The separating was transferred 17 h with 150 mA of current. The transfer was performed at 4°C. Once the transfer was carried out, the membrane was saturated with BSA 5% in TBS (Tris-HCl 50 mM, NaCl 150 mM, pH 7.5) for 3h at room temperature. The antibodies used to detect the proteins of interest were diluted in BSA 1% in TBS. The following primary antibodies were used:

Rabbit anti-MCU (Sigma)

Mouse anti-FLAG (Sigma)

Rabbit anti-AKT total (Cell Signaling Tecnology)

Rabbit anti-Thr308 AKT (Cell Signaling Tecnology)

Rabbit anti-Ser473 AKT (Cell Signaling Tecnology)

Mouse anti-GADPH (GenTex)

Rabbit anti-PP2A tot (Cell Signaling Tecnology)

Rabbit anti-Tyr307 PP2A (Cell Signaling Tecnology)

Rabbit anti-PP2A-B ((Cell Signaling Tecnology)

Rabbit anti-PP2A-C (Cell Signaling Tecnology)

Rabbit anti-GSK3β tot (Santa Cruz Biotecnology)

Rabbit anti-phospho GSK3β (Santa Cruz Biotecnology)

All the primary antibody incubations were carried out overnight at 4°C.Following the incubation, membranes were washed three times with TBST (TBS and 0.1% Tween 20, pH 7.4, 10 minutes each wash). Secondary antibodies were diluted in blocking solution and incubated with the membrane for 1 hour at room temperature. Secondary antibodies used were:

Anti mouse (Pierce), dilution 1:2000

Anti rabbit (Pierce), dilution 1:2000

Secondary antibody in conjugated to a reporter enzyme, namelyhorseradish peroxidase (HRP). When in the presence of a chemiluminescent agent, they can interact and the reaction product produces luminescence in proportion to the amount of protein. Prior to the detection, membrane was washed three times (15 minutes each wash).

4.7.3. Chemiluminescent detection

Membranes were exposed to LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone) for 1 min. This incubation causes the generation of luminous signal due to the oxidation of the substrate by horseradish peroxidase bound to the secondary antibody. The light, emitted at λ_{max} 340 nm, was detected by a CCD camera (Image Station 440 CF, Kodak).

4.7.4. Densitometry

Images of the acquired western blots were analysed using the Image J software. This program allows the quantification of the optical density of bands or dots that is directly proportional to the protein content.

4.8. In vivo MCU overexpression

The MCU overexpression was performed using an adeno associated virus type 9, injected at P8 in C57/BLJ6 mouse encoding for MCU-FLAG. The efficiency of the infection was evaluated performing an immunofluorescence staining using an anti-FLAG antibody.

4.9. Immunofluorescence

The efficiency of infection was assessed by staining LV cryosections with anti-FLAG antibody to detect the presence of MCU-FLAG encoding by the injected AVV9. Frozen crysections of the tissue fixed with paraformaldehyde 1% for 15 min were permeabilized with Triton 0.2% in PBS for 10 min. Then they were saturated with BSA 4% in PBS for 1 h. All these steps were performed at room temperature. Saturated cryosections were incubated with mouse anti-FLAG antibody (GeneTex) 1:100 in BSA 0.5% overnight at 4°C. The cells were washed 3 times with PBS for 10 min and then incubated with Alexa Fluor 647 goat anti-mouse IgG antibody (Life Technologies) 1:100 in BSA 0.5% for 30 min at room temperature. The washing steps were repeated. The cryosections were finally incubated with 10 µM Hoechst 33342 (Sigma) 10 min at room temperature and the coverslips were mounted onto glass slides using ProLong Gold Antifade Reagent (Life Technologies). Images were collected using a confocal microscope (Leica SP5), a 63 x oil objective and appropriate emission filters.

4.10. Statistics

Results are presented as mean ± standard error. When the experiment was repeated more than 3 times, comparisons between two groups of data were performed via 2-tailed unpaired Student t test. A P-value less than 0.05 was taken to indicate a significant difference that was then tagged with an asterisk.

5.AIM OF THE STUDY

Ischemia and post-ischemic reperfusion cause an irreversible alteration of cardiac myocytes structure and function eventually resulting in a loss of cell viability. A crucial role in determining this alteration has been attributed to mitochondria through a sequence of events where by a massive increase in intramitochondrial Ca^{2+} and reactive oxygen species (ROS) promote the opening of the mitochondrial permeability transition pore that causes cell death. However, mitochondrial Ca^{2+} and ROS are involved in physiological activity as well, in particular an elevation in mitochondrial Ca^{2+} is required for increasing respiration and ATP synthesis while a moderate increase in ROS is necessary to stimulate the activity of survival pathways. The advancement in the understanding of the role of mitochondrial Ca^{2+} uptake in the I/R injury in the heart has been limited for many years by the lack of knowledge of the responsible protein for mitochondrial Ca^{2+} uptake. Therefore, the link between the mitochondrial Ca^{2+} content in I/R injury was established by only pharmacological approaches using non-specific drugs. This scenario was radically changed by the discovery of the mitochondrial Ca^{2+}uniporter that now allows the use of genetic approaches to define the role of mitochondrial Ca^{2+} uptake in cardiac pathophisiology.

The aim of the present thesis was to characterize the role of MCU in cardiac injury induced by ischemia and reperfusion. The initial working hypothesis was that the deletion of MCU should have a reduce of the loss of cell viability that, on the other hand, was expected to increase in experimental models of MCU overexpression. We tested this hypothesis both in vitro of isolated cardiomyocytes and in intact heart overexpressing MCU by means of infection with AAV9. Surprisingly the result contradicted the initial hypothesis especially showing that MCU overexpression confers a robust protection against I/R injury. Consequently, we aimed at the elucidating the protective mechanism triggers by MCU overexpression. In this case the hypothesis was that MCU induced protection mimics the beneficial effect of ischemic preconditioning. Accordingly, we hypothesized that the increase in mitochondrial Ca^{2+} could induce a mild oxidative stress promoting the activation of survival kinases and in particular Akt. The result obtained both in NRVMs and perfused heart validated entirely our hypothesis. Finally, we aimed at demonstrating that the protection afforded by MCU overexpression is abolished by a further and massive elevation of mitochondrial calcium. This goal was achieved by co-expressing MICU1 with MCU.

The results obtained in this thesis provide evidence that the transition from protection to injury depends on the degree of elevation in mitochondrial Ca^{2+} and ROS. Indeed, while conforming that a massive rise in Ca^{2+} and ROS hampers the maintenance of cell

viability our results demonstrate that a slight increase in mitochondrial content of these two factors is a crucial step in eliciting cardioprotection.

6.RESULTS

6.1. Effects of MCU overexpression on NRVMs under normoxic conditions

By means of adenovirus infection in NRVMs we sought to determine whether an increase in the mitochondrial calcium uptake alters mitochondrial morphology and function. Initially, WB analysis confirmed that the expression level of MCU in NRVMs was increased 9-fold in NRVMs following adenovirus infection (Fig. 6.1.1).

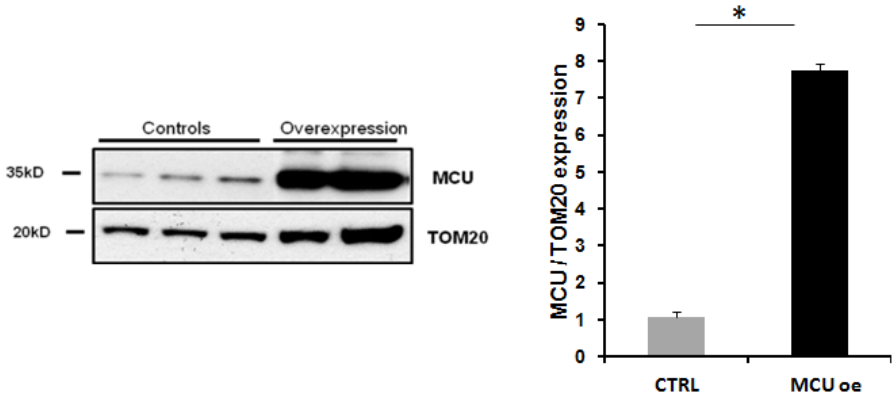


Figure 6.1.1. **Expression level of MCU in NRVMs**. Infection efficiency was determined by WB analysis. MCU overexpression is 9 fold increase in NRVMs after 24 hours virus exposure.

An increase in protein expression is not necessarily associated with an increase in protein activity. This potential mismatch could be due to an incorrect folding process or posttranscriptional processes occurring within a given cell. Therefore, to measure whether the overexpressed MCU was function mitochondrial Ca^{2+} uptake was assessed MCU overexpressing NRVMs. Fig. 6.1.2 shows that MCU overxpression increased the mitochondrial calcium uptake as comparison to control cells, confirming a previous report (Drago, De et al. 2012).

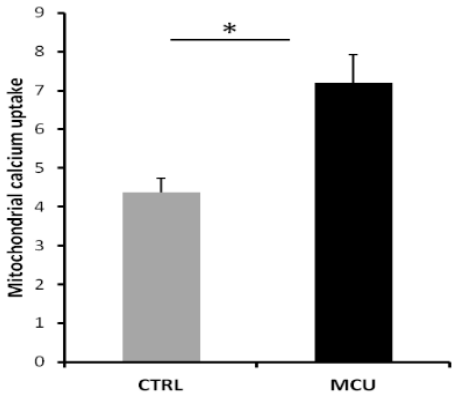


Figure 6.1.2. **MCU overexpression enhanced mitochondrial calcium uptake in NRVMs.** NRVMs were transfected with mtGcAMP, to evaluate the mitochondrial calcium uptake. The first ten frames were measured to determine the basal content of calcium in mitochondria. **p<0.05 vs control*

Following the demonstration that the adenoviral infection resulted in the overexpression of functional MCU, mitochondrial function was monitored by means of TMRM fluorescence. Fig. 6.1.3. shows that MCU overexpressing NRVMs did not display any significant change with respect to control cells.

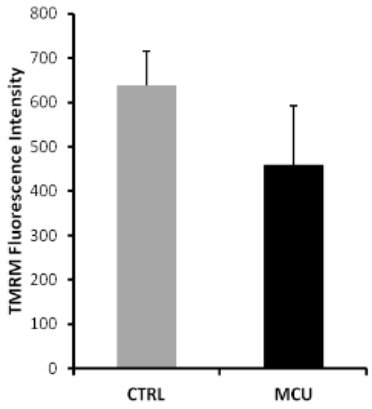


Figure 6.1.3. **MCU overexpression did not affect the mitochondrial membrane potential**. NRVMs were loaded with TMRM (25 nM) to monitor the mitochondrial membrane potential by fluorescence microscopy and the experiment was terminated by adding the uncoupling agent FCCP (4 µM) to fully depolarize mitochondria. The graph illustrates the difference in fluorescence intensities obtained before and after FCCP that renders the assessment of mitochondrial membrane potential independent of possible variations in the cellular (i.e., non-mitochondrial) uptake of TMRM.

Due to the relevance of oxidative stress in dysfunction of both mitochondria and cardiomyocytes and its link to intracellular [Ca^{2+}] increase, we evaluated ROS formation at basal state by using the fluorescent probe MTR which accumulates inside mitochondria, as described in the Material and Methods section. As shown in Fig. 6.1.4., fluorescence values in MCU overexpressing cells were superimposable to those recorded in control NRVMs.

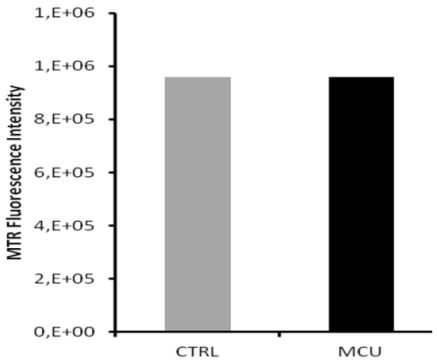


Figure 6.1.4. **<u>At basal state MCU overexpression did not affect ROS formation detected by MTR.</u>** NRVMs were loaded with Mitotracker Red CM-H_2XRos (MTR, 25 nM) for 30 minutes at 37°C.

To further characterize the impact of MCU overexpression on mitochondrial function under normoxic conditions, we evaluated oxygen utilization in NRVMs by means of the SeaHorse oxygraph. In fact, it has to be pointed out that within the mitochondrial matrix Ca^{2+} activates key enzymes involved in substrate oxidation, such as pyruvate, isocitrate and oxoglutarate dehydrogenase. Therefore, by activating these dehydrogenases an increase in mitochondrial [Ca^{2+}] increases the feeding electron to the respiratory chain. Consequently ATP synthesis is increased to match the increase in ATP demand dictated by a rise in cytosolic [Ca^{2+}], as in the case of an increase in force and/or frequency of contraction. In situ mitochondrial respiration of plate-attached NRVMs was monitored by using an extracellular flux analyzer (i.e., the SeaHorse setup). Five parameters of respiration were measured: (1) basal respiration, (2) respiration driving mitochondrial H+ leak (after oligomycin addition), (3) maximal respiration (after FCCP addition), (4) respiration supported by electron flow through complex II (after rotenone addition), (5) non-mitochondrial respiration (after antimycin A addition). MCU overexpressing cells showed the same basal respiration as control cells, and we did not detect any significant difference also in the respiration parameters (2), (4) and (5). However, when FCCP stimulated the maximal respiration by uncoupling the electron transport chain from ATP synthesis, mitochondria of MCU overexpressing cells displayed higher rates of oxygen consumption. This finding indicates an increase in the activity of the electron transport chain as a likely consequence of Ca^{2+}-induced stimulation of dehydrogenase activities.

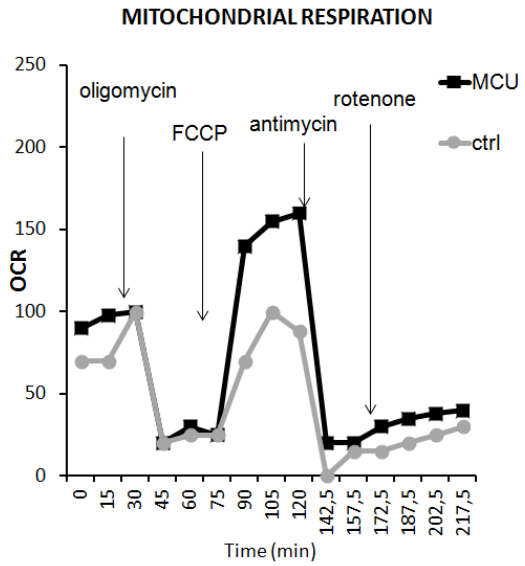


Fig. 6.1.5. **MCU overexpressing cells displayed a higher oxygen consumption rate under stress condition.** To provide a complete characterization of respiratory function, cells were stressed with different stimuli as follows: oligomycin to block ATP synthase, FCCP to uncouple the electron transport chain from ATP synthesis, rotenone and antimycin A to inhibit complex I and III, respectively. The measured oxygen consumption rate was subsequently normalized to the number of viable plated cells.

6.2. Effect of MCU overexpression on viability of NRVMs subjected to I/R injury.

Before the elucidation of the MCU molecular identity a general consensus existed that a large increase in mitochondrial [Ca^{2+}], such as that occurring upon post-ischemic reperfusion, is a determining factor in the loss of viability induced by I/R. In particular, the rise in matrix [Ca^{2+}] is likely to promote the opening of the mitochondrial permeability transition pore (PTP) that leads to the collapse of the membrane potential, cytochrome c release and cell death. In elaborating these notions, the role of mitochondrial Ca^{2+} uptake was defined by means of pharmacological inhibitors devoid of specificity.

The discovery of MCU allowed the genetic testing of the above-mentioned concepts. Surprisingly, the initial study on mice lacking MCU showed that the susceptibility to I/R injury was not at all affected (Pan, Liu et al. 2013). This conclusion was then argued by two papers showing that MCU deletion reduces I/R injury (Kwong, Lu et al. 2015). This discrepancy is not solved yet. Before these papers were published, we started investigating the effects of changes in MCU expression on the susceptibility to I/R injury in NRVMs. To this aim we performed a classical protocol of simulated I/R adding 5mM KCN for 60 minutes (i.e., simulated ischemia) followed by KCN washout (reperfusion) for 60 minutes. The loss of viability was evaluated by assessing the release of LDH in the

supernatant during the reperfusion period. Fig. 6.2.1 shows that MCU deletion by means of siRNA transfection increases the loss of cell viability in comparison to control cells.

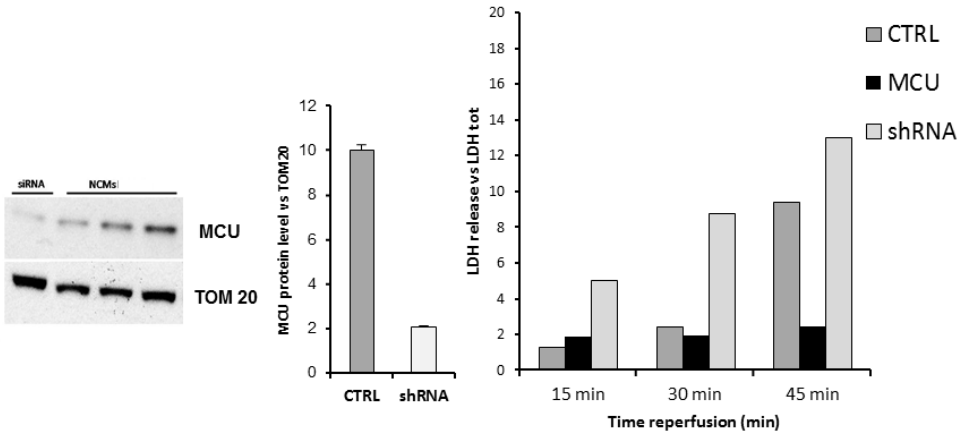


Figure 6.2.1**. <u>MCU deletion increased the loss of cell viability in I/R injury.</u>** MCU deletion was performed through shRNA transfection, and to assess the efficiency of the transfection WB was performed using anti-MCU antibody. After 48 hours of shRNA transfection , a simulated ischemia reperfusion has been performed. The release of LDH was evaluated using a spectrophotometer analysis on the supernatant collected in the post ischemic period.

This finding appears to confirm the results obtained in Finkel's laboratory showing a lack of protection in hearts lacking MCU (Pan, Liu et al. 2013). However, due to the ongoing controversy and, more importantly the results shown below the rest of the study was focused on the effects of MCU overexpression. In fact and surprisingly, as shown in Fig. 6.2.2 MCU overxpression strongly decreased the cell susceptibility to cell death occurring upon post-ischemic reperfusion.

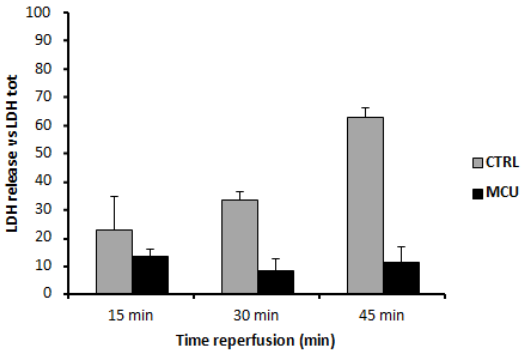


Figure 6.2.2. **<u>MCU overexpression reduced cell mortality in I/R injury</u>.** Cells were subjected to 60 min of ischemia (5mM KCN, 20mM DOG), followed by 60 min of reperfusion. Every 15 min the supernatant was collected to detect the release of LDH, evaluated using a spectrophotometer analysis.

Then we performed the same experiment using CsA, a desensitizer of the PTP. CsA is able to reduce the open probability of the PTP and consequently the occurrence of cell death induced by I/R (Di Lisa, Carpi et al. 2011). Confirming its cardioprotective efficacy the addition of 1 µM CsA at the onset of ischemia decreased the extent of LDH release. Interestingly, not only MCU overexpression afforded a higher degree of protection, but also its effect was additive to that of CsA when the two interventions were combined (Fig. 6.2.3).

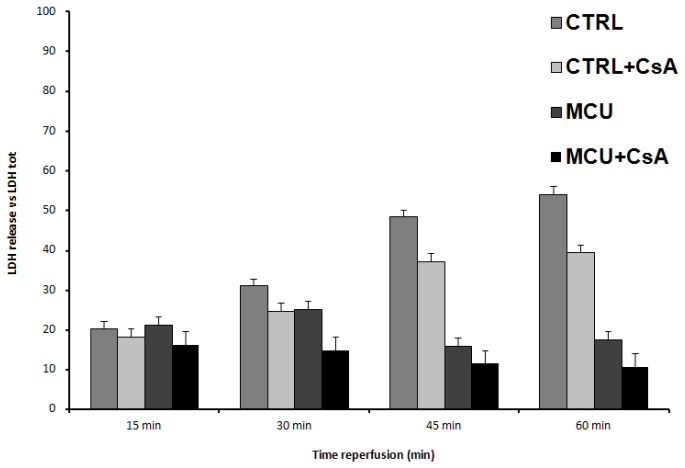


Figure 6.2.3. **MCU overexpression reduces cell mortality in I/R injury.** Cells were subjected to 60 min of ischemia and were incubated with 1µM CsA (5mM KCN, 20mM DOG), followed by 60 min of reperfusion. Every 15 min the supernatant was collected to detect the release of LDH, evaluated using a spectrophotometer analysis.

To override the limitations of simulated ischemia that is based on the non-physiological addition of cyanide, the effects of MCU overexpression were evaluated in a protocol of hypoxia-reoxygenation. To this aim we exploited a hypoxia chamber developed in the laboratory of Prof. N. Elvassore. This chamber equipped with a microfluidic chip is composed by three independent culture chambers and a chamber in an overall surface of 17x15 mm. The chip is divided into three layers: the glass coverslip makes the culture surface, a thin layer of highly gas permeable PDMS confines the culture chamber separating the liquid phase from the upper layer in which the gas flows. This system allows the necessary fast transitions from normoxic to anoxic conditions and viceversa to implement a reliable in vitro model of hypoxia/reoxygenation mimicking in vivo conditions. Preliminary experiments (not shown) were carried out to identify the optimal durations of ischemia and reperfusion to obtain the loss of viability in half of the plated cells. The optimal protocol was 3 hours of hypoxia (95% N_2 and 5%CO_2) followed by 3 hours of reperfusion (95% air, 5% CO_2). The percentage of dead cells was calculated as the ratio between propidium iodide positive cells (i.e., dead cells) and the total

number of cells stained by diethidium bromide. Confirming the data obtained with simulated ischemia, Fig. 6.2.4, shows that in NRVMs overexpressing MCU the extent of cell death induced by post-hypoxic reoxygenation was significantly lower than that obtained in wild type (wt) cells (41.82% ±8.37 vs 60.44% ±11.68, $p<0.05$).

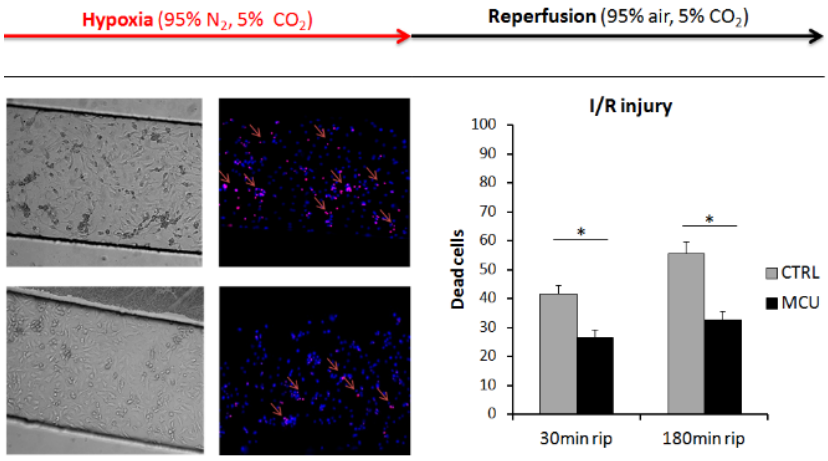


Figure 6.2.4. **MCU overexpression reduced cell deth in Hypoxia/Reoxygenation injury**. Cells were subjected to 3 hours of hypoxia followed by 3 hours of reperfusion. The percentage of viable cells was calculated as the ratio between propidium iodide positive cells (i.e., dead cells) and diethidium bromide stained cells (i.e., total cell number).

6.3. Effect of MCU overexpression on ROS levels in NRVMs subjected to I/R injury.

Upon reperfusion reintroduction of oxygen allows ATP synthesis, yet ischemia-induced alterations of the electron transport chain favour the mitochondrial generation of ROS providing a relevant contribution to I/R-induced injury. Indeed, the concomitant elevation in mitochondrial Ca^{2+} and ROS stimulates PTP opening that hampers the maintenance of cells viability. Counteracting Ca^{2+} overload and ROS levels represents an important strategy to protect the ischemic heart.

We hypothesized that the cardioprotective effect elicited by MCU overexpression might depend on a decreased ROS formation upon reperfusion. To test this hypothesis, mitochondrial ROS formation was monitored by means of Mitotracker Red (MTR) fluorescence. MTR is a reduced derivative of rosamine whose accumulation in mitochondria depends on the mitochondrial membrane potential (Ψm). Of note, the reduced probe does not fluoresce until it enters an actively respiring cell, where it is oxidized to the corresponding fluorescent mitochondrion-selective probe. Once inside mitochondria the chloromethyl moiety drives the covalent binding of MTR to mitochondrial proteins, so that the dye remains sequestered in these organelles. Therefore, following an increased formation of ROS, especially H_2O_2, MTR is oxidized with a parallel increase in fluorescence, directly proportional to the increase in

mitochondrial oxidative stress. Figure 6.3.1 shows that ROS levels increased in control NRVMs upon reperfusion, confirming the well-established occurrence of ROS accumulation when oxygenation is re-established after a prolonged period of ischemia. Interestingly, MCU overexpression resulted in a significant decrease in ROS accumulation validating our hypothesis.

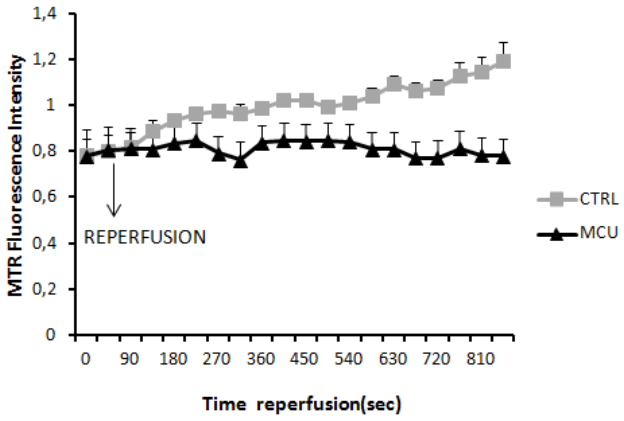


Figure 6.3.1. **Reprfusion-induced ROS formation was decreased in MCU overexpressing cells.** NRVMs were incubated at 37°C with MTR (25 µM) for 30 minutes. Cells were subjected to 60 minutes of simulated ischemia (5mM KCN, 20mM DOG). Upon reperfusion ROS formation was evaluated by monitoring MTR fluorescence.

MTR fluorescence is affected by several shortcomings the most relevant of which are as follows: (i) it is not entirely specific for H_2O_2; (ii) it is not specific for mitochondria. In fact, although in cardiomyocytes mitochondria are the most relevant site for ROS formation, MTR can be oxidized becoming fluorescent also outside of mitochondria; (iii) its accumulation depends on Ψm. Therefore, low fluorescence value could reflect mitochondrial depolarization rather than ROS formation.

To override MTR limitations, ROS measurements were repeated using the genetically-encoded probe HyPer (Huang and Sikes 2014) that offers the following major advantages: (i) it is ratiometric, so that its fluorescence is independent of intracellular levels; (ii) It is highly specific for H_2O_2. Indeed, its affinity for hydrogen peroxide is in the submicromolar range and its fluorescence is not affected by other oxidants tested, such as superoxide, oxidized glutathione, nitric oxide, and peroxinitrite; (iii) it does not generate ROS upon light exposure as is the case with MTR and many fluorescent small molecules (iv) being genetically-encoded it can be used for detection of fast changes of H_2O_2 levels in different cell compartments under various physiological and pathological conditions. Thus, HyPer can be targeted to mitochondria to provide a specific measurement of H_2O_2 generated within the mitochondrial matrix. We exploited the

mitochondrially targeted HyPer (i.e., HyPer mit) to evaluate mitochondrial ROS formation in NRVMs during post-ischemic reperfusion. The results illustrated in Fig. 6.3.2 confirmed that MCU overexpression resulted in a decreased ROS formation during reperfusion.

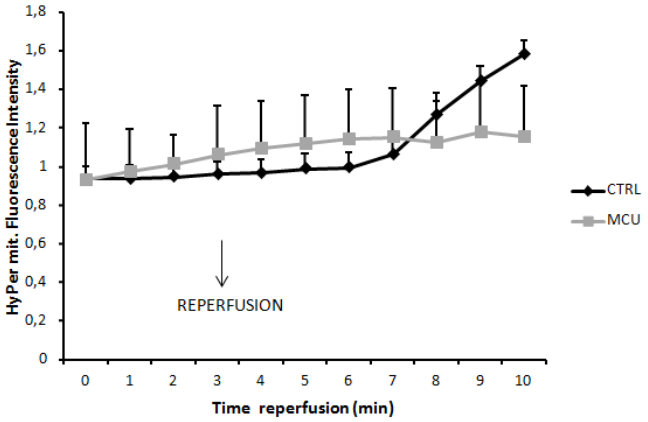


Figure 6.3.2**. <u>ROS formation was decreased in MCU overexpressing cells during reperfusion.</u>** NRVMs were transfected with mitochondrial HyPer and subjected to the simulated I/R protocol 48 hours later. In particular, cells were subjected to 60 minutes of ischemia (5mM KCN, 20mM DOG) and ROS formation was evaluated measuring the fluorescence intensity of HyPer during reperfusion.

On the other hand, as shown in Fig. 6.3.3, MCU deletion did not modify the increase in mitochondrial ROS formation occurring during reperfusion. This finding confirms the notion that I/R induced increase in cell mortality correlates with the sudden rise in ROS levels that characterizes the reperfusion phase.

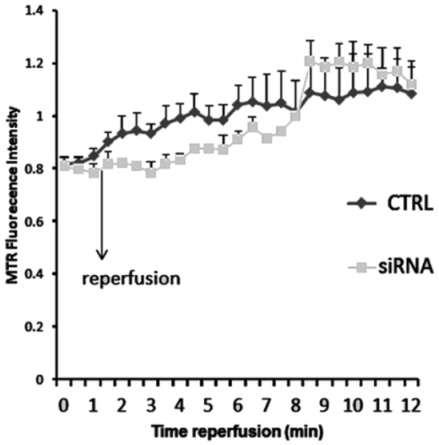


Figure 6.3.3. **<u>MCU deletion affected ROS formation occurred in the post ischemic reperfusion</u>**. NRVMs deleted MCU through shRNA transfection were subjected to I/R injury. Cells were loaded with MTR for 30 min at 37°C. The fluorescence intensity was detected to investigate the burst of ROS occurred in the reperfusion.

6.4. MCU overexpression determined Akt pathway activation

Overall, the evidence obtained with both MTR and Hyper suggests that the protection elicited by MCU overexpression against I/R-induced cell death is associated with and likely depends on a decrease in mitochondrial ROS formation during reperfusion. However, it was necessary to clarify the mechanism whereby an increase in mitochondrial Ca^{2+} reduces the extent of ROS formation and cell death during post-ischemic reperfusion. We focused our attention on Akt activation for the following reasons: (i) several cardioprotective strategies are associated with the stimulation of Akt activity (Fujio, Nguyen et al. 2000); (ii) in skeletal muscle MCU overexpression determines an increase in Akt activation (Mammucari, Gherardi et al. 2015) although the link was not elucidated..

Akt has been shown to play a critical role in cell growth, transcription regulation and cell survival. Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K) in response to stimulation of receptor tyrosine kinases and G-protein coupled receptors (GPCRs). In 1986 a seminal study demonstrated that a powerful cardioprotection can be obtained by performing several brief periods of ischemia (usually 3 cycles of 5 min of ischemia followed by 5 min of reperfusion) prior to the prolonged lethal ischemic insult (Murry, Jennings et al. 1986). This phenomenon was termed ‘ischemic preconditioning’ (IPC). Although the mechanisms involved in IPC protection are still matter of debate, several reports indicate the crucial role of an increased phosphorylation of Akt causing its activation. Akt is thought to mediate protection by phosphorylation of a number of target proteins such as GSK-3β, endothelial nitric oxide synthase (eNOS), the proapoptotic Bcl-2 family member BAD, and caspase 9. We hypothesized that Akt phosphorylation is increased in in MCU overexpressing condition in NRVMs. This hypothesis was validated by the WB analysis illustrated in Fig. 6.4.1 showing that AKT phosphorylation is significantly increased in NRVMs overexpressing MCU as compared to control cells under normoxic conditions (1,94± 0.027 vs 1±0.01 in MCU overexpressing NRVM and control cells, respectively; $p< 0.05$).

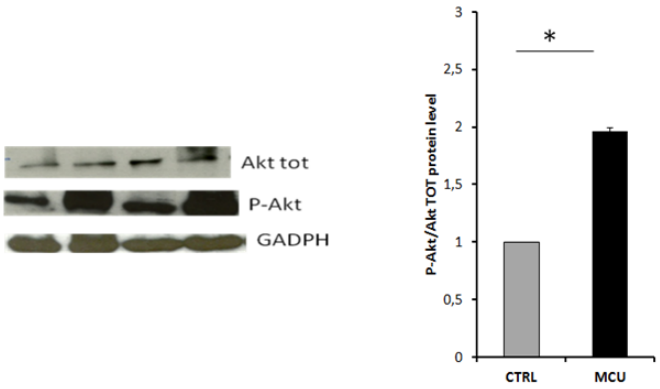


Figure 6.4.1. **MCU overexpression resulted in Akt activation.** MCU overexpression induced an higher Akt phosphorylation. Western blot with anti-pAkt (Ser473) antibody of lysates from NRVMs cells infected with MCU-flag adenovirus.

An important process downstream of AKt stimulation is GSK-3β phosphorylation resulting in its inactivation. GSK-3β phosphorylates and inactivates a number of enzymes involved in metabolism, such as acetylCoA-carboxylase, ATP citrate lyase, and pyruvate dehydrogenase. Moreover, phosphorylation and inactivation of GSK-3β has been reported to be antiapoptotic. In several models inactivation of GSK-3β has been reported to both reduce the extent of apoptosis and play a pivotal role in cardioprotection (Clarke, Khaliulin et al. 2008). To support the evidence of Akt activation, we evaluated also GSK-3β phosphorylation. Figure 6.4.2. shows that GSK-3β is more phosphorylated when MCU is overexpressed in NRVMs (1,45±0.054 vs 1±0.043 in MCU overexpressing NRVM and control cells, respectively; $p<0.05$).

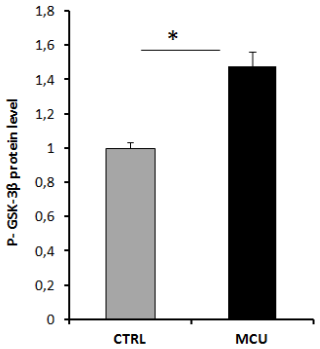


Figure 6.4.2. **MCU overexpression increased GSK-3β phoshorylation.** MCU overexpression induced a higher degree of GSK-3β phosphorylation. Western blot with anti-pGSK3β (Ser9) antibody was carried out in lysates from NRVMs infected with MCU-flag adenovirus.

6.5. MCU overexpression determined a slight increase in ROS formation

A large body of evidence demonstrates the deleterious role of reperfusion-induced ROS formation in promoting PTP opening and loss of viability. On the other hand, however, oxidants can also trigger signalling pathways involved in protective mechanisms, and antioxidants abrogate IPC-induced cardioprotection (Halestrap, Clarke et al. 2007). We hypothesized that in normoxic NRVMs the slight increase in mitochondrial [Ca^{2+}] induced by MCU overexpression might cause a mild rise in ROS that is eventually responsible for Akt activation and cardioprotection. To test this hypothesis mitochondrial ROS formation was assessed by means of HyPer mit, especially for its higher sensitivity as compared to MTR. Using this approach, Figure 6.5.1. shows that cells overexpressing MCU displayed a slight increase in ROS formation with respect to control cells (1,37± 0,064 vs 1 ± 0.067 in MCU overexpressing NRVM and control cells, respectively; p< 0,05).

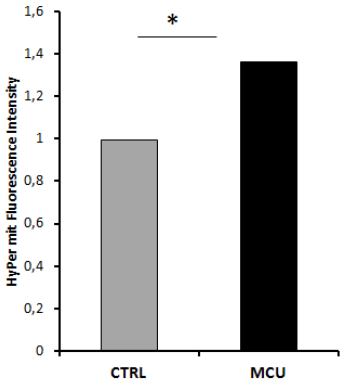


Figure 6.5.1. **MCU overexpression determined a slight increase in ROS formation.** Cells overexpressing MCU transfected with HyPer mit showed a slight increase in ROS formation in comparison to control cells.

Next we assessed the levels of ROS upon antioxidant treatment. As expected, mercaptopropylglicine (MPG) abrogated the rise of ROS levels in NRVMs overexpressing MCU (Fig. 6.5.2.).

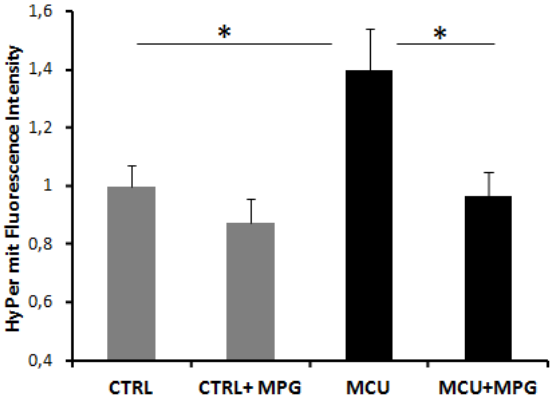


Figure 6.5.2. **Antioxidant treatment reduced ROS formation in MCU overexpressing cells.** MPG was added in the culture media at the concentration of 500μM. Cells were transfected with HyPer mit, and ROS formation was evaluated measuring the fluorescence intensity of the probe.

6.6. ROS formation is involved in Akt pathway

MCU overexpression determines a slight increase of ROS that is detectable using a ratiometric and very sensitive probe for hydrogen peroxide. In addition, we observed that MCU overexpression induces Akt activation that is likely responsible for the decreased susceptibility to I/R injury. We hypothesized that Akt activation was the result of a redox process triggered by the slight increase of ROS formation in MCU overexpressing NRVMs. The results in Fig. 6.6.1. validate this hypothesis showing that the addition of MPG prevented the increase in ROS levels induced by MCU overexpression. Therefore, Akt activation appears to result from a mild increase in mitochondrial ROS levels in response to a slight elevation of mitochondrial [Ca^{2+}] induced by MCU overexpression.

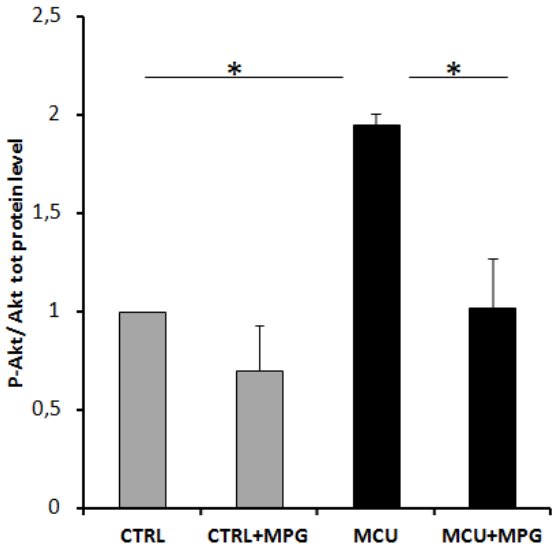


Figure 6.6.1 **Antioxidant treatment reduced Akt phosphorylation in MCU overexpressing cells.** NRVMs overexpressing MCU through adenovirus infection were treated with MPG 500µM. After 48 hours of treatment, Western blot analysis was performed with anti-pAkt (Ser473) antibody.

6.7. PP2A is involved in Akt activation

Akt activity is not directly modulated by ROS. We hypothesized that the ROS-dependent Akt activation in NRVMs overexpressing MCU depends on redox sensitive kinases or phosphatases upstream of Akt. In particular, an inhibition of Ser/Thr phosphatases may lead to Akt activation. We focused our attention on PP2A which, along with other Ser/Thr phosphatases, dephosphorylates Thr308 and Ser473 in Akt downregulating its activity.

Protein phosphatase 2A comprises a family of serine/threonine phosphatases containing a well conserved catalytic subunit, which is highly regulated by means of phosphorylation and methylation. PP2A plays a prominent role in the regulation of specific signal transduction cascades (Seshacharyulu, Pandey et al. 2013). The core enzyme is a dimer (PP2AD), consisting of a 36 kDa catalytic subunit (PP2AC) and the regulatory A with a molecular mass of 65kDa. A third regulatory B subunit can be associated with this core structure. In vitro, the catalytic subunit of PP2A can be phosphorylated by the tyrosine kinases pp60, pp56, as well as by the epidermal growth factor and insulin receptors. The phosphorylation occurs on Tyr 307 that is located in the conserved C-terminal part of PP2AC and results in the inactivation of the enzyme. In vivo, phosphorylation occurs in response to growth stimulation, insulin and TNFα. ROS have been shown to inhibit protein phosphatases 2A in a process that is abrogated by

antioxidant treatment in neurons (Chen, Liu et al. 2009). In addition, PP2AC is inhibited by phosphorylation.

To test whether ROS could abrogate PP2A activity in our model of MCU overexpression, firstly we evaluated the protein expression of the PP2AC subunit, that conserved the catalytic domain that was not modified by MCU overexpression (Fig. 6.7.1.).

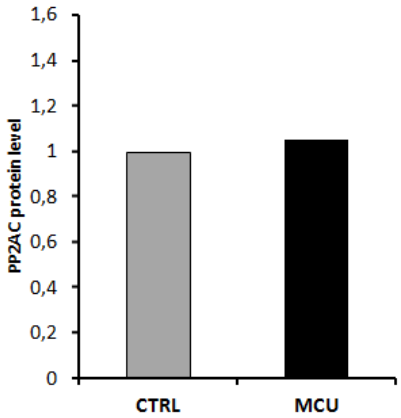


Figure 6.7.1. **MCU overexpression did not affect PP2A$_C$ protein level**. Western blot analysis was performed with anti-PP2A$_C$ antibody in NRVMs overexpressing MCU in comparison to control cells.

Then, we assessed the extent of Tyr307 phosphorylation in PP2AC that inhibits PP2A activity. Fig. 6.7.2. shows that in normoxic NRVMs MCU overexpression resulted in an increase in PP2AC phosphorylation as compared to control cells (2.84 ±0.26 vs 1±0.021 in MCU overexpressing NRVM and control cells, respectively).

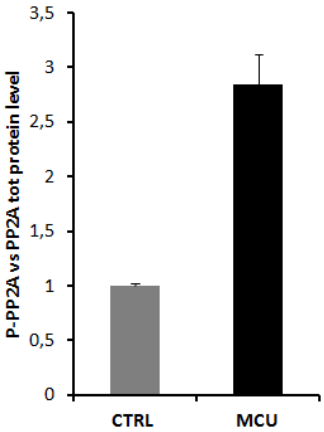


Figure 6.7.2. **MCU overexpression affected PP2A activity.** Western blot analysis was performed with anti-phosphoPP2A$_C$ antibody in NRVMs overexpressing MCU in comparison to control cells.

Overall, these results suggest that MCU induces a preconditioning-like effect resulting from a slight increase in mitochondrial ROS levels in response to an increase in mitochondrial Ca^{2+} uptake. These ROS are involved in Akt activation mediated by phosphatase inhibition. The increased activity of Akt is likely to provide a relevant contribution to the decreased susceptibility to cell death induced by MCU overexpression. For instance, the inactivated phosphorylation of PP2A could be catalyzed by Src kinase which is known to be directly activated by ROS (Chiarugi 2008). However, Further studies are needed to (i) elucidate the link between ROS and PP2A phosphorylation and (ii) provide a conclusive evidence of the protective role of Akt activation assessing the effects of MCU overexpression in NRVMs devoid of Akt.

6.8. Effect of MCU and MICU1 co-expression in NRVMs

Our current understanding of the mitochondrial contribution to I/R injury attributes a pivotal role to Ca^{2+} overload and excessive ROS formation. However, the data described above provide evidence that a limited increase in mitochondrial Ca^{2+}uptake triggers a preconditioning-like protection whereby a mild oxidative stress stimulates pro-survival processes, such as Akt activation. However, the present data do not rule out the notion that a large rise in mitochondrial [Ca^{2+}] is a deleterious event that compromises cell survival. Accordingly, we sought to investigate whether a further increase in mitochondrial Ca^{2+}uptake abolishes the protective effects afforded by MCU overexpression. To this aim we investigated the effect of MCU and MICU1 co-expression in NRVMs.

MICU1 was the first discovered member of the MICU family (Mallilankaraman, Doonan et al. 2012) and is described as a gatekeeper for the channel activity. At low [Ca^{2+}] MICU1 keeps MCU close, so that it could be considered as an inhibitor of MCU function. However, its deletion decreases mitochondrial Ca^{2+}uptake suggesting an activating role. Indeed MCU-MICU1 co-expression has been reported to increase mitochondrial [Ca^{2+}] under caffeine stimulation as compared to the overexpression of only MCU in HeLa cells.

MICU1-MCU co-expression was obtained by means adenovirus infection in NRVMs that were subjected to simulated I/R. As previously described, LDH release in the supernatant was assessed to evaluate cell death during post-ischemic reperfusion. Cells overexpressing MCU-MICU1 showed a significant increase in cell mortality of 40% in comparison to overexpression of MCU only. Therefore, MICU1 abolished MCU-induced protection (Fig. 6.8.1).

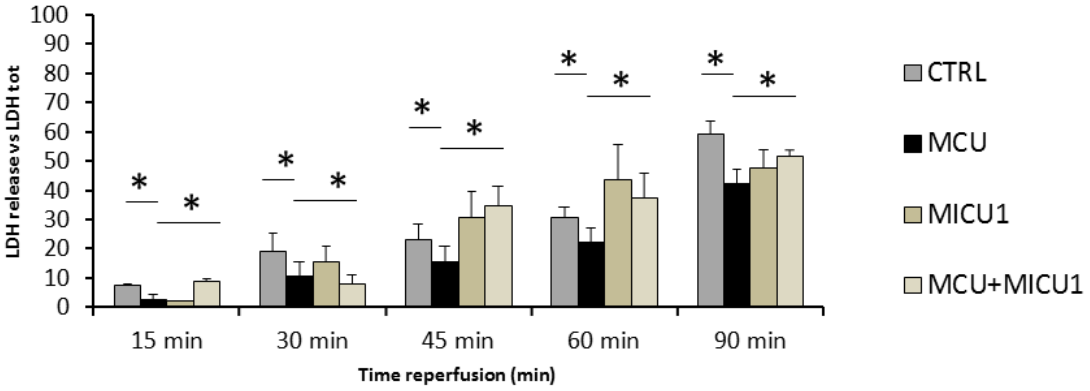


Figure 6.8.1. **MCU-MICU1 co-expression abrogated the cardioprotection afforded by MCU overexpression**. NRVMs overexpressing MCU-MICU1 through adenovirus infection were subjected to I/R injury. Loss of viability was evaluated as the percentage of total LDH activity released in the supernatant during reperfusion.

6.9. MCU MICU1 overexpression affects ROS formation in NRVMs

Since excess ROS production is a major cause of injury in ischemic hearts, oxidative stress was analyzed in NRVMs during post-ischemic reperfusion. To this aim NRVMs were transfected with HyPer mit and ROS formation was detected evaluating the changes in fluorescence intensity. A large increase in ROS formation was detected in MICU1-MCU co-expressing cells and was mostly abrogated by overexpression of MCU only.

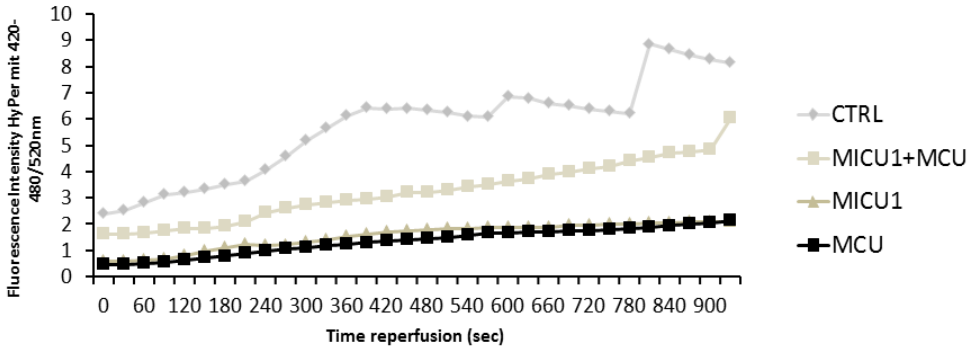


Figure 6.9.1. **MCU-MICU1 increased ROS formation during post ischemic reperfusion**. NRVMs were transfected with HyPer mit and subjected to I/R injury. Change of fluorescence intensity was evaluated to monitor the ROS level in the post ischemic reperfusion.

6.10. MCU-MICU1 mitochondria were more susceptible to oligomycin-induced depolarization

To further investigate if the increase in the mitochondrial calcium uptake due to MCU-MICU1 co-expression affects the mitochondrial function we evaluated Ψ_m using TMRM. It is worth pointing out that a defective electron transport chain may not lead to a detectable decrease in Ψ_m if this defect is compensated by the reversal of FoF1-ATP synthase. By inverting its normal activity FoF1-ATP synthase hydrolyzes ATP while pumping proton out of the matrix in order to maintain the proton gradient. Thus, to check whether mitochondria were dysfunctional in MCU-MICU1 overexpressing cells due to an excessive mitochondrial [Ca^{2+}], Ψ_m was measured with TMRM after treatment with oligomycin, an inhibitor of the ATP synthase. Figure 6.10.1. shows that oligomycin addition caused a rapid drop of Ψ_m only in NRVMs overexpressing MCU-MICU1 revealing a latent mitochondrial dysfunction compensated by the inverse operation of FoF1 ATP synthase.

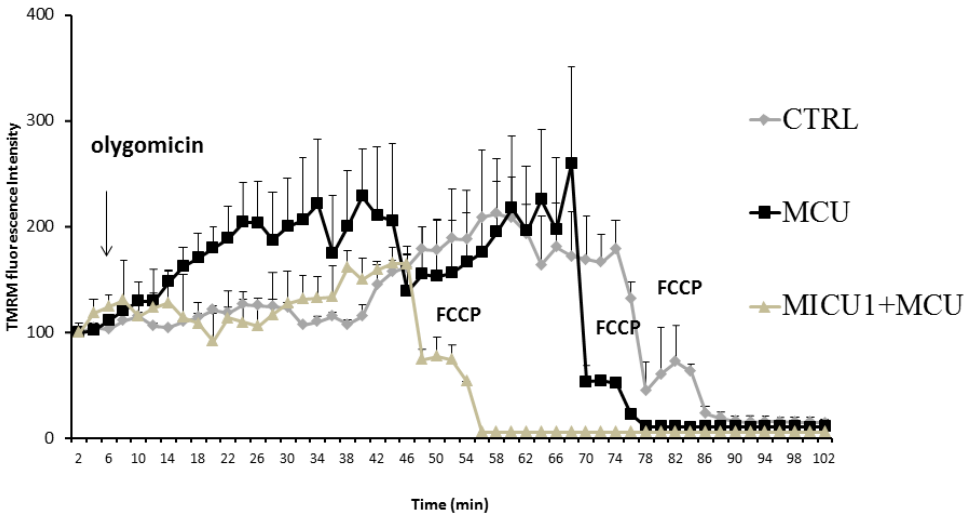


Figure 6.10.1. **Mitochondrial dysfunction in MCU-MICU1 cells indicated by the drop in TMRM fluorescence upon oligomycin addition.** TMRM fluorescence was monitored following the addition of 3 µM oligomycin addition. When indicated 4 µM FCCP was added to fully depolarize mitochondria. Mitochondria susceptibility to oligomycin-induced membrane depolarization was quantified as the percentage of all analyzed cells whose fluorescence had decreased more than 50% before FCCP addition.

6.11. MCU-MICU1 co-overexpression and Akt activation

These data strongly support that the excessive rise in mitochondrial [Ca^{2+}] caused by MCU-MICU1 co-overexpression results in mitochondrial dysfunction. In addition, cells overexpressing MCU-MICU1 displayed a higher susceptibility to I/R injury as compared to MCU overexpression only. To further demonstrate that a rise in mitochondrial [Ca^{2+}] abolishes the protection elicited by MCU overexpression we investigated the effect of

MCU-MICU1 co-overexpression on Akt activity. As previously shown a slight increase in mitochondrial [Ca^{2+}] due to MCU overexpression is associated with a mild increase in ROS levels that trigger Akt activation. Conversely, when also MICU1 was overexpressed, the increase in Akt phosphorylation was no longer detectable. This finding illustrated in Fig. 6.11.1. explains why protection is lost in NRVMs overexpressing MCU and MICU1.

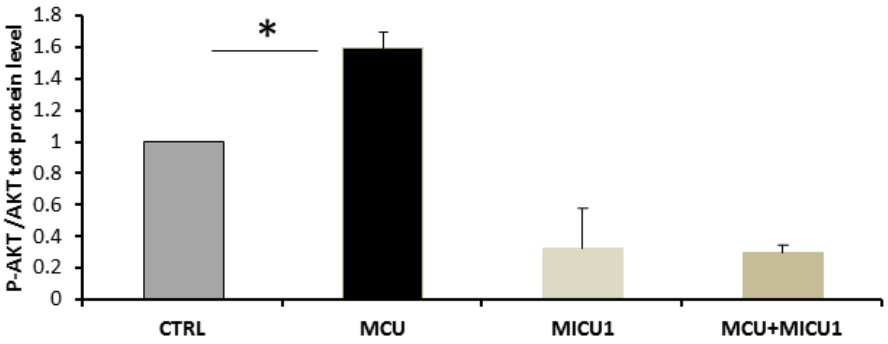


Figure 6.11.1. **Akt activation was abrogated in MCU-MICU1 co-expressing cells**. NRVMs were homogenized and proteins analyzed by Western Blot as described in Materials and Methods. Phosphorylated Akt and total Akt were detected using specific antibodies. Band intensities were quantified by densitometry using the ImageJ software.

Since Akt is negatively regulated by PP2A (as discussed in previous sections), we analyzed PP2A phosphorylation in MCU-MICU1 cells to correlate the decrease in Akt phosphorylation with PP2A activity. Figure 6.11.2. shows that MCU-MICU1 co-expression abolished the increase of Tyr307 phosphorylation in PP2A caused by MCU overexpression only. This finding implies that PP2A is no longer inhibited when MICU1 is co-expressed with MCU explaining the decrease in Akt phosphorylation/activity.

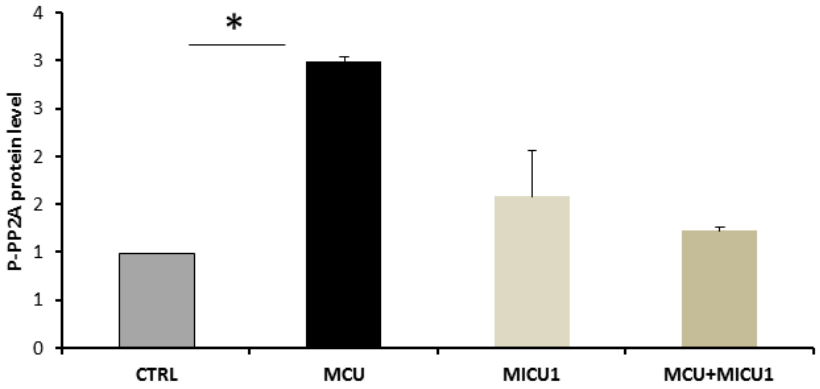


Figure 6.11.2. **MCU-MICU1 co-expression abolished the increase in PP2A phosphorylation induced by MCU overexpression only.** NRVMs were lysed and protein extracts underwent Western Blot analysis. Phosphorylated PP2A and total PP2A were detected using specific antibodies. Band intensities were quantified by densitometry using the ImageJ software.

6.12. MCU overexpression in mouse heart

To translate the findings obtained in vitro into a more complex in vivo setting, we investigated cardiac injury induced by I/R-in mice overexpressing MCU in the heart. To this aim, C57/BL6 mice were injected i.p. with an Adeno-associated virus (AAV9) encoding for the MCU-flag at P8 (8 days after birth). This virus presents a CMV promoter that is generally used to drive protein expression in mammalian cells. As shown in Fig. 6.12.1. P8 was found to represent the optimal time for maximizing the degree of heart infection.

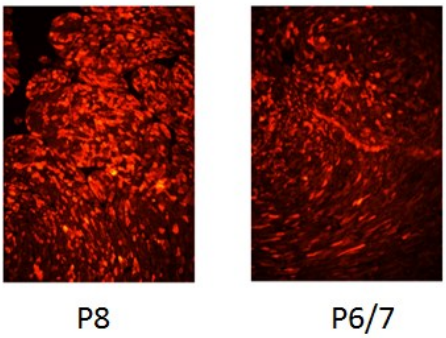


Figure 6.12.1. **Cardiac infection efficiency at different time points of injection**. Heart cryosections were incubated with an anti-flag antibody to evaluate the virus efficiency. The injection efficiency reached 65,3% vs ctrl.

After 4 months from the single injection, we evaluated the efficiency of virus infection using IF staining. We obtained an infection efficiency of 65,3% of MCU-flag expression in animals injected with the virus as compared to the control animals injected with PBS. The MCU overexpression did not affect the survival probability of the animals, as well as their morphology and development.

6.13. MCU overexpression protected hearts from I/R injury

To translate the effects observed in vitro in isolated cells, hearts were isolated and subjected to I/R injury using the classic model of Langendorff perfusion. After their isolation, hearts were stabilized by normoxic perfusion for 10 min. Then, global ischemia was induced by interrupting the coronary flow for 40 min (I-40). Finally, the coronary flow was restored for 30 min (reperfusion, R-30). During this latter period the effluent was collected to measure the release of LDH as a marker of cell death. At the end of reperfusion hearts were quickly immersed into PBS containing 0.5% Triton X100 and homogenized for assessing the residual content of lactate dehydrogenase (LDH). The data obtained in hearts infected with the AAV9 encoding for MCU-flag were compared with those observed in control hearts (injected with PBS). Fig. 6.13.1. shows that also in

intact hearts MCU overexpression resulted in a significant decrease of LDH release during reperfusion as compared to wt hearts (17,71 ±7.71% vs 30.16 ±10.35% in MCU overexpressing and control hearts, respectively; *p<0,05*).

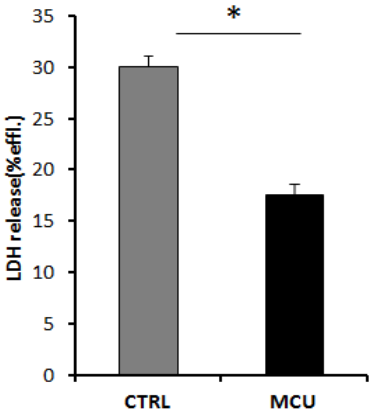


Figure 6.13.1. **MCU overexpression reduced the loss of viability in the intact heart subjected to I/R**. Hearts were subjected to a perfusion protocol of I/R (40min of no flow global ischemia followed by 30min of reperfusion). The effluent was collected to evaluate the release of LDH as a marker of cell death. MCU overexpression reduces by 43% the release of LDH occurring during reperfusion.

6.14. MCU overexpression reduced ROS formation in intact hearts subjected to I/R injury

Based upon the information collected in vitro, we investigated whether MCU expression affects ROS formation also in intact hearts. Oxidative stress was evaluated in heart cryosections stained with DHE. This probe, once inside the cell, can be oxidized by ROS to the fluorescent compound ethidium and irreversibly binds to nuclear DNA causing a further increase in fluorescence intensity. Fig. 6.14.1. shows that ROS levels are significantly decreased in MCU overexpressing hearts with respect to control hearts (74,12 ±5,68 vs 100 ±6,89 AU in MCU overexpressing and control hearts, respectively; *p<0.05*).

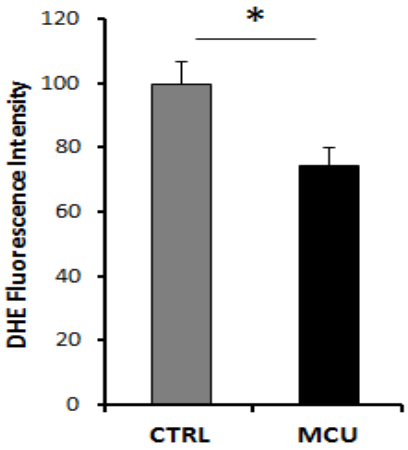


Figure 6.14.1. **MCU overexpression reduced ROS formation in hearts subjected to I/R injury.** Mice were subjected to the I/R protocol described in the text. Left ventricle sections were stained with DHE and visualized by fluorescence microscopy. Fluorescence intensity was quantified using ImageJ software.

Since DHE staining evaluates ROS levels, we added the evaluation of oxidative alterations of intracellular components. To this aim we analysed the oxidation of tropomyosin (Tm). This modification has been shown to occur in isolated rat hearts subjected to post-ischemic reperfusion (Canton, Neverova et al. 2004). The oxidative alteration of Tm is functionally relevant, since it is linearly related to contractile impairments in both cardiac and skeletal muscles (Canton, Menazza et al. 2011). The densitometric analysis shown in Fig. 6.14.2. indicates that MCU overexpression decreased the extent Tm oxidation induced by post-ischemic reperfusion (1 ±0.5 vs 0.276 ±0.23 in MCU overexpressing and control hearts, respectively). However, due to large variations between samples and the limited number of samples, the data have not yet reached the statistical significance.

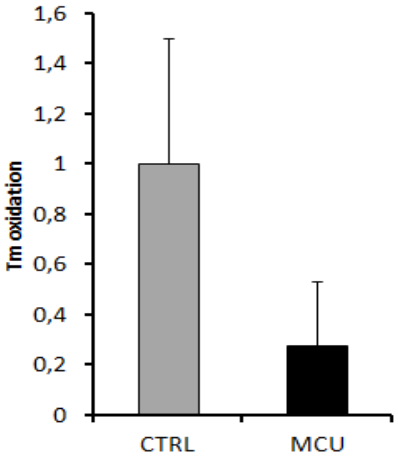


Figure 6.14.2. **Tm oxidation was decreased in MCU overexpressing hearts subjected to I/R injury**. Myofibrillar proteins were extracted from hearts subjected to I/R injury. MCU overexpressing, n=3; sham, n=2. As detailed in the Methods section, extracts were denatured in the absence (non-reducing conditions) or presence (reducing conditions) of β-mercaptoethanol and then analyzed by anti-Tm immunoblot. Densitometry analysis was quantified using ImageJ software.

6.15. MCU overexpression correlated with Akt activation in intact heart

To investigate the possible mechanism that determines a reduction of cell mortality in hearts overexpressing MCU, we analysed Akt activation in intact hearts as described in the previous sections detailing the in vitro experiments. Fig. 6.15.1. shows that MCU overexpression caused a 3-fold increase in Akt phosphorylation as compared to the control group (3,18 ±0,12 vs 1 ±0,03 in MCU overexpressing and control hearts, respectively; *p< 0,05*).

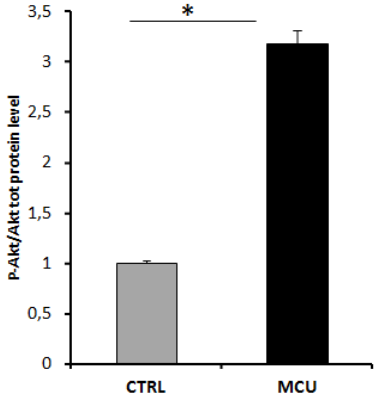


Figure 6.15.1. **Akt was activated in MCU overexpressing hearts**. Left ventricles were homogenized and proteins analyzed by Western Blot as described in Materials and Methods. Akt phosphorylation (Ser 473) was detected using specific antibodies. Band intensities were quantified by densitometry using ImageJ software.

6.16. MCU overexpression determines an increased in PP2A phosphorylation in intact hearts.

The in vitro experiments described above demonstrate that Akt activation is associated with an increased phosphorylation of PP2A that causes its inactivation. Accordingly, we analyzed the phosphorylation status of PP2A also in vivo. Fig. 6.16.1 shows that MCU overexpression increased the degree of PP2A phosphorylation as compared to control hearts (1,26 ±0,249 vs 1 ±0,18 in MCU overexpressing and control hearts, respectively).

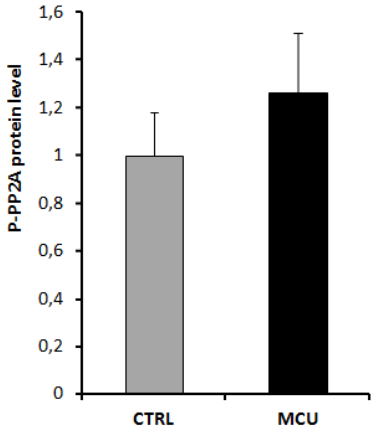


Figure 6.16.1. **PP2A activity was decreased in MCU overexpressing hearts**. LV tissue was homogenized and proteins analyzed by Western Blot as described in Materials and Methods. PP2A phosphorylation (Tyr 307) was detected using specific antibodies. Band intensities were quantified by densitometry using ImageJ software.

Therefore, also in intact hearts MCU overexpression causes the inactivation of PP2A associated with Akt activation that eventually is likely to be responsible for the protection against I/R injury observed in MCU overexpressing hearts.

7.CONCLUSIONS

The results achieved in this study provide novel evidence regarding the role of MCU in the cardiac injury induced by ischemia and reperfusion. Although molecular mechanism is still under investigation, the prevailing concept is that during reperfusion the rise of both Ca2+ and ROS is a causative effector in determining cell death. In fact, Ca2+ and ROS synergize promoting the opening of the PTP. The PTP opening induces a sudden change in the permeability of the mitochondrial membrane leading to mitochondrial membrane depolarization, release of cytochrome *c* and depletion of ATP. These alterations make the survival of the cell unlikely. The deleterious role of an excessive mitochondrial Ca2+ uptake has been suggested by using Ruthenium derivates, that lack selectivity. The reason for the pharmacological approach was the lack of knowledge of the molecular identity of the mitochondrial Ca2+ uniporter. The elucidation of the MCU structure has made possible exploiting genetic approaches to evaluate the role of mitochondrial Ca2+ uptake in the injury induced by I/R.

Here we have demonstrated that the deletion of MCU expression did not protect from the loss of cell viability in NRVMs subjected to a simulated I/R in accordance with the result obtained by Finkel et all. In addition, also the burst of ROS occurring during post-ischemic reperfusion was not reduced. These results suggest that MCU inhibition, which might allow Ca2+ to accumulate in the cytosol, is unlikely to produce a beneficial outcome and could trigger the adverse outcome. To further investigate the role of the mitochondrial Ca2+ uptake we evaluated the effect of MCU overexpression in NRVMs. Cells overexpressing MCU were subjected to both simulated I/R (5mM KCN, 20mM DOG) and hypoxia/reoxygenation showing an increase in cell viability in comparison to control cells. The protection effect was associated with a reduction in ROS formation occurring during post ischemic reperfusion. This finding was translated into an in vivo model overexpressing MCU by means of adeno-associated virus 9 encoded for MCU-flag. Hearts isolated from MCU overexpressing mice were subjected to I/R injury using the Langendorff model. Hearts overexpressing MCU showed a significant decrease of both cell death and oxidative stress.

Taken together these data suggest that promoting MCU activity provides a robust protection against I/R injury. To clarify the mechanistic link between MCU expression and cell susceptibility to I/R injury, we hypothesized that the mild increase in Ca2+ ascribable to MCU overexpression, elicits a preconditioning-like effect. To validate this hypothesis ROS levels were measured at the basal state. MCU overexpressing cells showed a slight increase in ROS levels, that might activate survival pathways. Indeed, we found that both in NRVMs and intact heart Akt was more phosphorylated when MCU was overexpressed. Moreover, MCU overexpression was associated with the

inactivation of PP2A, a phosphatase that negatively regulates Akt. The relevance of ROS formation was demonstrated showing that the antioxidant treatment with MPG abolished Akt activation. Therefore , the slight increase in mitochondrial Ca2+ induced by MCU overexpression caused a mild oxidative stress, that triggers self-defense cellular mechanism. Although we identified Akt activation through PP2A inactivation, other signaling pathways could be activated by either mechanism initiated by the increase of mitochondrial Ca2+ level, such as an increase in ATP formation, and/or redox processes related to mild increase in mitochondrial ROS. For instance, the inactivated phosphorylation of PP2A could be catalyzed by Src kinase which is known to be directly activated by ROS (Chiarugi 2008). Another redox sensitive phosphatase that is upstream of Akt is PTEN that is involved in insulin signalling (Schiaffino and Mammucari 2011). Future studies will aim at defining the phosphatases inactivated in redox dependent manner that might explain the increase in Akt activation. In addition, it is to demonstrate that the protection afforded by MCU overexpression is not detectable in cardiomyocytes lacking Akt.

Even if our results demonstrate that a slight increase in mitochondrial Ca2+ uptake affords cardioprotection, we also provide evidence that a large elevation in mitochondrial Ca2+ hampered the resistance to I/R injury. This latter part of our study lends support to the common concept linking an excessive level of mitochondrial Ca2+ with cardiomyocyte injury. To obtain this evidence MICU1 was overexpressed together with MCU in NRVMs. The consequent further elevation in mitochondrial Ca2+ abrogated the protection induced by MCU overexpression alone against the increase in ROS formation and cell death caused by I/R injury. The co-expression abolished also the effects of MCU overexpression alone on intracellular signaling. In fact, the increase in phosphorylation of Akt and PP2A was prevented by MICU1-MCU co-expression. These findings are likely to explain the mechanism by which the co-expression of MICU1-MCU results in a different outcome as compare to MCU alone .

Taken together these observations suggest to take in consideration the role of the cytosolic Ca2+. The decreased susceptibility shown in MCU overexpression could be due to the mitochondrial buffering capacity that avoid the activation of several Ca2+-dependent enzymes, such as calpain, CaMK and PLA2, that have already been described to aggravate cardiac injury. On the other hand, the activation of these enzymes could explain the unexpected adverse outcome resulting from MCU deletion.

8.REFERENCES

Adam-Vizi, V. and A. A. Starkov (2010). "Calcium and mitochondrial reactive oxygen species generation: how to read the facts." J Alzheimers Dis **20 Suppl 2**: S413-426.

Allen, D. G. and C. H. Orchard (1987). "Myocardial contractile function during ischemia and hypoxia." Circ.Res. **60**(2): 153-168.

Baughman, J. M., F. Perocchi, H. S. Girgis, M. Plovanich, C. Belcher-Timme, Y. Sancak, X. R. Bao, L. Strittmatter, O. Goldberger, R. L. Bogorad, V. Koteliansky and V. K. Mootha (2011). "Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter." Nature.

Bernardi, P. (1999). "Mitochondrial transport of cations: channels, exchangers, and permeability transition." Physiol.Rev. **79**(4): 1127-1155.

Bernardi, P., A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa and M. A. Forte (2006). "The mitochondrial permeability transition from in vitro artifact to disease target." FEBS J **273**(10): 2077-2099.

Bers, D. M. (2002). "Cardiac excitation-contraction coupling." Nature **415**(6868): 198-205.

Bienert, G. P. and F. Chaumont (2014). "Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide." Biochim Biophys Acta **1840**(5): 1596-1604.

Bolli, R. (1998). "Causative role of oxyradicals in myocardial stunning: a proven hypothesis. A brief review of the evidence demonstrating a major role of reactive oxygen species in several forms of postischemic dysfunction." Basic Res Cardiol **93**(3): 156-162.

Brill, A., A. Torchinsky, H. Carp and V. Toder (1999). "The role of apoptosis in normal and abnormal embryonic development." J Assist Reprod Genet **16**(10): 512-519.

Brookes, P. S., Y. Yoon, J. L. Robotham, M. W. Anders and S. S. Sheu (2004). "Calcium, ATP, and ROS: a mitochondrial love-hate triangle." Am.J.Physiol Cell Physiol **287**(4): C817-C833.

Cadenas, E. and K. J. Davies (2000). "Mitochondrial free radical generation, oxidative stress, and aging." Free Radic.Biol.Med. **29**(3-4): 222-230.

Canton, M., S. Menazza, F. L. Sheeran, P. Polverino de Laureto, F. Di Lisa and S. Pepe (2011). "Oxidation of myofibrillar proteins in human heart failure." J.Am.Coll.Cardiol. **57**(3): 300-309.

Canton, M., I. Neverova, R. Menabò, J. E. Van Eyk and F. Di Lisa (2004). "Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts." Am.J.Physiol Heart Circ.Physiol **286**(3): H870-H877.

Carafoli, E., W. X. Balcavage, A. L. Lehninger and J. R. Mattoon (1970). "Ca2+ metabolism in yeast cells and mitochondria." Biochim Biophys Acta **205**(1): 18-26.

Carafoli, E., C. S. Rossi and A. L. Lehninger (1965). "Uptake of Adenine Nucleotides by Respiring Mitochondria during Active Accumulation of Ca++ and Phosphate." J Biol Chem **240**: 2254-2261.

Carpi, A., R. Menabò, N. Kaludercic, P. Pelicci, F. Di Lisa and M. Giorgio (2009). "The cardioprotective effects elicited by p66(Shc) ablation demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury." Biochim.Biophys.Acta **1787**(7): 774-780.

Chaudhuri, D., Y. Sancak, V. K. Mootha and D. E. Clapham (2013). "MCU encodes the pore conducting mitochondrial calcium currents." Elife **2**: e00704.

Chen, L., L. Liu, J. Yin, Y. Luo and S. Huang (2009). "Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of protein phosphatase 2A and 5, leading to activation of MAPK pathway." Int J Biochem Cell Biol **41**(6): 1284-1295.

Chen, W., S. Gabel, C. Steenbergen and E. Murphy (1995). "A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart." Circ Res **77**(2): 424-429.

Chiarugi, P. (2008). "Src redox regulation: there is more than meets the eye." Mol Cells **26**(4): 329-337.
Clarke, S. J., I. Khaliulin, M. Das, J. E. Parker, K. J. Heesom and A. P. Halestrap (2008). "Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation." Circ.Res. **102**(9): 1082-1090.
Csordas, G., T. Golenar, E. L. Seifert, K. J. Kamer, Y. Sancak, F. Perocchi, C. Moffat, D. Weaver, S. de la Fuente Perez, R. Bogorad, V. Koteliansky, J. Adijanto, V. K. Mootha and G. Hajnoczky (2013). "MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter." Cell Metab **17**(6): 976-987.
Czerski, L. and G. Nunez (2004). "Apoptosome formation and caspase activation: is it different in the heart?" J Mol Cell Cardiol **37**(3): 643-652.
De Stefani, D., A. Raffaello, E. Teardo, I. Szabo and R. Rizzuto (2011). "A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter." Nature **476**: 336-340.
del Monte, F., D. Lebeche, J. L. Guerrero, T. Tsuji, A. A. Doye, J. K. Gwathmey and R. J. Hajjar (2004). "Abrogation of ventricular arrhythmias in a model of ischemia and reperfusion by targeting myocardial calcium cycling." Proc Natl Acad Sci U S A **101**(15): 5622-5627.
Denton, R. M. and J. G. McCormack (1990). "Ca2+ as a second messenger within mitochondria of the heart and other tissues." Annu Rev Physiol **52**: 451-466.
Di Lisa, F., A. Carpi, V. Giorgio and P. Bernardi (2011). "The mitochondrial permeability transition pore and cyclophilin D in cardioprotection." Biochim.Biophys.Acta **1813**(7): 1316-1322.
Dosenko, V. E., V. S. Nagibin, L. V. Tumanovska and A. A. Moibenko (2006). "Protective effect of autophagy in anoxia-reoxygenation of isolated cardiomyocyte?" Autophagy **2**(4): 305-306.
Drago, I., S. D. De, R. Rizzuto and T. Pozzan (2012). "Mitochondrial Ca2+ uptake contributes to buffering cytoplasmic Ca2+ peaks in cardiomyocytes." Proc.Natl.Acad.Sci.U.S.A **109**(32): 12986-12991.
Forbes, R. A., C. Steenbergen and E. Murphy (2001). "Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism." Circ Res **88**(8): 802-809.
Fujio, Y., T. Nguyen, D. Wencker, R. N. Kitsis and K. Walsh (2000). "Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart." Circulation **101**(6): 660-667.
Giorgio, M., E. Migliaccio, F. Orsini, D. Paolucci, M. Moroni, C. Contursi, G. Pelliccia, L. Luzi, S. Minucci, M. Marcaccio, P. Pinton, R. Rizzuto, P. Bernardi, F. Paolucci and P. G. Pelicci (2005). "Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis." Cell **122**(2): 221-233.
Giorgio, V., S. von Stockum, M. Antoniel, A. Fabbro, F. Fogolari, M. Forte, G. D. Glick, V. Petronilli, M. Zoratti, I. Szabo, G. Lippe and P. Bernardi (2013). "Dimers of mitochondrial ATP synthase form the permeability transition pore." Proc Natl Acad Sci U S A **110**(15): 5887-5892.
Golstein, P. and G. Kroemer (2007). "Cell death by necrosis: towards a molecular definition." Trends Biochem Sci **32**(1): 37-43.
Gorlach, A., K. Bertram, S. Hudecova and O. Krizanova (2015). "Calcium and ROS: A mutual interplay." Redox Biol **6**: 260-271.
Gottlieb, R. (2005). "ICE-ing the heart." Circ Res **96**(10): 1036-1038.
Griffiths, E. J. and A. P. Halestrap (1993). "Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts." J.Mol.Cell.Cardiol. **25**(12): 1461-1469.
Griffiths, E. J., C. J. Ocampo, J. S. Savage, G. A. Rutter, R. G. Hansford, M. D. Stern and H. S. Silverman (1998). "Mitochondrial calcium transporting pathways during hypoxia and reoxygenation in single rat cardiomyocytes." Cardiovasc.Res. **39**(2): 423-433.

Griffiths, E. J., C. J. Ocampo, J. S. Savage, M. D. Stern and H. S. Silverman (2000). "Protective effects of low and high doses of cyclosporin A against reoxygenation injury in isolated rat cardiomyocytes are associated with differential effects on mitochondrial calcium levels." Cell Calcium **27**(2): 87-95.
Halestrap, A. P., S. J. Clarke and I. Khaliulin (2007). "The role of mitochondria in protection of the heart by preconditioning." Biochim.Biophys.Acta **1767**(8): 1007-1031.
Hamacher-Brady, A., N. R. Brady and R. A. Gottlieb (2006). "The Interplay between Pro-Death and Pro-Survival Signaling Pathways in Myocardial Ischemia/Reperfusion Injury: Apoptosis Meets Autophagy." Cardiovasc.Drugs Ther. **20**(6): 445-462.
Han, D., F. Antunes, R. Canali, D. Rettori and E. Cadenas (2003). "Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol." J Biol Chem **278**(8): 5557-5563.
Hawkins, B. J., M. Madesh, C. J. Kirkpatrick and A. B. Fisher (2007). "Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling." Mol Biol Cell **18**(6): 2002-2012.
Hoffman, N. E., H. C. Chandramoorthy, S. Shanmughapriya, X. Q. Zhang, S. Vallem, P. J. Doonan, K. Malliankaraman, S. Guo, S. Rajan, J. W. Elrod, W. J. Koch, J. Y. Cheung and M. Madesh (2014). "SLC25A23 augments mitochondrial Ca(2)(+) uptake, interacts with MCU, and induces oxidative stress-mediated cell death." Mol Biol Cell **25**(6): 936-947.
Holmstrom, K. M. and T. Finkel (2014). "Cellular mechanisms and physiological consequences of redox-dependent signalling." Nat Rev Mol Cell Biol **15**(6): 411-421.
Hoyer-Hansen, M., L. Bastholm, P. Szyniarowski, M. Campanella, G. Szabadkai, T. Farkas, K. Bianchi, N. Fehrenbacher, F. Elling, R. Rizzuto, I. S. Mathiasen and M. Jaattela (2007). "Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2." Mol Cell **25**(2): 193-205.
Huang, B. K. and H. D. Sikes (2014). "Quantifying intracellular hydrogen peroxide perturbations in terms of concentration." Redox Biol **2C**: 955-962.
Hüser, J., C. E. Rechenmacher and L. A. Blatter (1998). "Imaging the permeability pore transition in single mitochondria." Biophys.J. **74**: 2129-2137.
Imahashi, K., C. Pott, J. I. Goldhaber, C. Steenbergen, K. D. Philipson and E. Murphy (2005). "Cardiac-specific ablation of the Na+-Ca2+ exchanger confers protection against ischemia/reperfusion injury." Circ Res **97**(9): 916-921.
Kaludercic, N., A. Carpi, R. Menabo, F. Di Lisa and N. Paolocci (2011). "Monoamine oxidases (MAO) in the pathogenesis of heart failure and ischemia/reperfusion injury." Biochim.Biophys.Acta **1813**(7): 1323-1332.
Kim, J. S., Y. Jin and J. J. Lemasters (2006). "Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion." Am.J.Physiol Heart Circ.Physiol **290**(5): H2024-H2034.
Kwong, J. Q., X. Lu, R. N. Correll, J. A. Schwanekamp, R. J. Vagnozzi, M. A. Sargent, A. J. York, J. Zhang, D. M. Bers and J. D. Molkentin (2015). "The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart." Cell Rep **12**(1): 15-22.
Lam, S. S., J. D. Martell, K. J. Kamer, T. J. Deerinck, M. H. Ellisman, V. K. Mootha and A. Y. Ting (2015). "Directed evolution of APEX2 for electron microscopy and proximity labeling." Nat Methods **12**(1): 51-54.
Lesnefsky, E. J., Q. Chen, S. Moghaddas, M. O. Hassan, B. Tandler and C. L. Hoppel (2004). "Blockade of electron transport during ischemia protects cardiac mitochondria." J Biol Chem **279**(46): 47961-47967.
Li, X., P. Fang, J. Mai, E. T. Choi, H. Wang and X. F. Yang (2013). "Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers." J Hematol Oncol **6**: 19.

Lowe, S. W. and A. W. Lin (2000). "Apoptosis in cancer." Carcinogenesis **21**(3): 485-495.
Mallilankaraman, K., P. Doonan, C. Cardenas, H. C. Chandramoorthy, M. Muller, R. Miller, N. E. Hoffman, R. K. Gandhirajan, J. Molgo, M. J. Birnbaum, B. S. Rothberg, D. O. Mak, J. K. Foskett and M. Madesh (2012). "MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival." Cell **151**(3): 630-644.
Mammucari, C., G. Gherardi, I. Zamparo, A. Raffaello, S. Boncompagni, F. Chemello, S. Cagnin, A. Braga, S. Zanin, G. Pallafacchina, L. Zentilin, M. Sandri, D. De Stefani, F. Protasi, G. Lanfranchi and R. Rizzuto (2015). "The mitochondrial calcium uniporter controls skeletal muscle trophism in vivo." Cell Rep **10**(8): 1269-1279.
Melendez-Hevia, E., T. G. Waddell and M. Cascante (1996). "The puzzle of the Krebs citric acid cycle: assembling the pieces of chemically feasible reactions, and opportunism in the design of metabolic pathways during evolution." J Mol Evol **43**(3): 293-303.
Mocanu, M. M., G. F. Baxter and D. M. Yellon (2000). "Caspase inhibition and limitation of myocardial infarct size: protection against lethal reperfusion injury." Br J Pharmacol **130**(2): 197-200.
Murphy, E., H. Cross and C. Steenbergen (1999). "Sodium regulation during ischemia versus reperfusion and its role in injury." Circ Res **84**(12): 1469-1470.
Murphy, E., M. Perlman, R. E. London and C. Steenbergen (1991). "Amiloride delays the ischemia-induced rise in cytosolic free calcium." Circ Res **68**(5): 1250-1258.
Murry, C. E., R. B. Jennings and K. A. Reimer (1986). "Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium." Circulation **74**(5): 1124-1136.
Nishihara, M., T. Miura, T. Miki, M. Tanno, T. Yano, K. Naitoh, K. Ohori, H. Hotta, Y. Terashima and K. Shimamoto (2007). "Modulation of the mitochondrial permeability transition pore complex in GSK-3beta-mediated myocardial protection." J.Mol.Cell.Cardiol. **43**(5): 564-570.
Pan, X., J. Liu, T. Nguyen, C. Liu, J. Sun, Y. Teng, M. M. Fergusson, Rovira, II, M. Allen, D. A. Springer, A. M. Aponte, M. Gucek, R. S. Balaban, E. Murphy and T. Finkel (2013). "The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter." Nat Cell Biol **15**(12): 1464-1472.
Petronilli, V., D. Penzo, L. Scorrano, P. Bernardi and F. Di Lisa (2001). "The mitochondrial permeability transition, release of cytochrome *c* and cell death. Correlation with the duration of pore openings *in situ.*" J.Biol.Chem. **276**: 12030-12034.
Piper, H. M., Y. Abdallah, S. Kasseckert and K. D. Schluter (2008). "Sarcoplasmic reticulum-mitochondrial interaction in the mechanism of acute reperfusion injury. Viewpoint." Cardiovasc.Res. **77**(2): 234-236.
Piper, H. M., K. Meuter and C. Schafer (2003). "Cellular mechanisms of ischemia-reperfusion injury." Ann Thorac Surg **75**(2): S644-648.
Plovanich, M., R. L. Bogorad, Y. Sancak, K. J. Kamer, L. Strittmatter, A. A. Li, H. S. Girgis, S. Kuchimanchi, J. De Groot, L. Speciner, N. Taneja, J. Oshea, V. Koteliansky and V. K. Mootha (2013). "MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling." PLoS One **8**(2): e55785.
Raffaello, A., D. De Stefani, D. Sabbadin, E. Teardo, G. Merli, A. Picard, V. Checchetto, S. Moro, I. Szabo and R. Rizzuto (2013). "The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit." EMBO J **32**(17): 2362-2376.
Rasmussen, T. P., Y. Wu, M. L. Joiner, O. M. Koval, N. R. Wilson, E. D. Luczak, Q. Wang, B. Chen, Z. Gao, Z. Zhu, B. A. Wagner, J. Soto, M. L. McCormick, W. Kutschke, R. M. Weiss, L. Yu, R. L. Boudreau, E. D. Abel, F. Zhan, D. R. Spitz, G. R. Buettner, L. S. Song, L. V. Zingman and M. E. Anderson (2015). "Inhibition of MCU forces extramitochondrial adaptations governing physiological and pathological stress responses in heart." Proc Natl Acad Sci U S A **112**(29): 9129-9134.

Rasola, A. and P. Bernardi (2011). "Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis." Cell Calcium **50**(3): 222-233.
Rees, D. M., A. G. Leslie and J. E. Walker (2009). "The structure of the membrane extrinsic region of bovine ATP synthase." Proc Natl Acad Sci U S A **106**(51): 21597-21601.
Rizzuto, R., S. D. De, A. Raffaello and C. Mammucari (2012). "Mitochondria as sensors and regulators of calcium signalling." Nat.Rev.Mol.Cell Biol. **13**(9): 566-578.
Rouslin, W., J. L. Erickson and R. J. Solaro (1986). "Effects of oligomycin and acidosis on rates of ATP depletion in ischemic heart muscle." Am.J.Physiol. **250**(3 Pt 2): H503-508.
Sancak, Y., A. L. Markhard, T. Kitami, E. Kovacs-Bogdan, K. J. Kamer, N. D. Udeshi, S. A. Carr, D. Chaudhuri, D. E. Clapham, A. A. Li, S. E. Calvo, O. Goldberger and V. K. Mootha (2013). "EMRE is an essential component of the mitochondrial calcium uniporter complex." Science **342**(6164): 1379-1382.
Schiaffino, S. and C. Mammucari (2011). "Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models." Skelet Muscle **1**(1): 4.
Seshacharyulu, P., P. Pandey, K. Datta and S. K. Batra (2013). "Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer." Cancer Lett **335**(1): 9-18.
Sohal, R. S. and R. G. Allen (1985). "Relationship between metabolic rate, free radicals, differentiation and aging: a unified theory." Basic Life Sci **35**: 75-104.
Steenbergen, C., M. E. Perlman, R. E. London and E. Murphy (1993). "Mechanism of preconditioning. Ionic alterations." Circ Res **72**(1): 112-125.
Steller, H. (1995). "Mechanisms and genes of cellular suicide." Science **267**(5203): 1445-1449.
Stephanou, A., B. Brar, Z. Liao, T. Scarabelli, R. A. Knight and D. S. Latchman (2001). "Distinct initiator caspases are required for the induction of apoptosis in cardiac myocytes during ischaemia versus reperfusion injury." Cell Death Differ **8**(4): 434-435.
Takagi, H., Y. Matsui and J. Sadoshima (2007). "The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart." Antioxid Redox Signal **9**(9): 1373-1381.
Tong, H., W. Chen, C. Steenbergen and E. Murphy (2000). "Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C." Circ Res **87**(4): 309-315.
Zweier, J. L., J. T. Flaherty and M. L. Weisfeldt (1987). "Direct measurement of free radical generation following reperfusion of ischemic myocardium." Proc.Natl.Acad.Sci.U.S.A. **84**(5): 1404-1407.