# REGULATION OF MITOCHONDRIAL FUNCTION IN ISCHEMIA/ REPERFUSION: LOOKING FOR THERAPEUTIC STRATEGIES IN FATTY LIVERS

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# *Regulation of Mitochondrial Function in Ischemia/Reperfusion: Looking for Therapeutic Strategies in Fatty Livers*

DIssertação de Doutoramento na área cientifica de Biologia,
especialidade de Biologia Celular, orientada pelos Professores Doutores *Carlos Manuel Marques Palmeira, Rodrigo Antunes da Cunha e Anabela Pinto Rolo e apresentada ao Departamento de Ciências da Vida* da Universidade de Coimbra

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Regulation of mitochondrial function in ischemia/reperfusion: looking for therapeutic strategies in fatty livers

## Abstract

Non-alcoholic fatty liver diseases (NAFLD) are associated with obesity and type II diabetes, which are rapidly increasing worldwide due to a fat rich diet and lack of exercise. Fatty liver is characterized by the accumulation of fat in the cytoplasm of hepatocytes derived from adipocytes, intestinal absorption or hepatic lipogenesis. Clinical data has shown that fatty livers are more susceptible to ischemia/reperfusion (I/R) injury, leading to diminished liver function. Different hypothesis, such as impaired ATP synthesis, microcirculation, Kupffer cell dysfunction and increased leukocytical adhesion have been proposed for the increased susceptibility of fatty organs to ischemic injury. Fatty degeneration, which induces a series of alterations in mitochondria, renders these organelles intrinsically more susceptible to I/R injury. Impairment of oxidative phosphorylation and induction of the mitochondrial permeability transition (MPT) are critical determinants for mitochondrial dysfunction induced by prolonged periods of ischemia followed by reperfusion.

Regarding this, and taking into account the increased incidence of this pathology, we aimed to protect fatty livers from I/R damage. Ischemic preconditioning (IPC) is a phenomenon in which brief periods of ischemia make tissues resistant to injury resulting from prolonged ischemia and reperfusion. Thus, it became crucial to define the functioning mechanisms of preconditioning (PC) to allow drug targeting in a pharmacological treatment in fatty livers, as a strategy to reduce I/R injury observed in transplantation and hepatic resections.

The first strategy presented demonstrates that selective blocking of $A_1$Rs with DPCPX abolished the protection achieved by ischemic preconditioning, and $A_1$R agonist CCPA reproduced the beneficial effects. This fact confirms the participation of this receptor in protection induced by IPC in lean livers. This involves preservation of OXPHOS efficiency and increase resistance to MPT onset. Induction of the MPT by I/R leads to cell death by uncoupling oxidative phosphorylation, inducing mitochondrial swelling and collapsing mitochondrial membrane potential, thus blocking cellular ATP formation. IPC induces the increase of HSP70 that may further protect mitochondria and prevent the MPT, thus maintaining the ability to generate ATP and recover from I/R insult. We demonstrate that modulation of the MPT is

achieved in preconditioning (ischemic and pharmacological) in a GSK-3β-dependent manner, which is essential to achieve protection in I/R.

GSK-3β inhibition prevents impairment of hepatic mitochondrial function and depletion of cellular ATP. Pharmacological preconditioning with indirubin-3'-oxime, an inhibitor of GSK-3β, in conditions of hepatic I/R, protects the liver by maintaining mitochondrial calcium homeostasis, thus preserving mitochondrial function and hepatic energetic balance. I/R induces translocation of GSK-3β from the cytosol to the mitochondria, increasing mitochondrial GSK-3β activity. Indirubin-3'-oxime preserves the level of inhibition of GSK-3β activity on mitochondria, by preserving the mitochondrial pool of phospho-Ser9-GSK-3β, that is decreased in I/R.

Although fatty livers exhibit an underlying state of mitochondrial dysfunction that enhances damage after I/R, treatment with indirubin-3'-oxime was also able to preserve the efficiency of mitochondrial function and prevent I/R damage in fatty livers. We demonstrated that active GSK-3β (in I/R situation) promotes an enhancement of CypD phosphorylation, resulting in increased binding of CypD to the ANT, sensitizing mitochondria to calcium-induced MPT. Therefore, indirubin-3'-oxime has great therapeutic potential in the clinical setting of hepatic ischemia/reperfusion, acting as pharmacological preconditioning in lean and fatty livers.

The last protective strategy highlights the importance of MPT modulation against I/R injury in fatty livers, involving the preventive axis SIRT3-CypD-MPT. Obesity decreases the cellular NAD+/NADH ratio, thereby contributing to a decrease in SIRT3 activity, thus increasing the acetylation of mitochondrial proteins and decreasing mitochondrial efficiency. We demonstrated that the increase in CypD acetylation in I/R animals was accompanied by an inhibition of SIRT3 activity. Conversely, adding $NAD^+$ to fatty livers prior to I/R significantly increases SIRT3 activity and decreases CypD acetylation, activity and binding to ANT, increasing the threshold to MPT induction. $NAD^+$ modulates mitochondrial function and preserves oxidative phosphorylation, being protective against injury in a model of hepatic warm I/R in fatty livers.

**Resumo**

As doenças do fígado gordo não-alcoólicas (NAFLD) estão associadas à obesidade diabetes tipo II, estão a aumentar rapidamente em todo o mundo devido a uma dieta rica em gorduras e falta de exercício. Um fígado gordo é caracterizado pela acumulação de gordura no citoplasma de hepatócitos, derivada de adipócitos, da absorção intestinal ou da lipogénese hepática. Os dados clínicos mostram que os fígados gordos são mais suscetíveis à lesão por isquemia/reperfusão (I/R), levando à diminuição da função hepática. Diferentes hipóteses, tais como síntese de ATP debilitada, microcirculação alterada, disfunção das células de Kupffer e aumento da adesão leucocitária têm sido propostas para o aumento da susceptibilidade de órgãos gordos à lesão isquémica. Este tipo de degeneração causada pela acumulação de gordura, induz uma série de alterações na mitocôndria, tornando estes organelos mais suscetíveis à lesão por I/R. Diminuição da eficiência fosforilativa e indução da transição de permeabilidade mitocondrial (MPT) são determinantes críticos para a disfunção mitocondrial induzida por períodos prolongados de isquémia seguidos de reperfusão.

Tendo em conta o aumento da incidência desta patologia, o nosso objetivo é aumentar a resistência dos fígados gordos aos danos por I/R. Pré-condicionamento isquémico (IPC) é um fenómeno em que breves períodos de isquemia tornam os tecidos resistentes à lesão decorrente de isquemia e reperfusão prolongadas.

Assim, tornou-se crucial definir os mecanismos subjacentes ao pré-condicionamento (PC) para permitir a identificação de alvos para drogas num tratamento farmacológico em fígados gordos como uma estratégia para reduzir a lesão por I/R observada no transplante e ressecções hepáticas.

A primeira estratégia apresentada demonstra que o bloqueio seletivo de $A_1$Rs com DPCPX aboliu a proteção obtida pelo pré-condicionamento isquémico, enquanto que um agonista de $A_1$R, o CCPA, reproduziu os seus efeitos benéficos. Este fato confirma a participação deste receptor na proteção induzida pelo IPC em fígados magros. Isto envolve a preservação da eficiência fosforilativa e aumenta a resistência ao MPT.

A indução da MPT por I/R leva a morte celular por desacoplamento da fosforilação oxidativa, induzindo entumescimento mitocondrial e o colapso do potencial de membrana mitocondrial, bloqueando assim a

formação de ATP. O IPC induz o aumento de HSP70 protegendo da indução do MPT, mantendo assim a capacidade para gerar ATP e recuperar do insulto por I/R. Demonstrámos que a modulação da MPT é alcançada em pré-condicionamento (isquémico e farmacológico) via modulação da GSK-3β.

A inibição de GSK-3β previne a disfunção mitocondrial e depleção do ATP celular. Pré-condicionamento farmacológico com indirubina-3'-oxima, um inibidor de GSK-3β, em condições de I/R hepática, protege o fígado através da manutenção da homeostase do cálcio mitocondrial, preservando assim a função mitocondrial e o equilíbrio energético hepático. A I/R induz a translocação de GSK-3β do citosol para a mitocondria, aumentando a actividade da GSK-3β mitocondrial. A indirubina-3'-oxima preserva o nível de inibição da actividade da GSK-3β nas mitocondrias, preservando o "pool" mitocondrial de fosfo-Ser9-GSK-3β, que diminuí em I/R.

Embora os fígados gordos exibam um estado subjacente de disfunção mitocondrial que aumenta os danos depois de I/R, o tratamento com indirubina-3'-oxima também foi capaz de preservar a eficiência da função mitocondrial e prevenir os danos da I/R em fígados gordos. Demonstrámos que a GSK-3β ativa (em situação de I/R) promove um aumento da fosforilação da CypD, resultando num aumento da ligação entre CypD e ANT, sensibilizando as mitocondrias à MPT induzida pelo cálcio. Portanto, a indirubina-3'-oxima tem um grande potencial terapêutico no estado clínico de isquemia/reperfusão hepática, atuando como pré-condicionamento farmacológico em fígados magros e gordos.

A última estratégia de proteção destaca a importância da modulação da MPT contra a lesão da I/R em fígados gordos, envolvendo o eixo preventivo SIRT3-CypD-MPT. A obesidade diminui a o conteudo de $NAD^+$ disponivel, contribuindo assim para uma diminuição na actividade da SIRT3,o que aumenta a acetilação de proteínas mitocondriais e diminui a eficiência mitocondrial. Demonstrámos que o aumento da acetilação da CypD em animais em situação de I/R foi acompanhada por uma inibição da actividade da SIRT3. Por outro lado, a adição de $NAD^+$ a fígados gordos antes da I/R aumenta significativamente a actividade da SIRT3 e diminui a acetilação, actividade e ligação entre a ANT e CypD, aumentando a restência ao MPT. O $NAD^+$ modula a função mitocondrial e preserva a fosforilação oxidativa, sendo protectivo contra danos num modelo de I/R hepática, em fígados gordos.

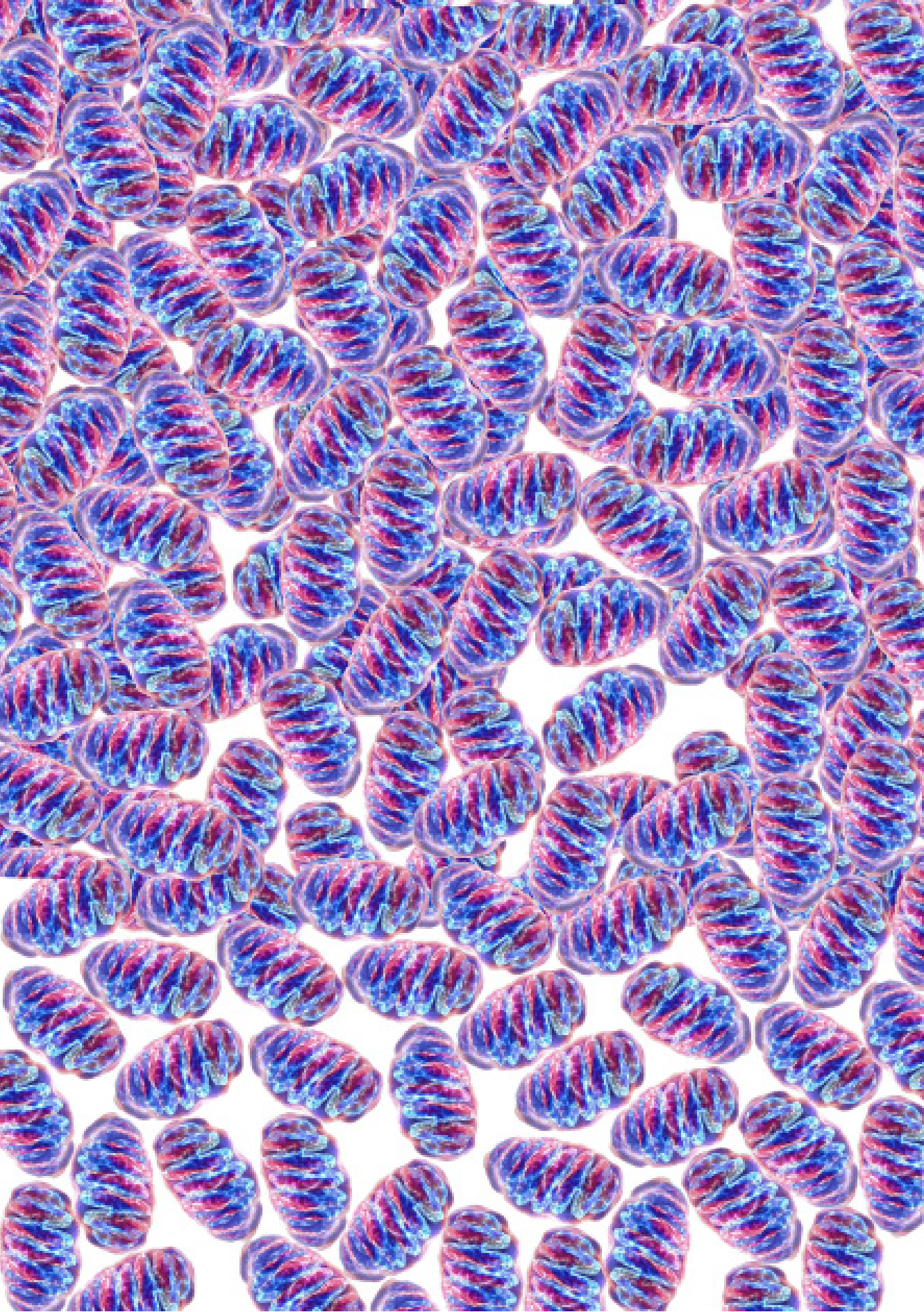
# LIST OF ABBREVIATIONS

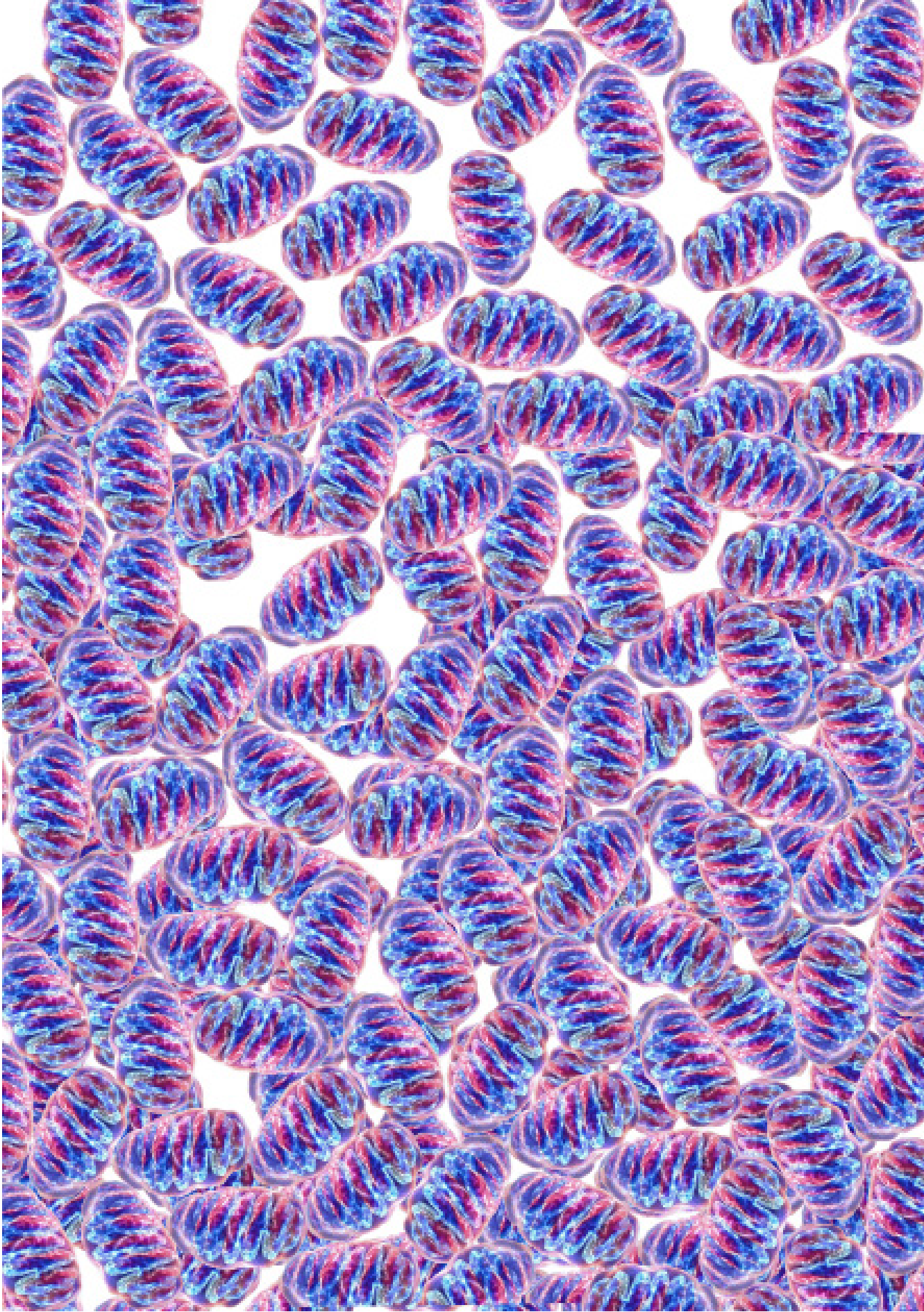
List of abbreviations:

•**OH**, hydroxyl radicals
**$A_1R$** adenosine receptors, subtype receptor 1
**$A_{2A}R$**, A2A subtype receptor
**ADP**, adenosine diphosphate;
**AICAR**, 5-amino-4-imidazole carboxamideriboside
**AIF**, apoptosis-inducing factor
**Akt**, protein kinase B
**ALT**, alanine aminotransferase
**AMP**, adenosine monophosphate
**AMPK**, Adenosine Monophosphate- Activated Protein Kinase
**ANT**, adenine nucleotide translocator
**Apaf-1**, apoptotic protease activating factor 1
**ApoB**, Apolipoprotein B
**AST**, aspartate aminotransferase
**ATP**, adenosine triphosphate;
**BAG-1**, Bcl-2 associated athanogene
**Bax**, Bcl-2–associated X protein
**Bak**, Bcl-2 homologous antagonist /killer
**Bcl-2**, B cell lymphoma - 2
**BPR**, benzodiazepines peripheral receptor
**BSA,** bovine serum albumin
**$Ca^{2+}$**, calcium
**CCPA**, 2-chlloro-N6-cyclopentyladenosine
**CDD**, choline-deficient diet
**CK**, Creatine kinase
**CoQ**, coenzyme Q
**CyA,** cyclosporin Ay
**CypD**, cyclophylin D
**cyt *c***, cytochrome *c*
**DAG**, diacylglycerol
**DCCD** , N, N'-dicyclohexylcarbodiimide
**DIABLO**, Direct IAP-binding protein with low PI
**DPCPX** , 8-cyclopentyl-1,3-dipropulxanthine
**ECD**, extended criteria donor
**EGTA,** ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
**eNOS**, nitric oxide synthase, endothelial form

**ER**, endoplasmic reticulum
**ERK**, extracellular signal-regulated kinase
**ETC**, electron transport chain
**FCCP**; carbonylcyanide-p-trifluoromethoxyphenylhydrazon
**GSK-3β**, glycogen synthase kinase 3β;
**GTP**, guanosine triphosphate
**$H_2$DCFDA** , 2',7'-dichlorodihydrofluorescein diacetate
**$H_2O_2$**, hydrogen peroxide
**HDL**, high-density lipoprotein
**HEPES** , N-[2-hydroxyethyl]piperazine-N'--[2-ethanesulphonic acid]
**HIF-1α**, hypoxia inducible factor-1α
**HK**, hexokinase
**HO-1**, heme oxygenase-1
**HSP**, heat shock proteins
**I/R**, ischemia/reperfusion
**IAPs**, Inhibitor of Apoptosis Proteins
**ICAM-1**, intercellular adhesion molecule-1
**IF1**, inhibitor F1 subunit
**IL**, interleukin
**IL-1Ra**, interleukin-1 receptor antagonist
**Ind**, indirubin-3'-oxime;
**iNOS**, nitric oxide synthase, inducible form
**I/R**, ischemia/reperfusion
**IκB-α**, inhibitor of kB-α
**KC**, Kupffer cells
**LC3**, microtubule - associated protein 1 light chain 3.
**LDH**, lactate dehydrogenase
**MAPK** ,mitogen-actived protein kinase
**mCa**, mitochondrial Ca2  channels
**MCU**. mitochondrial Ca2+ uniporter
**MPO**, myeloperoxidase
**MPT**, mitochondrial permeability transition
**mRyR**, ryanodine receptor
**mtDNA**, mitochondrial DNA
**$NAD^+$** , nicotinamide adenine nucleotide, oxidized form
**NADH**, nicotinamide adenine nucleotide, reduced form
**NADP,** nicotinamide adenine nucleotide phosphate, oxidized form
**NADPH,** nicotinamide adenine nucleotide phosphate, reduced form
**NAFLD**, non-alcoholic fatty disease
**NASH,** non-alcoholic steatohepatitis

**NFκB**, nuclear factor κ B
**NO**, nitric oxide
**NOS**, nitric oxide synthase
**O**, singlet oxygen molecules
**$O_2$•-**, superoxide anions
**OXPHOS**, oxidative phosphorylation
**PC**, preconditioning,
**PDK1**, phosphoinositide-dependent proteinkinase-1
**phospho-ser9-GSK3β,** glycogen synthase kinase 3β phosphorilated at ser9 residue
**$P_i$C**, phosphate carrier
**PKC**, protein kinase C
**Post**, postconditioning
**RaM,** rapide mode of calcium uptake
**RCR**, respiratory control ratio;
**RFUs**, relative fluorescence units
**ROS**, reactive oxygen species
**RR,** ruthenium red SIRT3, sirtuin 3
**SMAC**, second mitochondria-derived activator of caspases
**SOD,** superoxide dismutase
**SR**, sarcoplasmic reticulum
**stat-3**, signal activator of transcription 3
**TBS,** Tris-buffered saline
**TIM/TOM complex,** multi-subunit complexes present in both membranes
**TNF-α**, tumor necrosis factor- α
**$TPP^+$**, tetraphenylphosphonium;
**UCP**, uncoupling proteins
**UW**, University of Wisconsin
**VDAC**, voltage dependent anion channel
**VLDL's**, Very Low Density Lipoproteins
**X/XOD**, xanthine/xanthine oxidase
**ΔΨ**, mitochondrial membrane potential



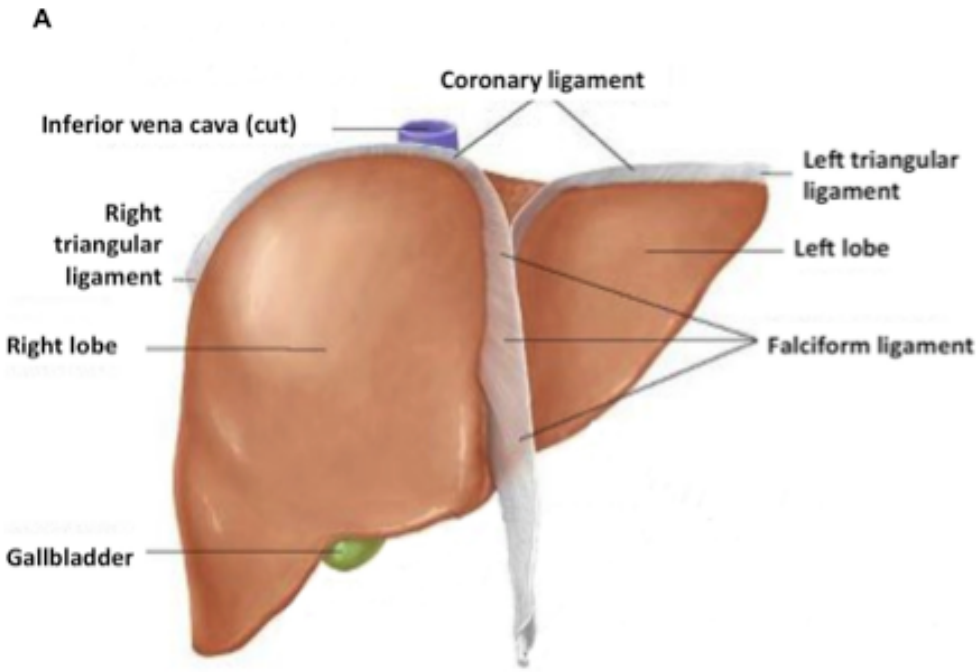


## 1.1 Liver

Liver is a vital organ, the largest internal organ of body, representing 2-5% of the body weight for an adult. It is divided in sectors and segments; each of them has its own branch of portal veins, hepatic artery and bile duct. Both the left and right parts have an independent blood supply. This organization facilitates some hepatic interventions such as hepatic resections and techniques applied in liver transplantation.

### 1.1.1 Liver general structure

Livers' structure is adapted to meet multiple and complexes functions (Figure 1.1). The liver has four lobes of unequal size and shape, which include two major lobes (left and right) and two minor lobes (median and caudate).



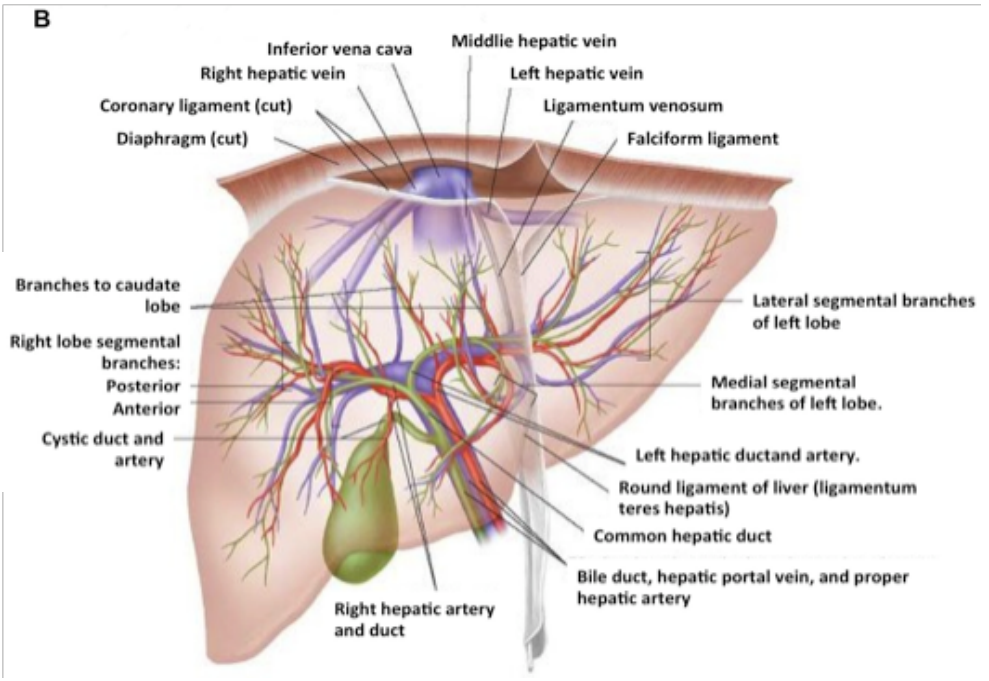



**Figure 1.1- Liver Anatomy: A) Anterior view; B) Portal vein, hepatic artery and biliary duct system** (adapted from Lippincott Williams & Wilkins, 2008).

The hepatic lobules are the basic functional units of the liver. The lobules have a hexagonal shape with a portal triad (portal vein, hepatic artery and hepatic duct) at each corner. In the center of each lobule there is the central vein that coalesce in hepatic veins from where it radiates columns of hepatocytes distributed in the form of cords. The spaces between the hepatic cords are blood channels called hepatic sinusoids. A cleflike, the bile canaliculus, lies between the cells within each cord and it's through it that the bile flows from the hepatocytes toward hepatic triad and exits the liver through the hepatic ducts. The sinusoids are lined with two cell populations: endothelial cells and Kupffer cells (KC). The perisinusoidal space or Space of Disse (the space between hepatic cords and sinusoids) is connected by lymphatic vessels that are responsible to remove the liquid excess.

### 1.1.1.1 Hepatic cells

**Hepatocytes** - The hepatocytes are the cells of the hepatic parenchyma and represent approximately 90% of the total cellular population. These cells are central to the intermediary metabolism of the body, so they have come to be considered the prototype cell in biochemistry. This high metabolic activity is related to the large amounts of mitochondria in these cells.

Hepatocytes have a wide variety of functions, including bile production to digest and absorb fat; storage and interconversion of nutrients, proteins, lipids and vitamins; detoxification of endogenous and exogenous toxic compounds to the organism; phagocytosis and synthesis of blood components, such as, plasma proteins, lipoproteins, glucose, fatty acids, cholestherol and phospholipids. Possibly all hepatocytes are able to perform all functions, however, there are differences in structure and in enzymatic activity depending on the concentration of oxygen and solutes in blood. The characteristics of blood (concentration of oxygen and solutes) can be different depending on the distance of the hepatocyte until the portal vein. The proportion of hepatocytes that are in contact with the sinusoid is called the sinusoidal region, which is responsible for the liver's exocrine function, the secretion of bile. (Cingolani et al.,2000 ; Ganong, 2001)

**Cells of hepatic sinusoid** - Endothelial cells and Kupffer cells are non parenchymal cells, significant in the total cellular population.

**Endothelial cells-** The endothelial cells form the wall of the hepatic sinusoids and are separated from hepatocytes by the "Space of Disse". This creates an extravascular compartment into which the hepatocytes project microvilosities, increasing the contact area between the hepatocyte and the vascular space. The presence of pores allows the exchange of fluids and macromolecular compounds. Vasoconstrictors and vasodilators derived from the sinusoidal endothelium, such as endothelin and nitric oxide (NO), are important to control hepatic blood flow in both physiological and pathological situations. Certain stimuli cause the release of a series of mediators by the endothelial cells that are actively involved in the inflammatory response. (Pocock et al.,2002; Zakim et al 2002)

**Kupffer cells** - The Kupffer cells are located mainly in the wall of hepatic sinusoids. They act as a protective barrier because of their ability to eliminate by endocytosis, many potentially harmful substances to the body, such as microorganisms, endotoxins, immune complexes, tumor cells, lipids, etc. When activated, the Kupffer cells begin to generate

several cytotoxic mediators such as cytokines, reactive oxygen species (ROS) and proteases. (Cingolani et al.,2000 ; Ganong, 2001; Pocock et al.,2002; Zakim et al 2002)

**Stellate cells** - These cells are located in close contact with endothelial cells and are the main deposit of vitamin A to the body. They synthesize extracellular matrix proteins (different types of collagen), are important mediators of tissue repairing processes (healing) in various liver diseases and have the capacity to promote and amplify the inflammatory response. ( ; Ganong, 2001; Pocock et al.,2002; )

#### 1.1.1.2 Hepatic vascular system

The liver has a dual blood supply: the portal vein carries blood rich in nutrients but poor in oxygen from the intestine, the spleen and the pancreas; the hepatic artery transports blood rich in oxygen coming from the heart. Blood will finally reach hepatic sinusoids, which is where takes place the exchange between blood and hepatocytes. The great porosity of sinusoidal walls facilitates this exchange of nutrients. From the blood, the hepatocytes can take up the oxygen and nutrients, which are stored, detoxified, used for energy, or used to synthesize new molecules. These molecules are released into the hepatic sinusoids or into bile canaliculi. Blood in the hepatic sinusoids flows to the central vein, where it exits the lobule and then exits the liver through the hepatic veins and finally into the inferior vena cava just below the diaphragm. So, blood flows from the triad toward the center of each lobule while bile flows away from the center of the lobule toward the triad. Bile flows across the bile canaliculi via the hepatic triad and exit the liver through the hepatic ducts (Cingolani et al.,2000 ; Ganong2001; Pocock et al.,2002; Zakim et al., 2002). (Figure 1.2).

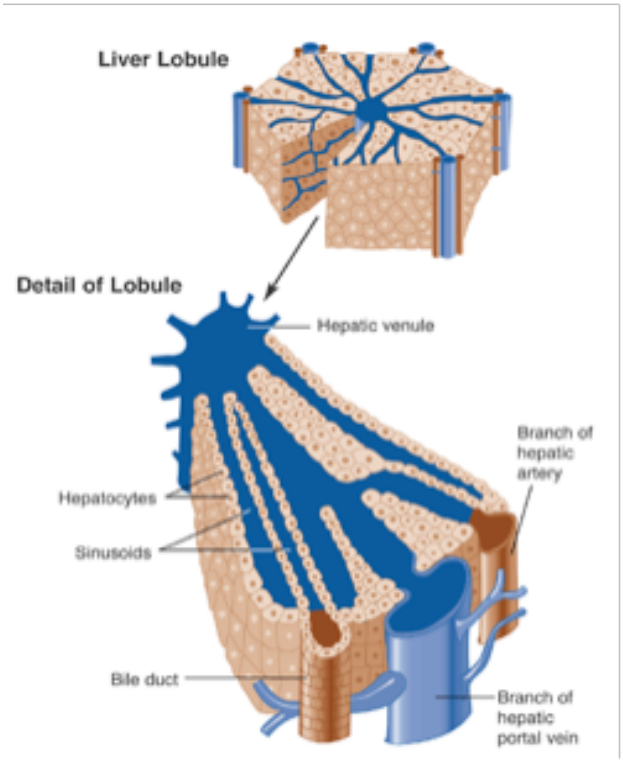



**Figure 1.2- The structure of the liver's functional units, or lobules.**
Blood enters the lobules through branches of the portal vein and hepatic artery, then flows through small channels called sinusoids that are lined with hepatocytes. The hepatocytes remove toxic substances, from the blood, which then exits the lobule through the central vein (adapted from Ross et al. ,1995).

### 1.1.2 Liver general function

The liver is the organ responsible for regulating the energetic levels of the body. It also performs important digestive and excretory functions, stores and processes nutrients, synthesizes new molecules and detoxifies harmful chemicals. Hepatocytes serve as a glycogen store and are able to maintain blood glucose levels. They can also store fat, vitamins, copper and iron. Amino acids, carbohydrates, lipids and vitamins income to the liver from digestive tract via the portal vein and then are stored and metabolically converted, to be released into the blood and into the bile as required (Kodavanti and Mehendale, 1991). The bile is produced and secreted in liver and plays a role in digestive and excretory function. Liver's core activity in metabolism makes it an easy target for various toxic processes and liver problems can be quickly followed by problems in other organs. The liver can also produce its own compounds, participating in the

metabolism of many serum proteins (Table 1.1).

Hepatocytes also transform substances that cannot be used by most cells into more readily usable substances. Another very important function of the liver, to keep the normal body function, is the detoxification of harmful compounds by biotransformation. In view of this, it is a natural target for xenobiotic-induced injury, resulting in perturbation of homeostasis. The liver forms a major defense line to harmful substances, detoxifying many of them by altering their structure to make them less toxic or make their elimination easier. For example, hydrophobic substances are converted into water-soluble products to be excreted into the bile or urine. Liver also plays a role in phagocytosis: Kupffer cells remove final products of the endogenous metabolism and exogenous substances that are not desirable.

**Table 1.1 Description of liver functions.**

| | |
|---|---|
| **Synthesis** | |
| **Protein metabolism** | Amino acids synthesis |
| | Protein synthesis and degradation |
| | Albumin |
| **Carbohydrate metabolism** | Gluconeogenesis |
| | Glycogenolysis |
| | Glycogenesis |
| **Lipid metabolism** | Cholesterol synthesis |
| | Lipogenesis |
| | Lipoprotein synthesis |
| **Coagulation factors** | |
| **Fetal hematocyte production** | |
| **Bile** | |
| **Hormones (ex: angiotensinogen)** | |
| **Degradation** | |
| **Insulin and other hormones** | |
| **Bilirubin** | |
| **Detoxification of metabolic byproducts and xenobiotics** | |
| **Ammonia** | |
| **Storage (ex: glycogen, vitamins, metals, others)** | |
| **Immune activity (reticuloendothelial system)** | |

## 1.2 Mitochondria, a key organelle in cell function

Mitochondria are the powerhouses of the cells. Being the main adenosine triphosphate (ATP) source of the cell, mitochondria play a key role in the regulation of several aspects of cell biology such as molecular metabolism, redox status, calcium signaling, and programmed cell death (Schatz et al., 1995). As they are the descendant's aerobic bacteria, it permitted the evolution of the first eukaryotic cell capable to aerobic respiration, which was fundamental to evolution of more complex multicellular organisms.

In the past years, the view of mitochondria as a lonely participant in the cell working tirelessly to provide the energy required for life has changed, as newer approaches have allowed the examination of dynamic mitochondrial function and behavior in response to cellular signals within intact cells.

### 1.2.1 Mitochondrial structure

The number and morphology (size and shape) of mitochondria differ according to the cell type, but maintaining basic properties (Koolman and Röhm,1996) (Figure 1.3). Mitochondria are a double membrane-system: the outer membrane separates the mitochondrion from the cytosol and defines the outer perimeter and the inner membrane, that is invaginated, forms the cristae and defines the matrix of the organelle.

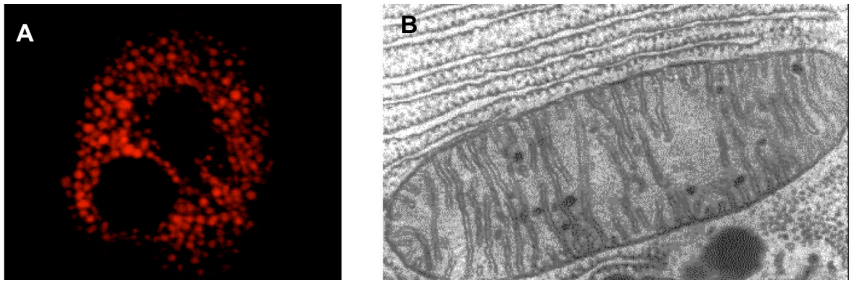



**Figure 1.3 - Visualization of mitochondria by electron (A) and fluorescence microscopy (B).**

The cristae are not simple invaginations of the inner membrane, but independent structures that are not always connected with the inner membrane (Manella et al., 2000; Scheffler et al., 2001; Frey et all., 2002). The surface area of cristae can be greater in the case of the tissues with the higher respiratory activity. Mitochondria are highly dynamic organelles that continuously change their shape through frequent fusion, fission and movement throughout the cell. These dynamics are crucial for the maintenance of cellular homeostasis, as perturbations in this balance are linked to apoptosis and metabolic diseases.

The outer and inner mitochondrial membranes have different permeability to various metabolites and ions, so as the outer membrane is permeable to ions and solutes up to 10 kDa, the inner membrane is highly proteinaceous and serves as a permeability barrier (Stryer, 1988; Crompton et al., 1999). Therefore, in the inner membrane, any movement of solutes is tightly controlled and allowed only through membrane proteins, channels highly selective and regulated. Some proteins are freely attached to the surfaces of the membranes and others are inserted as an integral part of the membrane.

The inner membrane has diverse elements with a role in metabolic pathways, such as components of the electron transport chain (ETC) and ATPsynthase (Hatefi et al., 1985), the di- and tricarboxylate carriers, the phosphate carrier, the adenine nucleotide translocase (ANT) and uncoupling proteins (UCP) (Caldwell et al., 2004). The large content in cardiolipin (diphosphatidylglycerol), reduces the permeability of the phospholipid bilayer to protons and thus enables a proton-motive force to be established across the inner membrane. This lipid is also important to the function of several mitochondrial proteins (Hoch, 1992; Paradies et al., 1998).

The outer membrane is composed of about half protein and half lipid and is rich in cholesterol. The voltage-dependent anion channel (VDAC) is the most abundant protein in the outer mitochondrial membrane. Associated with this membrane we also find mitochondrial hexokinase (HK), creatine kinase (CK) and benzodiazepines peripheral receptor (BPR).

There are still multi-subunit complexes present in both membranes - mitochondrial inner/ outer membrane transporter (TIM/TOM), that mediate the translocation of peptides into mitochondria (Paschen and Neupert, 2001).

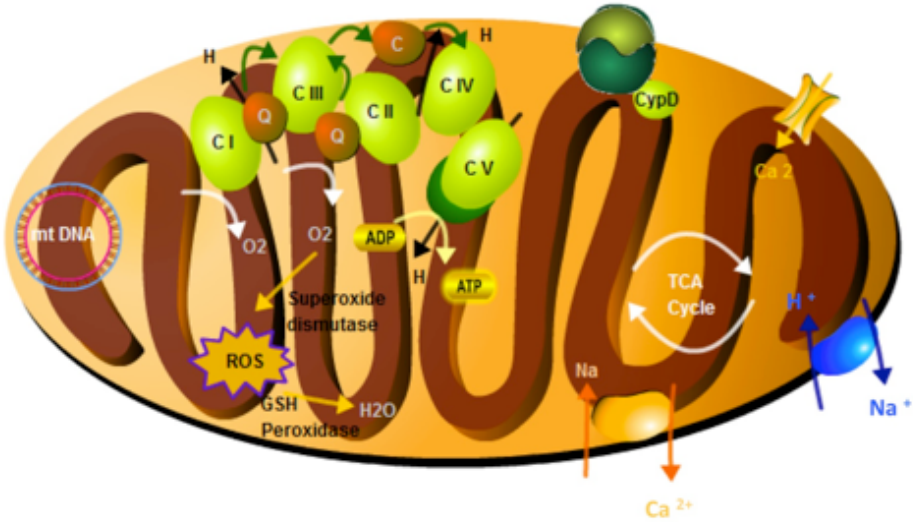



**Figure 1.4 - Mitochondrial function in cell metabolism**. The most prominent role of mitochondria is to produce ATP, through oxidatve fosforilation, and to regulate cellular metabolism. Components of the electron chain (CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV) and ATPsynthase (CV) are present in the inner membrane. Result of imperfectly coupled electron transport, mitochondrion is a major intracellular source of reactive oxygen species (ROS). $Ca^{2+}$ transport into the mitochondria occurs through ion channels, existing in the mitochondrial inner membrane. Present in the mitochondrial matrix are diverse enzymes part of the citric acid cycle or Krebs cycle (TCA cycle) and mitochondrial DNA (mtDNA).

Present in the mitochondrial matrix are diverse enzymes part of the citric acid cycle or Krebs cycle, amino acid metabolism, the urea cycle and the synthesis of heme groups, among others. It is also in the matrix that there is mitochondrial DNA (mtDNA) as well as transcription and translation systems, characteristic that makes mitochondria the unique organelle containing their own genome (Anderson, 1981) (Figure 1.4). Mutations in the mtDNA disrupt mitochondrial respiration and bioenergetics, resulting in cell death and dysfunction as major characteristics to the pathophysiology of mtDNA diseases (Moraes, 1996; Smeitink et al., 2001). However, most of the mitochondrial proteins are nuclear-encoded (Wallace, 1994).

### 1.2.2 The inner mitochondrial respiratory chain and energy production in mitochondria

In the eukariotic cells, the mitochondria is the primary organelle responsible for ATP generation associated with the oxidation of carbohydrates, fats and amino acids (Saraste,1999) . The process of oxidative phosphorylation (OXPHOS) occurs in the inner mitochondrial membrane and the complete sequence of events was described by the chemiosmotic theory proposed by Mitchell in 1961. This theory describes the process by which creation of an electrochemical gradient of protons across the inner membrane, supports the synthesis of ATP through the mitochondrial ATPsynthase (Mitchell,1961).

The ETC consists of five multi-subunits protein complexes located in the inner membrane (respiratory complexes and the $F_1F_0$-ATPsynthase) and two factors (cytochrome *c* (cyt *c*) and coenzyme $Q_{10}$(CoQ)) (Galluzi, 2010). The protein subunits can be grouped into four complexes: Complex I (NADH (nicotine adenine dinucleotide, reduced form) ubiquinone oxiredutase), Complex II (succinate dehydrogenase), Complex III (ubiquinone cytochrome c oxiredutase) and Complex IV (cytochrome c oxidase). ATP is then produced by the F1F0-ATP synthase, also known as complex V, a component of the OXPHOS system but not part of the ETC (Morais and De Strooper, 2010; Mattson et al., 2008).

The electron carriers of the respiratory chain are quinoid structures (flavins, quinones) and/or metal centers (centers FeS groups, heme centers, copper), driving electron ($e^-$) transfer to molecular oxygen occurs in the following sequence: complex I → ubiquinone → complex III → cytochrome c → complex IV → oxygen ($O_2^2$). Complex I accepts $e^-$ from NADH and transfers them to the “pool” of ubiquinone. Complex II accepts $e^-$ from succinate and also transfers them to the “pool” of ubiquinone. This molecule is reduced to ubiquinol, a highly mobile and hydrophobic molecule, will then be able to transfer $e^-$ to Complex III, again passing through the QH and Q states. After $e^-$ flow into Complex III, they are transferred to cytochrome c and then to Complex IV. Here, they are finally conducted to the final acceptor, $O_2$, which together with protons originates $H_2O$.

The redox potential $e^-$ the acceptor must be more positive than the redox potential of the donor. This phenomenon carries some advantages to the cell. At first place, the energy is not totally released in a single step, but sequentially, which allows a better use of that energy. Second, the e- flux occurs without excessive loss of e- or dangerously high dissipation

to the cell. At last, the flux takes place without energy consumption and the passage of the $e^-$ through the complexes, causes a conformational change in their structure, allowing them to eject protons from the matrix to the intermembrane space, in this state of high instability. The Complex II is an exception to this, because due to the fact of having a “redox” potential similar to that of ubiquinone, it does not reach a state capable of ejecting protons. There are human diseases caused by an inefficient electron transfer through the complexes I to IV, because of two main reasons, which are the inability to generate ATP and the production of toxic reactive oxygen species due to insults to the ETC enzymes (particularly Complexes I, II and III).

Thus, sequential transfer of electrons from the reducing equivalents to molecular oxygen and subsequent proton ejection, originates an electrochemical gradient (composed by a pH gradient (ΔpH) and an electrical gradient) between the matrix and the intermembrane space. Thus, there is a high concentration of protons in the intermembrane space that tends to be dissipated as soon as possible. As the inner membrane is quite impermeable to protons, this dissipation of the gradient occurs through ATPsynthase (Figure 1.5).

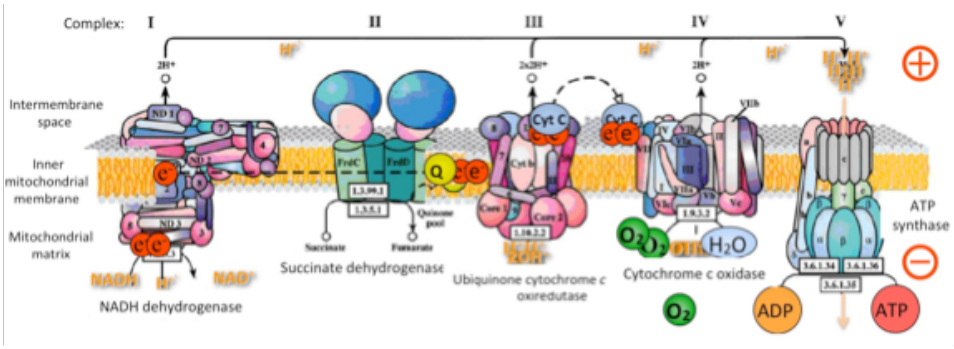



**Figure 1.5 - Schematic representation of the mitochondrial respiratory chain and ATPsynthase**. Represented are the four complexes of the respiratory chain and the F1Fo ATP-synthase. Complex I and complex II accept electrons from NADH and succinate, which flow to the ubiquinone pool, complex II, cytochrome *c*, complex IV to the final acceptor, molecular oxygen. Electron flow is coupled to proton ejection at complexes I, III and IV. Complex V harvests the resulting proton gradient to generate ATP (adapted from Mandavilli et al., 2002).

The ATPsynthase is a proteic complex composed by two components, the $F_0$ and the $F_1$. The $F_0$ component is a hydrophobic segment that crosses the inner mitochondrial membrane, forming a proton channel.

The $F_1$ component consists of five types of polypeptidic chains and is responsible for phosphorylating the ADP, being also capable, in severe circumstances to the mitochondrion, to hydrolyze ATP, in order to try to restore the proton gradient. Other subunits are also part of ATP synthase, including the regulatory protein of the proton flow and ATP synthesis, called inhibitor F1 subunit (IF1), and a protein that confers sensitivity to oligomycin. The ATPsynthase is specifically inhibited by oligomycin and DCCD (N, N'-dicyclohexylcarbodiimide) (Boyer, 1997; Boyer, 1998).

Two models for the organization of the mitochondrial respiratory chain have been proposed: the "fluid-state" model, where all OXPHOS complexes diffuse individually in the membrane and electron transfer depends on the random collision of the complexes and electron carriers; the "solid-state" model, which has recently received more attention, where the complexes together form supercomplexes or respirasomes (Dudkina et al. 2008; Schon et al, 2009). However, is a combination of these two models - the "plasticity" model – that has been the model mostly accepted. Work by Acin-Perz and colleagues, using mouse liver mitochondria, conclude that complex I is hardly found as an independent entity, being mostly associated with complex III in different supercomplexes (Acín-Peréz et al., 2008). Some of these associations contain CoQ, cyt *c*, and complex IV, with or without complex II, and are able to respire. There are two abundant forms of I/III supercomplexes that differ in their molecular mass and do not associate with complex IV, but may interact with complex V. Complex III is also mostly associated with other complexes in liver mitochondria. The association with complex I was already described but it can also form additional associations such as II/III/IV. Despite the association of complexes II and IV with other complexes, there is a big part that is found nonassociated with any of the other respiratory complexes (Acín-Pérez et al., 2008 ).

### 1.2.3 Mitochondrial generation of reactive oxygen species

Reactive oxygen species (ROS) is the collective term that generally describes a variety of molecules and free radicals derived from molecular oxygen: singlet oxygen molecules (O), superoxide anions ($O_2$•-), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (•OH). These are products of normal metabolism acting as "redox messengers" in intracellular signaling and regulation, allowing communication between the mitochondria and the cytosol (D'Autréaux et al. 2007; Mittler et al., 2011).

The mitochondrion is a major intracellular source of ROS, as a

result of imperfectly coupled electron transport associated with oxidative phosphorylation (Boveris et al., 1976; Takeshige and Minakami, 1979; Turrens et al., 1985). Result of this is a particular susceptibility of the mitochondria to oxidative damage. Electrons released from the mitochondrial electron transport chain incompletely reduce $O_2$ to form superoxide, which is converted into $H_2O_2$ by manganese superoxide dismutase (MnSOD) in the mitochondria.

The redox state of the respiratory chain is the primary factor for mitochondrial ROS generation (Skulachev, 1996; Lambert and Brand, 2004) (Figure1.6). The amplitude of the electrochemical proton gradient, which is known as respiratory control, regulates the overall rate of electron transport in the respiratory chain. When the electrochemical potential difference generated by the proton gradient is high (such as in high-fat or high-glucose states), or in conditions of inhibition of the ETC complexes, the life of superoxide generating electron transport intermediates, such as ubisemiquinone, is prolonged (Skulachev, 1998). This occurs because the transmembrane proton gradient and the membrane potential inherently govern the activity of the respiratory chain complexes as proton pumps. When sufficiently high, pH and potential inhibit the proton pumps.

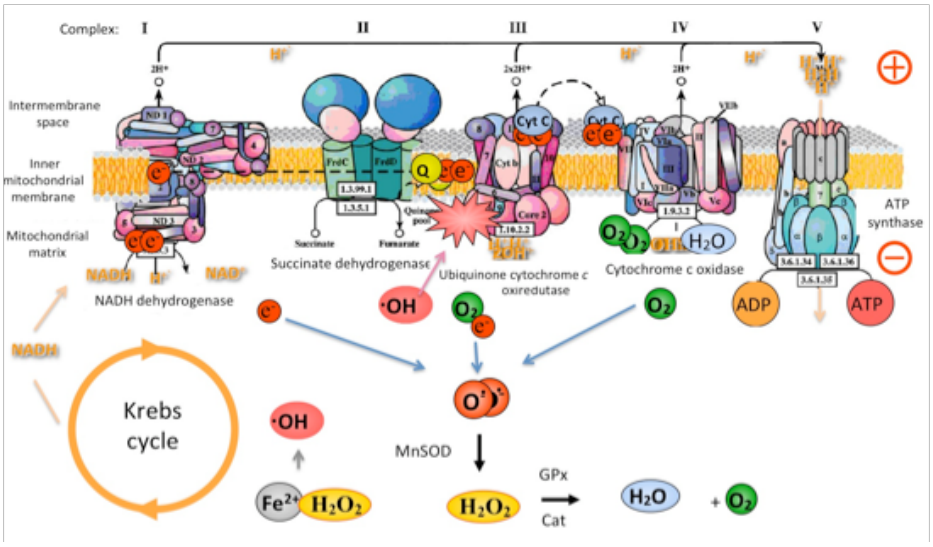



**Figure 1.6- Schematic representation of the mitochondrial generation of reactive oxygen species in the mitochondria.** The mitochondrial respiratory chain generates ROS mainly at the complexes I and III. Specifically, the e-flow from the Q pool to Complex III is the step where the major part of superoxide anion production occurs.The enzymatic dismutation catalyzed by the manganese superoxide dismutase (MnSOD) produces $H_2O_2$ and regulates the superoxide anion levels (adapted from Mandavilli et al.,2002).

### 1.2.4 Mitochondrial role in calcium homeostasis

Intracellular calcium ($Ca^{2+}$) is vital for cell physiology and the disruption of its homeostasis leads to various diseases. There are several proteins localized in different regions of the cell, which, working in coordination, guarantee an effective intracellular $Ca^{2+}$ dynamics (Figure 1.6).

At local micro-domains such as plasma membrane and endoplasmic/sarcoplasmic reticulum (ER/SR), $Ca^{2+}$ concentrations extremely differ between the entrance of the open $Ca^{2+}$ channel, where it is higher, and places very close to the channel, where it reaches very low concentrations (Parekh, 2003). Extra mitochondrial $Ca^{2+}$ concentrations, mitochondrial inner membrane potential and $Ca^{2+}$ concentrations in the mitochondrial matrix, determine the dynamics of mitochondrial $Ca^{2+}$ uptake. When the extra mitochondrial $Ca^{2+}$ concentrations are high (intracellular calcium stores and plasma membrane calcium channels), mitochondria sense and respond to the $Ca^{2+}$ transients by taking up $Ca^{2+}$.

This physiological increase in mitochondrial $Ca^{2+}$ serves as a key signal to regulate mitochondrial activities. Intramitochondrial enzymes involved in energy metabolism are activated after a calcium signal, so one of the main functions of mitochondrial calcium transport is to increase NADH production. Mitochondrial calcium uptake stimulates the activity of Krebs cycle and oxidative phosphorylation, and thus ATP synthesis (Denton, 2009; Balaban, 2009).

$Ca^{2+}$ transport into the mitochondria occurs through ion channels (Bernardi 1999; Ryu et al., 2010), existing in the mitochondrial inner membrane. Each of calcium influx plays a role via their unique characteristics in $Ca^{2+}$ affinity, kinetics and pharmacological properties.

The mitochondrial $Ca^{2+}$ uniporter (MCU) is a protein complex formed by a channel and a regulatory subunit. The MCU allows $Ca^{2+}$ transport down the electrochemical gradient into the mitochondrial matrix and without coupling to ATP hydrolysis or cotransport with other ion or molecule (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994). The calcium uniporter is regulated by a number of modulators: ruthenium red and other drugs (Noack and Greeff, 1971; Sastrasinh et al.,1982; Schellenberg et al.,1985) block mitochondrial Ca2+ uptake; inorganic phosphate (Pi) and spermine activate MCU and facilitate $Ca^{2+}$ transport (Rossi et al., 1973). Cytosolic $Ca^{2+}$ also regulates MCU in a calmodulin-dependent manner (Moreau et al., 2006; Putney and Thomas, 2006).

Another proposed mechanism for mitochondrial $Ca^{2+}$ uptake is the rapid mode (RaM) uptake. It was described in liver mitochondria that in response to a cytosolic $Ca^{2+}$ pulse, mitochondrial $Ca^{2+}$ concentrations increase very rapidly (Rizzuto et al.,1993; Rizzuto et al.,1994). Calcium conductivity through the RaM is many times higher than that through the uniporter. At higher extra mitochondrial $Ca^{2+}$ concentrations, $Ca^{2+}$ uptake is mediated by both MCU and RaM. However, MCU does not transport $Ca^{2+}$ when the calcium concentration is below its threshold (Sparagna et al.,1995). Like the calcium uniporter, the RaM is inhibited by RR (but is less sensitive) and stimulated by spermine. ATP and guanosine triphosphate (GTP) can activate RaM in liver but not in heart.

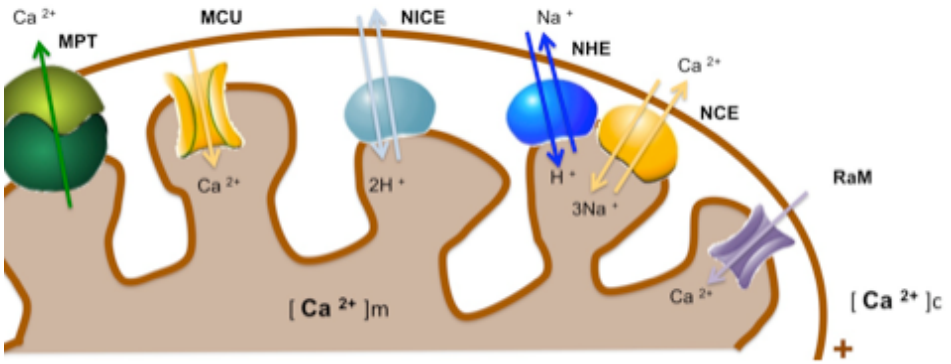



**Figure 1.7- Schematic diagram of calcium ($Ca^{2+}$) transport systems in the mitochondrial.** Calcium influx to the mitochondrial matrix is mediated by a uniporter transporter, powered by the electrochemical gradient (MCU). $Ca^{2+}$ can also enter mitochondria by the rapid calcium uptake mode (RaM). Mitochondrial $Ca^{2+}$ efflux is mediated by antiporter transporters: a $Na^{+}$- independent mechanism (NICE) and a $Na^{+}$-dependent mechanism (NCE). MPT represents another important mechanism for $Ca^{2+}$ release from mitochondria in physiological conditions.

Recently, other proteins have been reported as $Ca^{2+}$ transporters, for example, the ryanodine receptor (mRyR) (Beutner et al., 2001), uncoupling proteins UCP2 and UCP3 (Trenker et al.,2007) and new mitochondrial calcium channels voltage-dependent highly selective for $Ca^{2+}$ (mCa1,mCa2) (Michels et al., 2009).

The mitochondrial calcium efflux occurs via two different routes: dependent (Pfeifer and Gunter, 1990) and independent of sodium (Puskin et al., 1976). Both systems have different kinetic, but both require energy, since the calcium efflux occurs against the electrochemical gradient.

The output of mitochondrial calcium through $Na^+$-dependent antiporter is abundant in tissues such as heart with a stoichiometry of $3Na^+$:$1Ca^{2+}$ (Gunter et al., 1994). The sodium-independent mechanism is present in the liver and allows efflux of one calcium in exchange of two protons. This pathway saturates at low calcium loads and is extremely slow (Gunter et al., 1994).

The balance between $Ca^{2+}$ influx and efflux across mitochondrial inner membrane establishes mitochondrial $Ca^{2+}$ homeostasis. The mitochondrial $Na^+/Ca^{2+}$ exchanger normally serves as a major $Ca^{2+}$ efflux mechanism, but the transient opening of MPT represents another important mechanism for $Ca^{2+}$ release from mitochondria in physiological conditions.

### 1.2.5 The mitochondrial permeability transition

Under conditions of mitochondrial calcium overload, especially when accompanied by oxidative stress, elevated phosphate concentrations and adenine nucleotide depletion, a non-specific pore, the mitochondrial permeability transition (MPT), opens in the inner mitochondrial membrane. The MPT is a large conductance channel in the mitochondrial inner membrane abruptly increasing its permeability to all solutes of molecular weight up to about 1500 Da. This leads to an increase in mitochondrial matrix volume, due to water entry inside mitochondria, resulting in mitochondrial swelling associated with membrane depolarization and uncoupling of the mitochondria, calcium release and unfolding of the inner membrane cristae (Zoratti and Szabò, 1995; Bernardi, 1999; Crompton, 1999).

#### 1.2.5.1 Regulation of the mitochondrial permeability transition

Matrix $Ca^{2+}$ is an essential permissive factor for mitochondrial permeability transition induction. The matrix free $Ca^{2+}$ that favors MPT is difficult to define, since the concentration required is highly variable for different tissues and is influenced by various factors that change together with the $Ca^{2+}$ load (such as matrix Pi and pH).

Increasing $P_i$ concentrations decrease matrix $Ca^{2+}$, which should in turn decrease the probability of MPT induction; on the other hand, the mitochondrial accumulation of $P_i$ potentiates $Ca^{2+}$ uptake by counteracting matrix alkalinization established by $H^+$ extrusion in response to $Ca^{2+}$ entry into the matrix. Therefore, the large electrochemical gradient for $Ca^{2+}$ existing at the level of the inner membrane of respiring mitochondria favors

the matrix accumulation of both $Ca^{2+}$ and $P_i$. Therefore, $P_i$ is normally referred to as a MPT inducer, whose effect is explained as the result of buffering matrix pH, since the transition is potently inhibited at matrix pH below 7.0. The open probability decreases sharply below this pH value through reversible protonation of critical histidyl residues that can be blocked by diethylpyrocarbonate ( Bernardi , 1992). The MPT-regulatory histidyl residues are not located on cyclophilin D (CypD) , a proposed MPT component, because MPT modulation by matrix pH is not affected by CypD deletion (Basso et al., 2005). A recent study reports mitochondrial phosphate carrier (PiC) (Leunget al., 2008) and CypD (Basso et al., 2008) as mediators of the effects of Pi on the MTP. These results have modified the understanding of the role of Pi in MPT regulation.

MPT opening is favored by mitochondrial depolarization. By modifying the threshold potential, MPT inducers can increase the opening probability at physiological mitochondrial membrane potential ($\Delta\Psi$) values (Bernardi,1992). The dependence on $\Delta\Psi m$ is likely to be also related to redox processes, and especially to oxidative stress. Agents that promote an oxidized state of pyridine nucleotides and thiol cross-linkers are inducers of the MPT (Kowaltowski et al., 1996). On the other hand, antioxidants have a preventive role (Petronilli et al., 1994). The susceptibility to MPT induction might be increased by cellular responses triggered by intracellular $Ca^{2+}$ elevation such as generation of arachidonic acid by phospholipase $A_2$ and calpain activation (Penzo et al., 2004; Polster et al., 205; Di Lisa et al., 2007). So, conditions and xenobiotics that cause oxidative stress, adenine nucleotide depletion, increased $P_i$ concentrations and mitochondrial depolarization, will promote the onset of the MPT.

A major consequence of MPT induction is inhibition of oxidative phosphorylation, which unrestrained will lead to necrotic cell death. The permeability transition has also been pointed to be involved in apoptosis, through the release of pro-apoptotic factors.

### 1.2.5.2 Molecular composition of the MPT pore

Although the molecular identity of the MPT remains uncertain, several proteins have been implicated in either its structure or regulation. The original model for the MPT based upon biochemical and pharmacological studies had three components: the voltage-dependent anion channel in the outer membrane, the adenine nucleotide translocase in the inner membrane, plus cyclophilin D in the matrix (Figure 1.8) . VDAC, ANT, and CypD interact at membrane contact sites and reconstitution of this complex

in vesicles yields a $Ca^{2+}$-sensitive channel reminiscent of the MPT pore (Cromptom et al., 1998). This working model was consistent with inhibition and activation of the MPT by bongkrekic acid and atractyloside, ligands of ANT, and blockade by CsA, which binds to CypD (Cromptom et al., 1992). Actually, this model is discussed since recent genetic studies have questioned the validity of this paradigm.

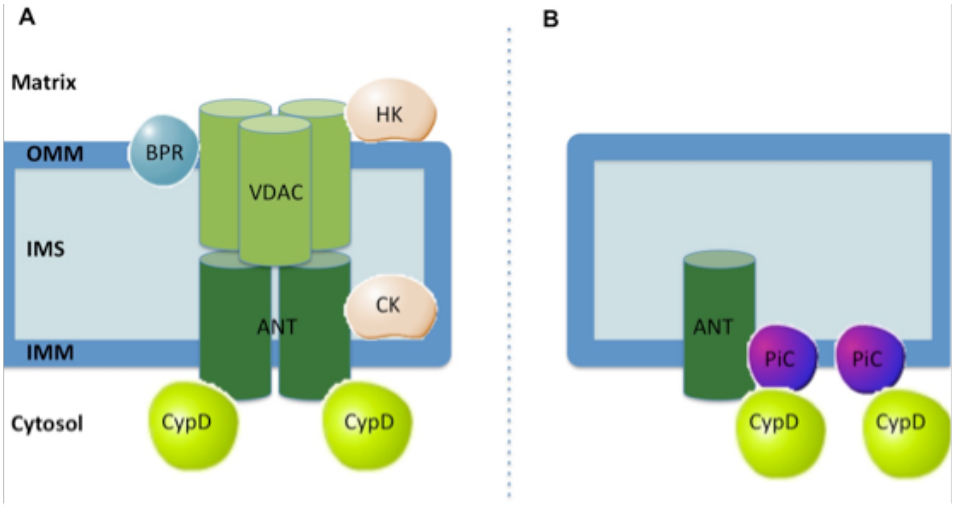



**Figure 1.8- Molecular models for the mitochondrial permeability transition (MPT) pore.** A) The original model for the MPT pore, consisting of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane (IMM), and cyclophilin-D (CypD) in the matrix. B) Revised models in light of recent findings in gene targeted mice. VDAC is no longer part of the model and it appears that an outer membrane component may not even be necessary for this process. ANT now appears to be more of a regulatory protein, and only CypD remains as an established component. In contrast, the mitochondrial phosphate carrier (PiC) has been added to model as a potential candidate for the pore-unit of the MPT pore.

#### 1.2.5.2.1 Voltage-dependent anion channel (VDAC)

Although VDAC has long been considered a key component of the MPT, the evidence supporting for its involvement is largely circumstantial. VDAC is the most abundant protein in outer mitochondria membrane and facilitates the efficient transport of ATP/ADP across the outer leaflet

(Blachly-Dyson et al.,2001; Rostovtseva et al., 2005).

Some works report that VDAC inhibitors and VDAC "blocking" antibodies can prevent MPT, but the specificity of these agents is uncertain (Cesura et al., 2003; Toth et al., 2006). On the other hand, Halestrap group showed that mitochondria and cell lacking all three VDAC isoforms showed normal MPT opening, further demonstrating that VDAC is not an essential component of the MPT (Halestrap et al.,2005).

#### 1.2.5.2.2 Adenine nucleotide translocase (ANT)

The ANT is the most abundant protein in mitochondria. The ANT family mediates the exchange of ATP and ADP across the inner mitochondrial membrane (ATP from the matrix to the cytosol, by exchange with an ADP molecule). There are three isoforms of ANT: ANT-1, ANT-2 and ANT-3 (Fiore et al., 1998). ANT-1 is the most abundant inner mitochondrial membrane protein. Some evidences suggest that ANT-1 or ANT-3 is a component of the MTP (Bauer et al., 1999; Pereira et al., 2007; Yang, Z. et al., 2007). ANT functions as a pore between two extreme conformations, in which the binding sites of the substrate are on cytosolic side of the inner membrane - c conformation - or on the side of the matrix - m conformation. C conformation is likely to change with the binding of Ca2+ inducing MPT. This does not apply in the conformation m (Crompton et al. 1999).

Pharmacological manipulation of the ANT with atractyloside or bongkrekic acid influences MPT (Haworth et al.; 2000; Akao et al., 2003). In an earlier model, the ANT was suggested to be the membrane component of the MPT. This model was disputed by results of gene targeting experiments demonstrating that the ANT is not essential for MPT formation. Studies by Kokoszka and colleagues, who have used mice in which ANT is not expressed, showed that it does not significantly alter MPT thus raising doubts about ANT's identity and if it is a necessary component of the MPT pore (Kokoszka et al., 2004). However, the same group has demonstrated that even though ANT might not be a component of the PTP per se, it can control susceptibility to MPT induction (Lee et al., 2009).

#### 1.2.5.2.3 Cyclophilyn D (CypD)

Cyclophilins are a family of proteins that catalyze the *cis-trans* isomerization of peptidic bonds. CypD is a water-soluble protein (18kDa) that is located predominantly in the mitochondrial matrix. CypD, facilitates a conformational change in the ANT, promoting MPT. Therefore,

suppression of the ANT-CypD interaction may contribute to the elevation of the threshold for MPT induction CypD deficient mitochondria and cells are resistant to $Ca^{2+}$ and oxidative stress-induced MPT and cell death. In CypD knockout mice, MPT induction requires greater $Ca^{2+}$ and is not blocked by cyclosporine A (CyA), a classical pharmacological MPT inhibitor (Basso et al., 2005; Baines et al., 2005; Nakagawa et al., 2005). Studies have shown that the potency of different CyA analogues to inhibit the MPT correlates with their ability to inhibit the peptidyl-prolyl cis-trans isomerase activity within the matrix that was subsequently identified as CypD (Halestrap and Davidson,1990; Griffiths and Halestrap, 1991).

The therapeutic value of maintaining the MPT in a closed state is highlighted by the resistance of CypD knockout mice (Ppif-/-) to a variety of diseases, and made CypD a drug target of high interest. However, pharmacological inhibitors of CypD, such as CyA, also inhibit the structurally-related cyclophilins CypA and CypB, which are critical for immune function.

#### 1.2.5.2.3.1 Regulation of CypD interaction with the ANT

CypD binding to the ANT increases sensitivity to $Ca^{2+}$, which decreases the threshold for MPT induction. This binding is dependent on CypD activity. Recently, a relationship between sirtuin-3 (SIRT3) and CypD has been established (Shulga et al., 2010). Sirtuins are NAD+-dependent protein deacetylases that mediate adaptive responses to a variety of stresses, including calorie restriction and metabolic stress. Sirtuin 3 (SIRT3) is localized in the mitochondrial matrix, where it regulates the acetylation levels of metabolic enzymes. Shulga showed that Sirt3 deacetylates and inactivates CypD causing its dissociation from the ANT (Shulga et al., 2010). Another published study performed on heart, showed that the decrease in Sirt3 activity leads to increased activation of the MPT in response to Ca2+increases, cardiac stress and aging, resulting in a decline in cardiac function (Hafner et al., 2010).

Besides acetylation, also phosphorylation of CypD favors pore opening. Rassola and colleagues proposed a model in which inhibition of glycogen synthase kinase-3β (GSK-3β) activity, prevents its association to CypD and CypD phosphorylation, leading to MPT desensitization, whereas the Ser/Thr phosphorylation of CypD favors pore opening (Rassola et al., 2010). GSK-3β was first identified as a regulator of glycogen metabolism, but is also an important regulator of cell function, including gene expression, cell cycle, survival and apoptosis. GSK-3β is

active under normal resting conditions when it is not phosphorylated, and its activity is regulated by several signaling pathways. GSK-3β activity is regulated by phosphorylation, has a high basal activity that is increased by phosphorylation of its Tyr216 residue, whereas phosphorylation at Ser9 decreases GSK-3β activity (Kockeritz et al., 2006). Under physiological conditions, total GSK-3β is nearly undetectable in mitochondria

**1.2.5.2.4- Mitochondrial phosphate carrier (PiC)**

Functional support was provided to the role of PiC as a MTP component by demonstrating that agents able to inhibit mitochondrial Pi transport activity also blocked MPT in isolated mitochondria (Petronilli et al., 1994; Fontaine et al.,1998; Cesura et al., 2003). It has also been shown that the mitochondrial phosphate transporter forms a complex with CypD. MPT-inducing agents enhance PiC–CypD interaction, whereas MPT-blocking compounds reduce it.

Recently, Halestrap proposed a new MPT model consisting of PiC and ANT, of which $Ca^{2+}$ sensitivity is regulated by CypD binding to PiC and by oxidant stress- induced dithiol cross-links within PiC and ANT molecules (Halestrap et al., 2009).

It has been suggested that several other proteins such as hexokinase, creatine kinase and the pro-apoptotic proteins such B cell lymphoma-2 (Bcl-2) may also associate with the MPT.

The BPR is an outer mitochondrial membrane protein which works as pharmacological target, as well as the target of some citotoxic compounds.

Creatine kinase is a protein from the intermembrane space, which binds to the VDAC–ANT complex, inhibiting the MPT. This enzyme allows the cell to use creatine-creatine phosphate as a form of energy available (Vieira et al., 2000).

The hexokinase is an outer membrane protein that can interact with the complex ANT-VDAC. Binding of HK to VDAC is promoted when the ANT is in the cytosolic conformation. The interaction between HKII and VDAC might cause a conformational change that favors closure or prevents the interaction between pro-apoptotic Bcl-2 family members and MPT pore components or other regulators.

Pro-apoptotic proteins such as Bcl-2–associated X protein (Bax) or Bak (Bcl-2 homologous antagonist /killer) do not appear to constitute the MPT pore per se, but have been suggested as proteins that can bind

to and regulate it. Recently, there are three main models relatively to involvement of this proteins: (1) pro-death Bcl-2 proteins form their own protein-permeant pore; (2) pro-death Bcl-2 proteins interact with VDAC to form a protein- permeant channel that specifically permeabilizes the outer membrane; and (3) pro-death Bcl-2 proteins bind to and induce MPT opening.

### 1.2.6 Cell death: necrosis *versus* apoptosis

Death of hepatocytes and other hepatic cells is a characteristic feature of diverse liver diseases. Cell death can occur as a consequence of massive damage (necrosis) or in a controlled form, through programed cell death (apoptosis).

#### 1.2.6.1 Necrotic cell death

Necrosis is most often the consequence of acute metabolic perturbation with ATP depletion. ATP depletion-dependent cytoskeletal alterations can lead to a moderate cellular swelling. The early phase of necrotic cell death is characterized by loss of plasma membrane integrity, which is usually anticipated by mitochondrial depolarization, lysosomal breakdown, ion deregulation and mitochondrial and cellular swelling (Herman et al., 1988; Niemaen et al., 1988,Gores et al.,1989; Zahrebelski et al., 1995) . As a consequence of plasma membrane permeabilization and cell lysis, the intracellular components are released and the damage-associated molecular patterns may modulate inflammation, which results in damage to the adjacent cellular population. The swelling is a prominent feature of necrosis.

The proton electrochemical gradient is maintained by reversal of the mitochondrial proton-translocating ATPase. This actively stimulates the hydrolysis of ATP produced by glycolysis, leading to general metabolic failure. Induction of the MPT as an irreversible process of mitochondrial damage is associated with a necrotic-like terminal degradation of the cell.

#### 1.2.6.2 Apoptotic cell death

Apoptosis represents the execution of a death program often initiated by quite specific stimuli. In apoptosis, individual cells dye separately and shrink rather than swell. There are several morphological and biochemical changes that can define apoptosis, such as plasma and

nuclear membrane blebbing, shrinkage of the cell, chromatin condensation and nuclear fragmentation (Wyllie et al., 1980). Fragmentation of the cell into membrane-defined bodies, termed apoptotic bodies, is a hallmark of apoptosis. The machinery of apoptosis is well conserved and it is composed of caspases (the proteases which execute cell death), adapter proteins (caspase activators), Bcl-2 family proteins and Inhibitor of Apoptosis Proteins (IAPs) (Muñoz-Pinedo, 2012).

Apoptosis can be triggered by a variety of intra- and extra-cellular stimuli. The intracellular stimuli (such as DNA damage, high levels of calcium, intracellular proteins, reactive oxygen intermediates) normally result in mitochondrial outer membrane permeabilization and release of pro-apoptotic factors (cytochrome c, second mitochondria-derived activator of caspases (SMAC)/ direct IAP-binding protein with low PI (DIABLO), apoptosis-inducing factor (AIF)) from the intermembrane space into the cytosol. This intrinsic pathway (or mitochondrial) of apoptosis, is initiated by the activation of pro-apoptotic BH3-only (Bid, Bim, Bad, PUMA, Noxa) and multi-domain (Bax and Bak) members of the Bcl-2 family, and antagonized by the anti-apoptotic members of the same family (Bcl-2, Bcl-xL, Mcl-1) (Nieminen et al., 1988). Bcl-2 is the founding member of a family of proteins that regulate apoptosis. Bcl-2 localizes to the mitochondria, where it reduces both apoptotic and necrotic cell death caused by several stressors. In addition to reducing cytochrome *c* release, Bcl-2 overexpression reduces acidification, as well as the rate of mitochondrial ATP consumption when ATP hydrolysis is stimulated. This is accomplished by limiting ATP entry into the mitochondria through VDAC or ANT or by direct inhibition of the $F_1F_0$-ATPase.

The balance between pro-/anti-apoptotic Bcl-2 proteins, is determinant in the response to apoptotic stimuli (Adams and Cory, 1998). Released cytochrome c associates with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome, which recruits procaspase 9 and facilitates its autoactivation (Trump et al., 1965). Caspases (such as caspase-3, -8, -9, -12) are a family of cysteine proteases, known as the effector molecules of apoptosis, because most of the morphological hallmarks of this type of cell death result from their activity (Chang and Yang, 2000). Caspases are constitutively expressed as inactive pro-caspases and are activated by a regulated proteolytic cleavage of inhibitory sequences. Caspase-9 triggers the activation of caspase-3 and leads to the execution and degradation phases, resulting in the morphological changes associated to apoptosis (such as cellular and nuclear collapse). Mitochondria play a key role in the apoptotic process, functioning as a link

between apoptotic mediators and effector molecule (Gross et al., 1999).

The extracellular stimuli through the binding of death receptors, such as tumor necrosis factor-α (TNFα) or Fas their receptors in the plasma membrane, can induce apoptosis (Kerr et al., 1979). This leads to the activation of procaspase-8, which cleaves and activates procaspase-3 and initiates the caspase cascade (Ashkenazi and Dixit, 1998). Additionally, caspase 8 can involve the mitochondrial pathway by cleaving the BH3-only protein Bid, whose mutilated fragment translocates to the mitochondrial outer membrane causing its permeability (Dickson et al.1992); Marsh, 1993). Kupffer cells activation and TNF-α production increase the sensitivity of hepatocytes to TNF-α-mediated apoptosis (Shimizi et al., 2000). ER stress could also partly contribute to apoptosis by releasing calcium into the cytosol and so activating procaspase-12.

#### 1.2.6.3 Necrapoptosis

A necroapoptosis theory proposed by Lemasters refers to a process that begins with the same death signal and ends in either necrosis or apoptosis depending on conditions (decline of cellular ATP levels or fat content) (Lemasters et al., 1999). It is proposed that an abrupt MPT onset causes ATP depletion, blockage of caspase activation and plasma membrane rupture, deviating cell death from apoptosis to necrosis.

### 1.3 Non-Alcoholic Fatty Liver Diseases (NAFLD)

Fatty liver or steatosis, is characterized by the accumulation of fat in the cytoplasm of hepatocytes derived from adipocytes, intestinal absorption or hepatic lipogenesis (Haque and Sanyal, 2002); Browning and Horton, 2004). Alcoholic steatosis is caused by alcohol consumption. Non-alcoholic fatty liver diseases (NAFLD) are associated with obesity and type II diabetes and is increasing rapidly in Western countries due to a fat rich diet and lack of exercise, reaching epidemic proportions with an incidence of 20% to 30% of individuals.

The mechanisms involved in the development of NAFLD are under intensive research. Deletion or over-expression of genes involved in lipid metabolism and modulation of appetite, or administration of modified diets, leads to the development of a steatotic phenotype.

Zucker fatty rats are a well-characterized model of obesity associated with over nutrition, which simulates very closely the most common cause of steatosis in the western countries (Figure 1.9). These

animals have a missense point mutation (fa/fa) of the leptin receptor, which impairs the ability of leptin to bind to the receptor in order to induce satiety. Different metabolic dysfunctions, such as insulin resistance, hyperglycemia or raised lipid levels have been described in these rats. Moreover, an important fact is that steatosis in Zucker rats is not associated with inflammation, in opposite to other models of steatosis using ethanol ingestion or a choline-deficient diet (CDD).

Data gathered from both humans and experimental animals suggest that fatty livers are more prone to a variety of insults such as ischemia/reperfusion (I/R) being hepatic steatosis a major risk factor for liver surgery and success in the transplant of fatty donor organs.

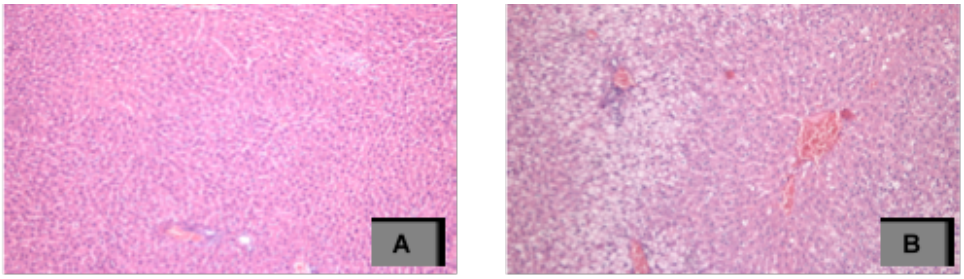



**Figure 1.9 - Hepatic histology of normal (A) and fatty livers (B) showing fat acumulation** (Teodoro et al., 2006).

### 1.3.1 The biochemistry of pathology

A deregulation in fatty acid's metabolism leads to an overproduction of triglycerides with subsequent fat accumulation inside the liver cells. Very Low Density Lipoproteins (VLDL's) are the main vehicle of triglyceride exportation from the liver to the peripheral adipose tissue. These lipoproteins are formed by triglycerides and cholesteryl esters and phospholipids surrounded by a protein called Apolipoprotein B (ApoB), and its synthesis is dependent upon the availability of triglycerides. So, alterations in one of these pathways may result in liver lipid metabolism perturbations (Haque & Sanyal, 2002).

The excessive abundance of free fatty acids causes a shift in metabolism, turning these molecules into the main energy source of the cell (Haque and Sanyal, 2002; McClain et al., 2004). So, degradation of glucose as main energy source is substituted by β-oxidation of fatty acids

(FFAs). Although FFA are used as a source of energy, continues to exist an overload in the cell, because the "pool" of FFAs in hepatocytes is in equilibrium with the level of FFAs in circulation, the synthesis of FFA's, the β-oxidation and the "pool" of cellular triglycerides. Such alteration increases lipid peroxidation, destabilizing the inner cell redox environment.

### 1.3.2 Mitochondrial dysfunction in NAFLD

Mitochondria is the dominant oxidative pathway for the disposition of fatty acids under normal physiologic conditions. In order to counteract excessive fat storage, there is a compensatory stimulation of mitochondrial β-oxidation of fatty acids. As the oxidative capacity of the mitochondria becomes impaired or is exceeded, cytosolic fatty acids accumulate activating alternative pathways in the peroxisomes and in microsomes (Johnson et al.,1996; Reddy and Mannaerts,1994). This increases ROS generation, destabilizes the redox environment of the cell causing cellular damage. As major ROS generators, mitochondria are often targets of high ROS exposure with deleterious consequences, such as oxidative damage to the ETC complexes and mtDNA (Ricci et al., 2008; Racheket al., 2009). All these effects may further block the flow of electrons in the ETC, leading to a vicious cycle in which accumulating oxidative damage affects the efficiency of mitochondria and further increases the rate of ROS production (Petrosillo et al., 2007).

There are consistent evidences for a central role of mitochondrial dysfunction in the pathophysiology of NAFLD, whatever its origin, such as drug-induced or associated with insulin-resistance. Impairment of hepatic ATP synthesis, reductions in the activities of complexes of the ETC and increased ROS production have been reported (Cortez-Pinto et al.,1999; Hensley et al., 2000). These biochemical changes are associated with ultrastructural abnormalities indicative of impaired oxidative phosphorylation. Liver mitochondria appear scarce in number, swollen and rounded with loss of cristae and presence of paracristalline inclusions (Chavinet al., 1999; Pérez-Carreras et al., 2003; Teodoro et al., 2006; Teodoro et al., 2008) .

### 1.3.3 Diagnosis

Most individuals with NAFLD have no symptoms and the diagnosis can be made with routine examination, such as biochemistry (Preiss and Sattar, 2008) .The patient with abnormal liver function tests, with raised

or high–normal fasting blood glucose, low HDL (high-density lipoprotein), high cholesterol and elevated fasting triacylglycerols is likely to have NAFLD.

The only proven techniques to confirm diagnosis and different stages of NAFLD are invasive, including a liver tissue biopsy. The percentage of fat present on livers defines the level of hepatic steatosis, which is graded as mild, moderate or severe (Kleiner et al., 2005). Steatosis is usually macrovesicular and the hepatocytes have a single large intra cytoplasmic fat droplet or smaller well-defined droplets moving the nucleus to the cell periphery. When there are groups of hepatocytes with centrally placed nuclei and numerous lipid droplets in the cytoplasm, in addition to macrovesicular steatosis, it is defined as mixed steatosis (Brunt and Tiniakos, 2010).

Although NAFLD is considered a relatively benign condition, this pathology can progress to more severe stages of liver disease including NASH (non-alcoholic steatohepatitis), with or without fibrosis, cirrhosis and occasionally hepatocellular carcinoma (Preiss et al., 2008). These stages can be reached due to additional cellular events that promote oxidative stress and production of inflammatory cytokines causing cell death, inflammation and fibrogenic response.

### 1.3.4 Liver Transplantation

Over the past decade, the gap between the number of patients awaiting an organ and the number of available organs has dramatically increased.

Actually, the main restriction of hepatic transplantation is the shortage of donors. To minimize the lack of organs available to transplant, several strategies have been developed to optimize the use of liver donors. These strategies have been designed to increase the number of organs that are transplanted by reevaluation and increase of the variables of acceptance of an organ for transplantation and the development of various alternative and conventional transplant techniques.

In 2008, Portugal was the country that did more liver transplants in the world performing 25.8 per million of habitants, in other words, 274 transplants were done (Figure 1.10). This was only possible due to an increase of collection centers resulting in a higher number of donors by more than 50% compared to previous years. This has led to a high incentive to use extended criteria donor organs, which include organs distinguished by hepatic steatosis, old donor age, prolonged cold ischemia, or donation

after cardiac death. Many of the current research in liver transplantation are directed to the possible use of suboptimal or marginal organs as a strategy to increase the number of organs available for transplant. There is the special interest for steatotic organs, due to its high prevalence among the potential liver donors.

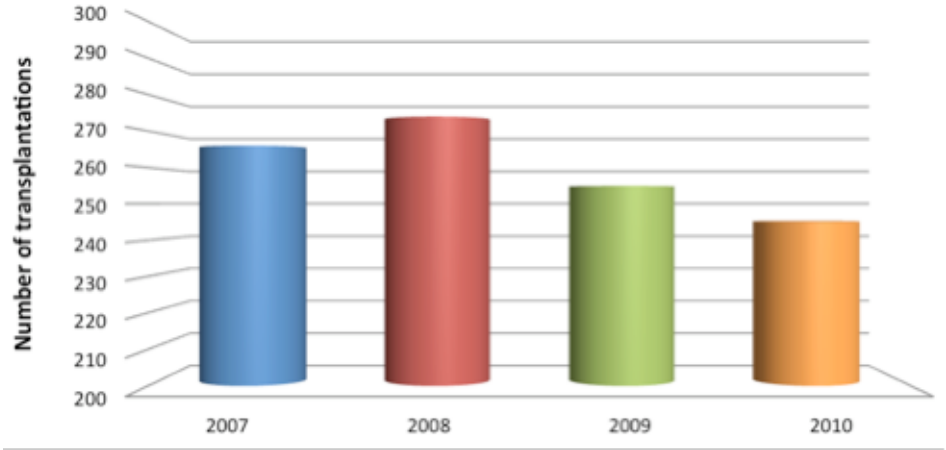



**Figure 1.10- Hepatic transplantations in Portugal 2007-2010** (data of the Sociedade Portuguesa de Tansplantação).

Hepatic steatosis is a major risk factor for liver surgery and success in the transplant of fatty donor organs, since it is associated with a significantly increased risk of primary graft nonfunction.

Operative mortality rate associated with steatosis after major liver resection has been reported as high as 14% compared with 2% for healthy livers (Behrns et al.,1998). Fatty liver grafts are more frequently used in the context of transplantation and they are associated with a primary non-function rate of 60% compared with 5% for non-steatotic grafts (Canelo et al.1999; D'Alexandro et al., 1999). Livers with 30% or more fat content have a high prohibitive rate of primary nonfunction, being 25% of liver donors rejected due to steatosis (He et al., 2009). Therefore, developing protective strategies to minimize the adverse effects of I/R injury in steatotic livers is of paramount importance (Degle Esposti et al., 2011).

## 1.4 Ischemia/reperfusion

Ischemia/reperfusion injury is a phenomenon whereby damage to a hypoxic organ is accentuated following the return of oxygen delivery. Toledo-Pereyra and collaborators, in 1975, recognized this form of injury

as a clinically important pathological disorder. Only in the mid-1980s the term “reperfusion injury” began to be used when an association was made between congestion, progressive thrombosis and/or graft necrosis, resulting in organ failure in transplanted liver. I/R may occur in many clinical situations such as transplantation, resection, trauma, shock, hemorrhage, and thermal injury.

The mechanism of organ damage after I/R has been extensively studied and consists of complex interactions of multiple pathways. The major contributors to I/R injury include production of ROS, release of proinflammatory cytokines and chemokines, and activation of immune cells to promote inflammation and tissue damage.

Warm I/R injury occurs in settings of transplantation, trauma, shock, and selective liver surgery in which hepatic blood supply is temporarily interrupted. There are two distinct phases that occur in warm I/R injury. The early phase occurs in the period less than 2 hours after reperfusion and is characterized by activation of immune cells and oxidative stress. Kupffer cells are a key cell type involved in the initial stages of I/R injury (Teoh et al., 2003; Jaeschke et al., 1990). The activation of KC leads to ROS generation that provokes moderate hepatocellular injury characterized by a mediate increase in serum transaminase levels, and a partial preservation of the hepatic structure. In addition to oxidative-mediated damage, oxidative stress leads to the production of several proinflammatory cytokines and chemokines, such as TNF-α, interleukin (IL)-12 and IL-1b Jaeschke et al.,1988; Wanner et al.,1996; Lentsch et al.,1999). These molecules have the role to start and perpetuate a later secondary inflammatory phase, which causes more damages to the liver. The expression of the cytokines is mediated by transcription factors, nuclear factor κ B (NF-κB) and hypoxia inducible factor-1α (HIF-1α).

The late phase, involved in warm I/R damage, occurs from 6 to 48h after reperfusion, and it is an inflammatory disorder mediated by neutrophils, attracted by chemokines released in the early stage and attached to the liver by adhesion molecules (Lentsch et al.,1998). TNF-α also has an essential role in the induction and release of neutrophil chemoattractants, especially CXC chemokines from KCs and hepatocytes. Neutrophils also cause some of the damage to hepatocytes by releasing ROS via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Jaeschke et al.,1990; Jaeschke et al.,1991). Because of that, knockout mice deficient in the gp91-phox component of NADPH are protected against I/R injury. Another neutrophil-mediated injury occurs through enzyme-mediated degradation of cellular membranes and matrix components. Neutrophils release proteases and

other cytotoxic enzymes through granule exocytosis, such as elastase, cathepsin G, heparanase, collagenase and other hydrolytic enzymes.

Damage associated with cold ischemia occurs exclusively in the transplant setting, when a donor graft is harvested. Cold storage aims to reduce metabolic activities of the graft while the organ awaits implantation. This requires the use of preservation solutions at low temperatures (2-4ºC), being the most used University of Wisconsin (UW). Some strategies have been developed to improve preservation solutions, to expand the preservation periods and to reduce the negative effects of preservation. Studies with animals are normally focused on storage times up to 18h, although some increase cold storage to 24h or more. In clinical situation, using human livers, these can be successfully stored at 4°C for up to 12 h in UW solution, but if storage periods are extended the graft failure is greatly increased (Furukawa et al.,1991; Ploeg et al.,1993; Porte et al.,1998).

While there are many common pathogenic mechanisms between warm and cold IR injury, there are also several important differences. Cold storage has specific effects on the various cellular subsets within the liver graft, such as endothelial cells, which are more susceptible to cold storage. Several studies showed that endothelial cells have higher mortality than hepatic parenchymal cells at the same time point, which means that it occurs independently of apoptosis and coagulative necrosis (Caldwell-Kenkel et al.,1991; Gao et al.,1998). The cause of this higher damage of endothelial cells is the functional impairment of the liver after reperfusion. The endothelial cells that remain viable accentuate I/R injury by expression of adhesion molecules. Increasing cold storage times is closely related to increasing KC activation on reperfusion, which result in increased ROS and cytokine production (Caldwell-Kenkel et al.,1991).

For the study of hepatic IR injury three models can be used: in vivo models, in vitro cell culture systems and ex vivo intact organ models. There are two main in vivo models of hepatic IR injury: total hepatic ischemia and partial hepatic ischemia. When livers are subjected to total hepatic ischemia there is an occlusion of the hepatic artery, portal vein and common bile duct. In this case, the period of ischemia using this model is limited (20 min), as longer occlusion times result in high mortality. Kawamoto has demonstrated that global ischemic periods of more than 30 min cause irreversible hemodynamic instability and severe splanchnic congestion. In 1982, Yamauchi and colleagues described a model of partial hepatic ischemic (70%) where the hepatic artery and portal vein to the left and median liver lobes were occluded. This model was used in order to prevent mesenteric venous congestion by permitting portal decompression

through the right and caudate lobes. There are models that use the isolated perfused liver, in which the excised organ is perfused via the portal vein using a system with buffer as perfusate, where buffer flow rates can be adjusted. In vivo models are faithful to the clinical reality of IR injury, while cell culture systems fail to reproduce the dynamic conditions that liver cell types are exposed to in vivo during ischemia and I/R.

### 1.4.1 Mitochondrial alterations associated with I/R

One of the most important features of I/R injury is the alteration of energy metabolism due to impairment of mitochondrial function. Removal of oxygen during ischemia leads to the cessation of oxidative phosphorylation. This causes tissue ATP and creatine phosphate concentrations to decrease with a concomitant rise in ADP, AMP and Pi concentrations. Although glycolysis is activated, it is unable to meet the demand for ATP. Mitochondrial ATPase consumes ATP during ischemia to temporarily maintain mitochondrial membrane potential. Since maintenance of ion gradients across the plasma membrane and between cellular compartments depends on ATP-driven reactions, metabolic disruption by injurious stresses may quickly perturb cellular ion homeostasis.

Ischemia elevates intracellular $H^+$, $Na^+$, and $Ca^{2+}$ levels, induces osmotic stress and causes mitochondrial damage. Intracellular $Na^+$ concentrations rise during ischemia due to stimulated $Na^+$ influx via the $Na^+$–$H^+$ exchanger, caused by intracellular $H^+$ accumulation, and reduced $Na^+$ efflux via the $Na^+$–$K^+$ pump (Inserte et al., 2006; Inserte et al., 2002; Murphy et al., 2006). Although $Na^+$ overload stimulates $Ca^{2+}$ influx by the $Na^+$–$Ca^{2+}$ exchanger and depletion of ATP reduces $Ca^{2+}$ uptake by the endoplasmic reticulum, $Ca^{2+}$ level is kept modest during ischemia since acidosis inhibits the $Na^+$–$Ca^{2+}$ exchanger, and cytosolic $Ca^{2+}$ is taken up by the mitochondria as long as its membrane potential is maintained. Influx of extracellular $Ca^{2+}$ is responsible for irreversible cell injury as shown by studies in which removal of extracellular $Ca^{2+}$ protects against various hepatotoxicants (Farber et al.,1982; Schanne et al.,1979).

Immediately after an organ becomes ischemic there is an increase in ROS generation that is thought to play a major role in damaging the organ during ischemia and sensitizing it to reperfusion. The source of the ROS is unclear, as further discussed, and might involve Complexes I and III of the ETC or perhaps xanthine/xanthine oxidase (X/XOD), acting on xanthine formed from the degradation of adenosine. AMP is slowly converted into adenosine and then inosine and xanthine through a purine

degradation pathway.

The depletion of ATP combined with elevated intracellular $Ca^{2+}$ and ROS leads to a gradual decline in cellular integrity as degradative enzymes are activated and ATP-dependent repair processes are unable to operate. If the tissue remains ischemic for only short periods and the mitochondria remain sufficiently intact to generate ATP, tissue damage is slight and can be repaired upon reperfusion. However, a critical point is reached when recovery is not possible. In this case, although reperfusion restores ATP production, it will actually cause further damage to the organ due to the metabolic derangements accumulated during ischemia, causing cell death.

Reperfusion is associated with increased $Ca^{2+}$ but is also associated with a burst in ROS generation. Although ROS may be produced by xanthine oxidase and NADPH oxidase, probably the majority is formed by uncoupled mitochondria, mainly from mitochondrial complexes I and III of the ETC (Jaeschke et al.,1989; Turrens et al., 2003). When the respiratory chain is inhibited by lack of oxygen and then re-exposed to oxygen, ubiquinone can become partially reduced to ubisemiquinone. This can then react with the oxygen to produce superoxide that is reduced to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is removed by glutathione peroxidase or catalase, but if ferrous ions (or other transition metals such as copper) are present it will form the highly reactive hydroxyl radical through the Fenton reaction (Becker et al., 2004). Damage to mitochondrial lipids and proteins caused by ischemia favors ROS generation during reperfusion (Insert et al., 2008). ROS cause peroxidation of the unsaturated fatty acid components of the phospholipids, and especially cardiolipin of the inner mitochondrial membrane, impairing electron flow through the ETC (Petrosillo et al., 2003; Paradies et al.,2004). Furthermore, lipid peroxidation causes the release of reactive aldehydes such as 4-hydroxynonenal that modifies membrane proteins (Echtay et al., 2003). ROS also have direct effects on several respiratory chain components and other iron sulphur proteins such as aconitase. ROS can also cause thiol oxidation and inhibition of the ATPase and ANT. In addition, glutathione oxidation and protein modification is thought to have inhibitory effects on ion pumps and therefore exacerbate the effects of ATP deprivation on ionic homeostasis (Hool et al., 2006). Oxidative stress is enhanced by the depletion in superoxidase dismutase, glutathione peroxidase and reduced glutathione that occurs during reperfusion (Dhalla et al., 2000).

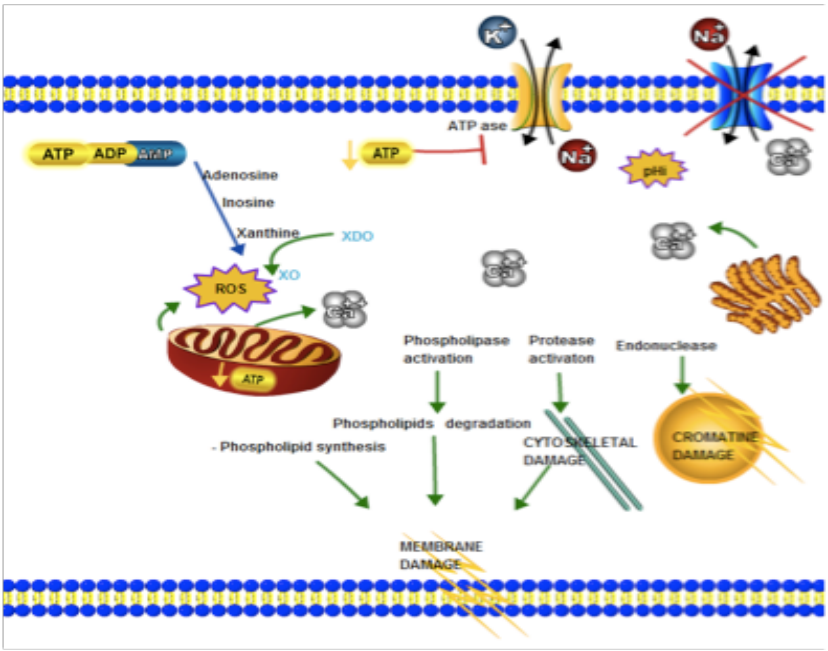



**Figure 1.11- The combined action of ROS and elevated [$Ca^{2+}$] cause cell death.** Rapid ATP depletion inhibits the Na+/K+ ATPase, which leads to a rise in intracellular [$Na^{+}$] and impairs the ability to restore the pHi. This increases intracellular [$Ca^{2+}$] since the $Na^{+}/Ca^{2+}$ antiporter is inhibited or reversed. The conversion of ATP to ADP and AMP is rapid and reversible. AMP is slowly converted into adenosine and then inosine and xanthine , that might be involved in ROS generation through the action of xanthine oxidase. The elevated [$Ca^{2+}$] leads to a gradual decline in cellular integrity, activating phospholipases, proteases and endonucleases that contribute to membrane, cytoskeletal and cromatine damage, respectively.

The combined effects of ROS and mitochondrial $Ca^{2+}$ overload play a critical role in the transition from reversible to irreversible reperfusion injury (Figure1.11). Mitochondria are the major target of these agents since they are potent inducers of the MPT, resulting in mitochondrial-initiated cell death. As a consequence of the MPT, mitochondrial depolarization, uncoupling of oxidative phosphorylation and large-amplitude mitochondrial swelling driven by colloid osmotic forces occur.

MPT induction causes the release of apoptotic factors, such as cytochrome *c* and the apoptosis-inducing factor, into the cytosol (Forbes et al., 2001; Murata et al., 2001). Additionally, the release of cytochrome *c* from the mitochondrial intermembrane space may also occur through channels formed by Bax, a proapoptotic Bcl-2 family (Borutaite et al., 2003). This may occur due to increased translocation of Bax to the mitochondria

in response to pro-apoptotic signals (Figure 1.12).

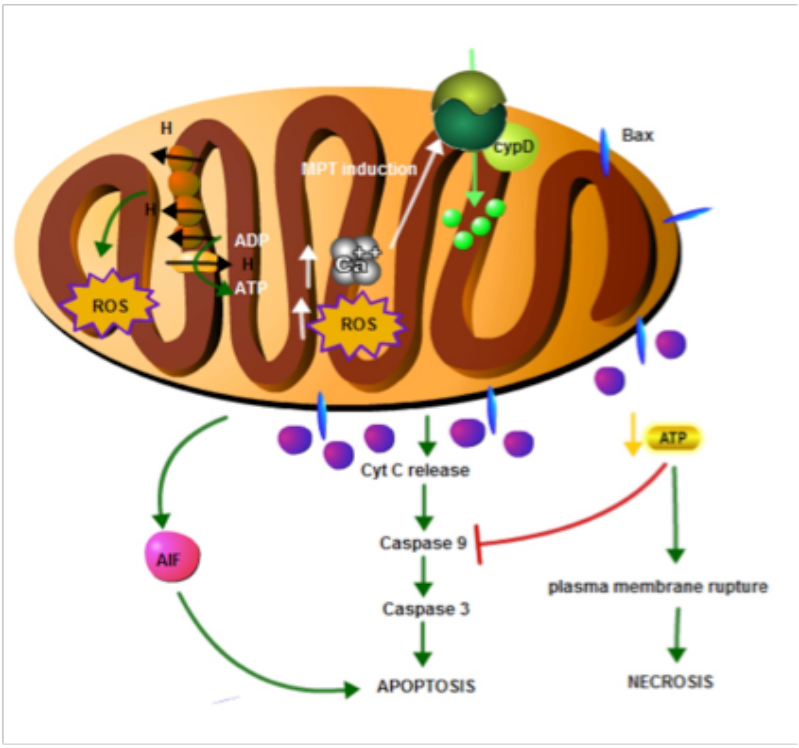



**Figure 1.12- Elevated ROS and calcium induce the MPT.** MPT induction causes the release of apoptotic factors, such as cytochrome c and the apoptosis-inducing factor (AIF). Cyt *c* release may also occur through channels formed by Bax, a proapoptotic Bcl-2 family protein. ATP depletion may block caspase activation, deviating cell death from apoptosis to necrosis.

There is still controversy about the significance of necrotic or apoptotic cell in parenchymal injury observed during hepatic reperfusion. Some investigators reported massive necrotic alterations as responsible for the main part of parenchymal injury. However, other works demonstrate that specific inhibition of apoptosis notably prevented parenchymal injury and improved animal survival after prolonged periods of ischemia. In conditions of ATP depletion, apoptosis can deviate to necrosis (necroapoptosis). It has been recently reported that autophagy, a catabolic pathway that crosstalk's with apoptotic and necrotic cell death pathways is involved in I/R. Autophagy is a catabolic pathway triggered by several stresses such as starvation and hypoxia that favors cell survival by preserving energy levels and eliminating damaged organelles (Huang et al., 2007; Scherz-Shouval et al., 2007). Autophagy has been shown to retard cell

death by suppressing ER stress (Choi et al., 2010) that is involved in the pathogenesis of fatty liver (Demetris et al.,2006; Arkadopoulos et al., 2009). ER stress inhibition has been shown to be protective under I/R in an experimental model of steatotic/ non-steatotic partial hepatectomy (Mosbah et al., 2010).

### 1.4.2 ROS generation associated with I/R independent on the ETC

There is controversy regarding sources of ROS generation during hepatic I/R. Experiments with allopurinol, an inhibitor of X/XOD, have indicated this system as a major ROS generation in the setting of I/R (Grattagliano et al.,1999). On the other hand, results from studies with the isolated perfused liver showed that the main ROS generator in I/R is mitochondria and not the X/XOD system. Inactivation of KC as also been shown to attenuate the increased vascular oxidant stress after 30 and 60 min of ischemia, while high doses of allopurinol didn´t afford protection (Metzger et al.,1988; Jaeschke et al.,1991). Besides KC, activation of neutrophils as also been implicated in reoxygenation injury.

A possible explanation for these conflicting results are the differences in experimental models and parameters, including the time of ischemia, which is particularly significant when assessing the relevance of mitochondria or X/XOD for ROS generation. Prolonged cold ischemia leads to a significant conversion of XDH to XOD. Therefore, in this case, X/XOD has a crucial role in hepatic I/R injury. But in shorter ischemic periods, such as 6h of cold ischemia, the major ROS generation occurs in the mitochondria (Fernandez et al., 2002). The pleiotropic range of effects of compounds tested, as protective against in I/R, also has to be taken into account. Allopurinol, besides inhibiting XOD, may also improve ischemia-induced mitochondrial dysfunction. In a warm hepatic I/R model, high doses of allopurinol preserved mitochondrial function. Besides time, the temperature of ischemia (4°C or 37°C) also influences oxidative stress in hepatocytes and the stimulatory state of KC after I/R, determining the relevant of intracellular vs vascular oxidative stress to hepatic I/R injury (Mochida et al.,1994).

### 1.4.3 The role of nitric oxide

The role of nitric oxide (NO) in I/R is complex, and it is difficult to distinguish between beneficial and harmful mediators in I/R injury.
NO is an important signaling molecule in the liver produced by nitric oxide

synthase (NOS) through the conversion of arginine. The inducible (iNOS) and the endothelial form (eNOS) are two isoforms of nitric oxide synthase (NOS) that are involved in NO production during I/R injury. The eNOS form is expressed in endothelial cells and eNOS-derived NO has been reported as protective. On the other hand, the iNOS form is regulated by many of the same cytokines, such as TNF-α and IL-1, contributing for I/R injury. Although the source of NO may determine whether NO plays a protective or damaging role, there are other factors, such as, timing, release or downstream activation of NO-responsive elements that may be decisive in NO effects.

### 1.4.4 The role of TNF-α and Nuclear factor κ B (NFkB)

The role of TNF-α in I/R injury is also complex and controversial and depends on the cell type and/or experimental or pathologic circumstances. Accordingly, TNF-α can be either protective or damaging during episodes of hepatic I/R: TNF-α may promote cell death or it may produce effects mediated by antioxidant, anti-apoptotic or anti-stress mediators, therefore promoting hepatoprotection and a proliferative biologic response (Bradham et al., 1998; Aggarwal et al., 2000; Dieh et al., 2000). For example, although the deleterious effect of TNF-α in local and systemic damage associated with hepatic I/R is well established, this mediator is also a key factor in hepatic regeneration which is very important in children liver transplantation, and pre-treatment with a low dose of TNF-α was highly protective against hepatic I/R injury (Peralta et al., 1999; Teoh et al., 2006). TNF-α contributes significantly to mitochondrial dysfunction by promoting ROS (possibly through direct effect on the ETC) and ROS production by iNOS. TNF-α also has the ability to induce cell death through caspase 8 activation (Wang et al., 2008; Hatano et al., 2007; Badiola et al., 2009). This leads to a partial block in the flow of electrons in the ETC and increases mitochondrial ROS formation that induces MPT.

The differential effects observed for TNF-α can be extrapolated from transcription factors, such as NFkB. Being able to modulate several pathways it is known that NFkB can induce both pro- or anti-apoptotic effects. Currently it is not very clearly whether the beneficial effects of NFκB activation against I/R result from reducing apoptotic cell death or if it is pro-inflammatory role that prevails in hepatic I/R. Suppression of NFkB activation in mice, following partial hepatic I/R, leads to a significant reduction in neutrophil recruitment and hepatocellular damage (Yoshidome et al., 2006). It has also reported that NFκB activation is crucial for hepatic

regeneration after rat liver transplantation (Bradham et al., 1999). Thus, one has to consider several aspects in order to better understand and clarify the dual role of NFkB in hepatic I/R situations. In first place, differences can be detected between the species used in different studies; for instance, some mechanisms that give protection against apoptosis may be different in rats and mice. Additionally, the experimental design used to evaluate the role of this transcription factor may also be important. As an evidence for this, it is reported that the use of adenoviral vectors containing a repressor to prevent NFκB activation may not accurately modulate the role of NFκB signaling in regenerating liver, since adenoviral vectors themselves cause increased TNF-α levels, DNA synthesis, and apoptosis in the liver before partial hepatectomy (Lieber et al.,1997; Limuro et al., 1998).

### 1.5 Causes for increased susceptibility of fatty livers to I/R

Different hypothesis have been proposed for the increased susceptibility of fatty organs to ischemic injury. These include impaired microcirculation, ATP depletion, Kupffer cell dysfunction, impaired mitochondrial function and increased adhesion of leukocytes, but the role that each of these mechanisms play in injury is not yet elucidated (Figure 1.13).

#### 1.5.1 Impaired microcirculation

Diverse authors consider that the alterations in hepatic sinusoidal microcirculation present in fatty livers as one of the principal factors for the higher susceptibility to I/R (Behrns et al., 1998; Caraceni et al., 1999).

The result of the accumulation of fat in the cytoplasm of hepatocytes is an enlargement of these cells. This can cause compression and distortion of sinusoidal vascular channels, conducting to an increase of hepatic intraportal resistance and a decrease of blood flow. Fatty livers present a decrease in blood flow at approximately 50% compared with livers without fat, which can induce chronic hypoxia (Bradham et al.,1998; Peralta et al.,
1999).

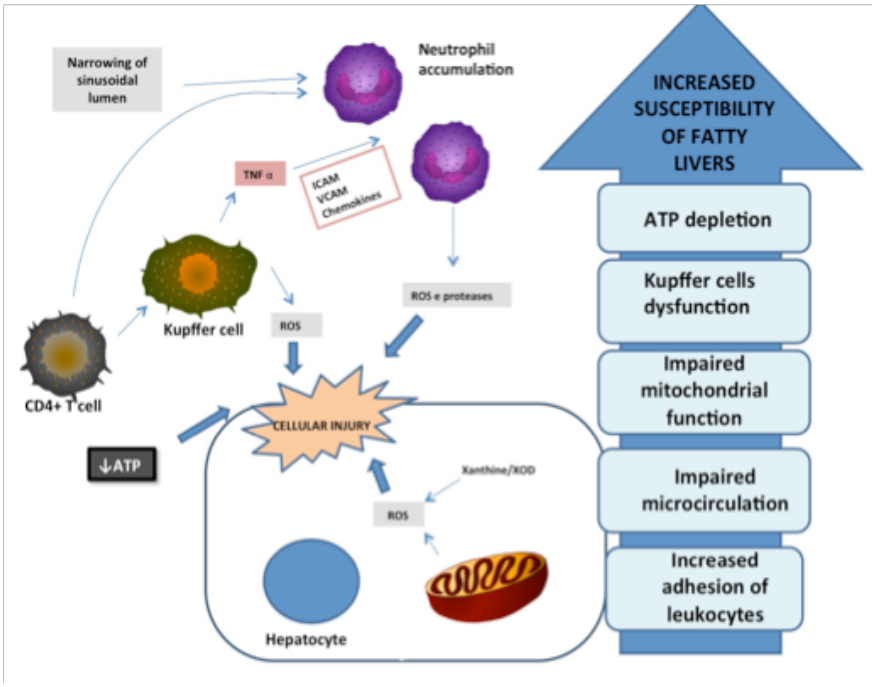



**Figure 1.13- Susceptibility of fatty livers to I/R injury.** Different hypothesis have been proposed for the increased susceptibility of fatty organs to ischemic injury: ATP depletion, Kupffer cell dysfunction, impaired mitochondrial function, impaired microcirculation and increased leukocyte adhesion.
Narrowing of sinusoidal lumen leads to activation and accumulation of neutrophils. The accumulated $CD4^+$ T-lymphocytes in the liver produce factors that amplify Kupffer cells activation and promote neutrophil recruitment into the liver. Kupffer cells are an important source of ROS and cytokines, such as TNF-alpha. Release of cytokines up-regulate the production of adhesion molecules (ICAM and vascular cell adhesion molecule [VCAM]) and chemokines, which promote neutrophil activation and accumulation. These neutrophils then extravasate, causing cellular injury by ROS and protease generation. ROS may derive from xanthine/xanthine oxidase (X/XOD) and mitochondria. ROS and ATP depletion lead the cellular injury.

Diverse authors propose that these alterations in hepatic sinusoidal microcirculation in fatty livers can amplify the negative effects produced by I/R and worsen the hepatic damage (Sun et al., 2001; Clemens et al., 1999).

So, in fatty livers, the damaged sinusoidal endothelium, the liberation of fat droplets during organ preservation, even as formation of little fibrin thrombi and cellular elements in blood, adhesion of neutrophils

and platelets to sinusoidal endothelium during reperfusion, the reduction of sinusoidal space can all contribute for major susceptibility to I/R injury (Fukumori et al., 1997; Selzner et al., 2000; Teramoto et al., 1993).

### 1.5.2 Kupffer cell dysfunction

Kupffer cells are the resident macrophages of the liver and are involved in the pathogenesis of liver parenchymal cell damage as seen during I/R injury. Diverse studies show that after I/R there are more KC and higher phagocytic activity of these cells in fatty livers than in normal livers (Wanner et al., 1996). KC produce substances that can modulate sinusoidal blood flow and are an important source of ROS and cytokines, such as TNF-α and IL-1, which increases during reperfusion. Inhibition of KC activity in models of fatty livers reduces hepatic IR injury and increases the survival after transplantation (Mosher et al., 2001).

### 1.1.5.3 Increased adhesion of leukocytes

The excess of fat in steatotic livers produces an alteration in membrane fluidity due to the reduced presence of cholesterol and polyunsaturated fatty acids (Fukumori et al., 1999). After cold preservation, there are alterations in sinusoidal endothelium of fatty livers that can induce an increase of infiltration an adhesion of neutrophils during reperfusion (Fukumori et al., 1999). However, it is not yet elucidated how neutrophils actually accumulate in the liver. It may involve adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin (Banga et al., 2005; Cutrin et al., 2002). However, several authors reported that the increased expression of ICAM-1 and P-selectin is not the main cause for neutrophil accumulation, but an extensive vascular injury during reperfusion disrupt the sinusoidal endothelial cell barrier allowing neutrophils to access hepatocytes (Jaeschke et al., 2003; Caldwell-Kenkel et al., 1991; Peralta et al., 2001).

### 1.5.4 Impaired mitochondrial function and ATP depletion

Steatosis is associated with a decreased ability of the liver to generate ATP. Ischemic injury causes low intracellular ATP levels, which are rapidly restored to normal levels in lean hepatocytes, but steatotic livers are unable to restore ATP contents after reperfusion (Teodoro et al., 2008; Teodoro et al., 2006). These low intracellular ATP levels might

be related to the failure of fatty hepatocytes to trigger apoptosis following ischemia. The difference in the ability to generate ATP and the mechanism of cell death between lean and fatty livers might be important because different strategies might be necessary to protect the fatty and lean livers (Vendemiale et al., 2001; Caraceni et al., 2004). The lower ATP content in fatty livers, associated with higher levels of oxidized lipids and proteins, is the probable cause for higher necrotic cell death following I/R injury, comparatively to lean livers. It is noteworthy that high ATP levels have been correlated with better post-transplantation outcomes (Lanir et al., 1988; Marni et al., 1988).

Various models of NAFLD show altered mitochondrial morphology, impaired mitochondrial bioenergetics, increased mitochondrial peroxides and decreased ATP content. Although the increased expression of UCP-2 in the livers of patients with fatty liver disease decreases ROS generation, it still compromises the capacity to address energy demands, which reflects impairment oxidative phosphorylation (Chavin et al., 1999). A possible cause for the loss of oxidative phosphorylation efficiency observed in fatty liver is the decreased ANT content. During I/R, oxidative phosphorylation activity is greatly exacerbated by fatty infiltration likely resulting from oxidative damage to lipid and proteins.

### 1.6 Protective strategies

In the past few years, data from several experimental investigations suggested that different drugs could be useful to prevent and/or reduce damage associated with I/R. Given that fatty livers are considerably more susceptible to acute stressors, such as I/R, and knowing that the incidence of this pathology is deeply increasing, there is an urgent need to find strategies against I/R injury in fatty livers (Figure 1.14).

Identification of the molecular mechanisms responsible for I/R injury allows focusing research on the genetic modulation of the involved pathways. This approach aims not only for a better knowledge of the mechanisms involved in IR injury, but also and especially for potential therapeutic applications (Ke et al., 2006). In order to suppress the ROS burst in IR injury, adenovirus, liposomes or polyethyleneglycol were used to transfect superoxide dismutase and catalase enzymes (Banga et al., 2005; Mari et al., 2002). Also the overexpression of Bcl-2 associated athanogene (BAG-1) and Bcl-2 using adenovirus has been tested to inhibit apoptosis (Selzner et al., 2003). These genetic tools have also been used to express some genes that provide cytoprotection, such as Heme

oxygenase-1 (HO-1), anti-inflammatory cytokine interleukin-13 (IL-13) and interleukin-1 receptor antagonist (IL-1Ra) (Coito et al., 2002; Harada et al., 2002; Pachori et al., 2004). In addition, some authors have tried strategies which purpose is the amelioration of the hepatic inflammatory response to I/R, namely by inhibiting NFκB pathway through adenoviral transfection of a mutant inhibitor of kB-α (IκB-α) (Banga et al., 2005; Lentsch et al., 2000). Supression of Fas by siRNA was shown to inhibit cellular apoptosis in vivo and may consequently mitigate I/R injury in rat liver transplantation (Li,X et al., 2007). Nevertheless, a gene therapy approach brings also some difficulties; for example, vector toxicity, problems regarding transfection efficiencies and protein expression at the appropriate time and site.

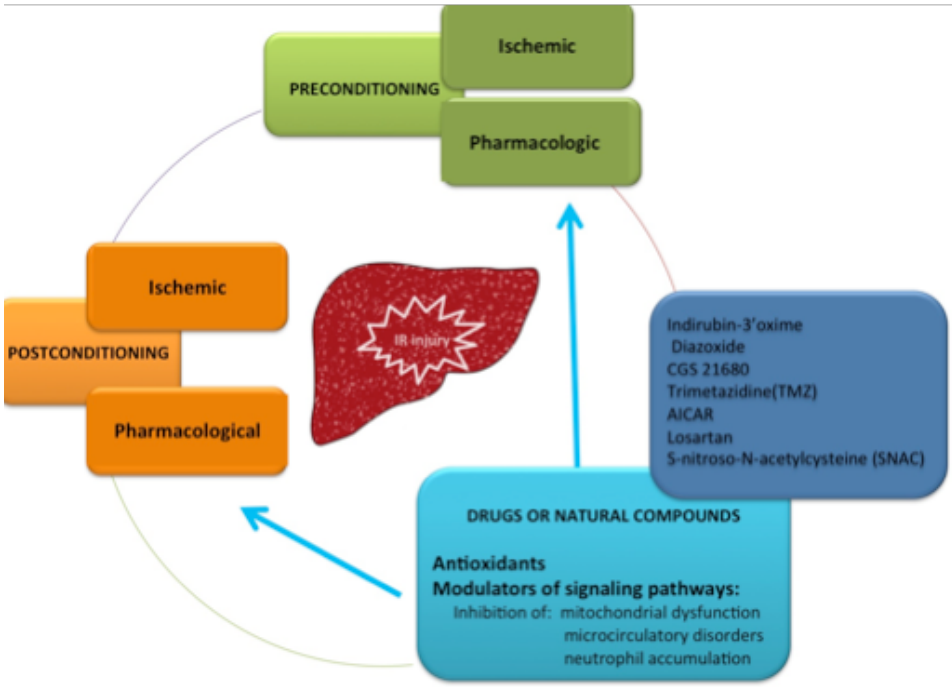



**Figure 1.14- Therapeutic strategies against Ischemia / Reperfusion injury.** Preconditioning and Postconditioning are endogenous adaptive mechanism of protection that can be actived surgical or pharmacologically. Indirubin-3'oxime, Diazoxide, CGS 21680, Trimetazidine (TMZ), 5-amino-4-imidazole carboxamideriboside (AICAR), Losartan, S-nitroso-N-acetylcysteine (SNAC) are some examples of compounds which may be used as pharmacological therapeutic.

A number of drugs and agonists have been shown to reduce I/R injury. Although the perfect drug does not exist at the moment, there are some synthetic and natural derivatives presenting metabolic and/or antioxidative effects that can directly or indirectly improve hepatic function after I/R (Varela et al., 2010). A drug that reduces I/R injury associated with hepatic surgery and transplantation would constitute a major medical breakthrough. Natural products are important therapeutic tools in several clinical areas since they are viable sources and resources for drug discovery and development. It is possible to relate the chemical structures of the pharmacological drugs and natural derivatives to their correspondent protective action against I/R injury. This can be of interest in order to create new natural remedies in I/R therapy. Also, brief intermittent periods of ischemia and reperfusion, before I/R, termed ischemic preconditioning (IPC), are also protective. One exciting new development with important clinical implications is the observation that protection can be initiated by the activation of signaling pathways after reperfusion.

It is a procedure based on the induction of intracellular protective reactions by the application of brief periods of ischemia and reperfusion, at the immediate onset of reperfusion. From the first report of Zhao et al., several studies have demonstrated that in other organs, such as heart, brain, kidney and liver, the method of postconditioning seems to be simple to apply and potentially effective in reducing reperfusion injury.

Zeng et al. shows that in the liver transplantation model, postconditioning (treatment immediately with six cycles of 60s of reperfusion and 60s of ischemia at the onset of reperfusion) can improve the liver function, reduce oxygen-free radicals production and cytokines expression, and leads to increased expression of HO-1 in liver.

### 1.6.1 – Preconditioning (PC)

Ischemic preconditioning refers to an endogenous adaptive mechanism of protection against a sustained ischemic insult by exposure to an initial, brief ischemic stimulus followed by a period of reperfusion. This phenomenon was first described in heart and brain and only later was evidenced in other organs including the liver.

Administration of chemical agents is another way to achieve PC, which is known as pharmacological PC. The pathways by which PC and compounds afford protection, usually converges on the activation of signaling cascades that involves phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), nitric oxide synthase (NOS), glycogen synthase kinase-

3β (GSK-3β) and protein kinase B (Akt). Although the precise targets and mechanisms are still unclear, mitochondria are a major target of these protective events.

PC strategy may find new surgical and/or pharmacologic therapeutic applications in complex hepatic resections in livers with underlying disease or steatosis, as well as in liver transplantation. PC may be beneficial for both patients receiving either steatotic or non-steatotic grafts, although biological mechanisms mediating PC effects can be different, as well as pharmacologic treatments (Peralta et al., 1999).

#### 1.6.1.1 Molecular basis and cellular mechanisms

The precise mechanism of PC-induced hepatoprotection is unknown but it is likely to be a receptor-mediated process, related with preservation of energy metabolism. IPC occurs in a biphasic pattern: an early phase, which wanes for several hours (early IPC), and involves direct modulation of cell function as a result of accumulation of adenosine and/or nitric oxide and activation of PKC, and a late phase (late IPC), which requires synthesis of multiple stress-response proteins, including heat shock proteins (HSP), NOS and HO-1 (Schulz et al., 2001; Takano et al., 1998; Massip-Salcedo et al., 2006).

There is an indication that PC prevents I/R-induced mitochondrial dysfunction by preserving the integrity of mitochondrial oxidative phosphorylation and increasing the resistance to MPT induction. Preservation of mitochondrial function by IPC favors the restoration of sufficient energetic levels in injured hepatocytes may thus be correlated with better post-transplantation outcomes (Lanir et al., 1988; Marni et al., 1988). A recent study has shown that induction of autophagy by PC limits necrosis in human recipients of fatty liver grafts, resulting in a decreased incidence of rejection episodes (Degle Esposti et al., 2011). It is suggested that the trigger of autophagy switches off necrosis in fatty livers subjected to I/R, by preserving ATP content and decreasing ROS generation.

Several mediators have been proposed to play a critical role in the protective pathways activated by PC, including adenosine, nitric oxide, oxidative stress, some HSP and TNF–α (Peralta et al., 1999; Carini et al., 2003; Morihira et al., 2006; Bolli et al., 2000; Teoh et al., 2003) (Figure 1.15).

#### 1.6.1.1.1 Adenosine receptors activation in preconditioning

Adenosine is a major homeostatic modulator in eukaryotic tissues since it is produced according to tissue stress or workload and protects from subsequent injury by decreasing metabolic rate. In particular, adenosine receptors have been shown to be responsible for the phenomena of PC in tissues such as the brain, heart or liver.

Adenosine receptors are G-protein-coupled receptors and there are 4 subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$. The $A_1$ and $A_3$ receptors are coupled to Gi/Go proteins and $A_{2A}$ and $A_{2B}$ are linked to Gs proteins.

All these receptors have been proved to be cardioprotective against ischemia/reperfusion-induced injury, even though different receptor subtypes may be related to distinct signaling pathways. $A_1$ adenosine receptors were also shown to protect lung and brain from this injury.

In the liver, Peralta et al. identified a protective pathway in which adenosine-induced protection involves the induction of the enzyme nitric oxide synthase. The protective effect of adenosine receptors has been demonstrated by the administtration of agonists and antagonists. The agonists reproduced the beneficial effects of PC and the antagonists suppress them. Several studies show the intervention of intracellular messengers on the protective effect of PC. PKC, AMP-dependent protein kinase (AMPK), p38, and some translator's signs and transcription factors activated by the release of adenosine are involved. The release of adenosine into the extracellular space causes the activation of $A_2$ receptors coupled to G protein. These activate a membrane phospholipase (phospholipase CorD) that generates inositol triphosphate (inducing the release of $Ca^{2+}$ from the intracellular mitochondrial reserves) and diacylglycerol (DAG), activates PKC.

PKC has an important role in cellular protection and regulates some biological processes such as metabolism, ions transport and gene expression. Several studies refer to this kinase as the head of the protective effects of PC in the liver (Inagaki et al., 2006). The activation of PKC promotes the phosphorylation of various molecules such as tyrosine kinase and mitogen-actived protein kinase (MAPK), including MAPK p38. The associated increase in the tolerance of hepatocytes and endothelial cells to I/R injury may thus imply the entry of these cells into cell cycle, promoting hepatic regeneration. The PKC activation can induce the activation of many transcription factors, such as NFκB, probably the main responsible for the protective effects during the late phase.

Increased synthesis of proteins such as HSP have been proposed as effectors of the protective effect of PC.

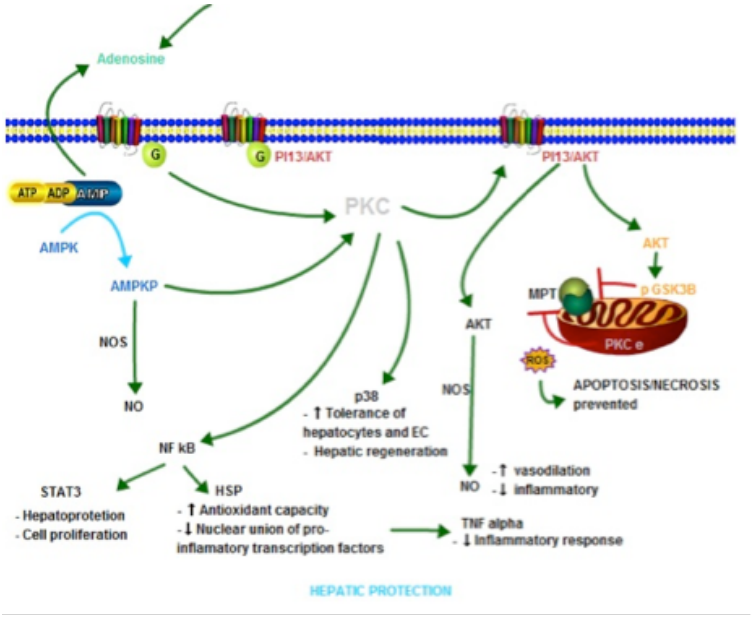



**Figure 1.15- Preconditioning pathway.** The release of adenosine causes the activation of G protein-coupled receptors and stimulation of phospholipase C, producing diacylglycerol that activates Protein kinase C (PKC). PKC actives the MAP p38 that promotes hepatic regeneration.PKC activation can also induce the activation of NFκB that is responsible for the protective effects during the late phase of preconditioning, through the activation of signal activator of transcription 3 (stat-3) or through synthesis of proteins such as heat shock proteins (HSP). HSP reduces the nuclear union of pro-inflammatory transcription factors and increases the antioxidant capacity of the cell. These effects may contribute to the reduction of TNF-alpha and attenuation of inflammatory response.PI3K/Akt pathway is activated by receptor tyrosine kinases, integrins, cytokine receptors, G protein coupled receptors and other stimuli that induce the production of phosphatidylinositol 3,4,5 triphosphates (PIP) by phosphoinositide 3-kinase (PI3K). Activated Akt is released from the plasma membrane and targets substrates in the cytosol, nucleus, and mitochondria. In the mitochondria, Akt is responsible for the phosphorylation of glycogen synthase kinase-3β (GSK-3β) on serine 9 thereby inhibiting GSK-3β and MPT induction, promoting cell survival. AKT also leads to enhanced activity of eNOS and augments NO.

PC associates with the synthesis of various forms of HSP such as HSP70, HSP72, HSP73 and HO-1 (Ye et al., 2011).The induction of HSP reduces the nuclear union of pro-inflammatory transcription factors and increases the antioxidant capacity of the cells. Both effects may contribute to the reduction of TNF-α and attenuation of inflammatory response in preconditioned livers. PC can decrease the transcription of genes such as c-fos and c-jun that are involved in the development of hepatic I/R injury (Saito et al., 2001). On the other hand, the activation of NFκB could induce the activation of signal activator of transcription 3 (stat-3) implicated in hepatoprotection and cell proliferation. Diverse studies have showed that PC protects fatty liver from IR and maintains hepatic oxygenation, tissue perfusion, and mitochondrial redox state (Hafez et al., 2010).

PI3K/Akt signaling to the mitochondria also plays a crucial role in the protection against cell death induced by I/R. Accumulation of PIP3 following P activation, leads to recruitment of Akt to the membrane, where Akt is converted to the active form by phosphoinositide-dependent proteinkinase-1 (PDK1)-mediated phosphorylation at Thr308. Activated Akt is released from the plasma membrane and targets substrates in the cytosol, nucleus, and mitochondria (Kockeritz et al., 2006; Matsui et al., 2005; Bijur et al., 2003). Ischemic and pharmacological PC, as discussed further on, enhances Akt phosphorylation and translocation to the mitochondria (Bijur et al., 2003). In the mitochondria, Akt phosphorylates GSK-3β on serine 9 (phospho-Ser9-GSK-3β ) and inactivates it.

### 1.7 Objectives of this thesis:

The main goal of this project was to define and approach protective strategies against I/R injury in fatty liver by an in vivo warm I/R protocol, with either lean or fatty livers, an approach allowing retrieving conclusions relevant for the distinct clinical settings.

Fatty livers cause an increased susceptibility to I/R injury, thus bearing additional risks of primary nonfunction subsequent to liver surgery or transplantation. There is an urgent need for strategies against I/R injury in fatty livers, due to the increased incidence of this pathology.

Defining IPC underlying mechanisms will allow drug targeting to induce the preconditioning response in fatty livers as a developing strategy to reduce hepatic injury observed in transplantation and hepatic resections. Understanding key aspects of mitochondrial dysfunction, is a necessary step towards the elaboration of new protective strategies against I/R injury, since mitochondria are a major target of the protective signalling cascades

induced by preconditioning, this is not surprising given their important role in the regulation of cell energetics and death, ROS, and calcium. Pharmacological modulation could maybe be improved by examining the mechanisms responsible for mitochondrial alterations in steatotic livers.

The hope of finding new surgical and pharmacological therapeutic applications provides a strong impetus to identify the mechanisms responsible for the failure of fatty livers ( Figure 1.16).

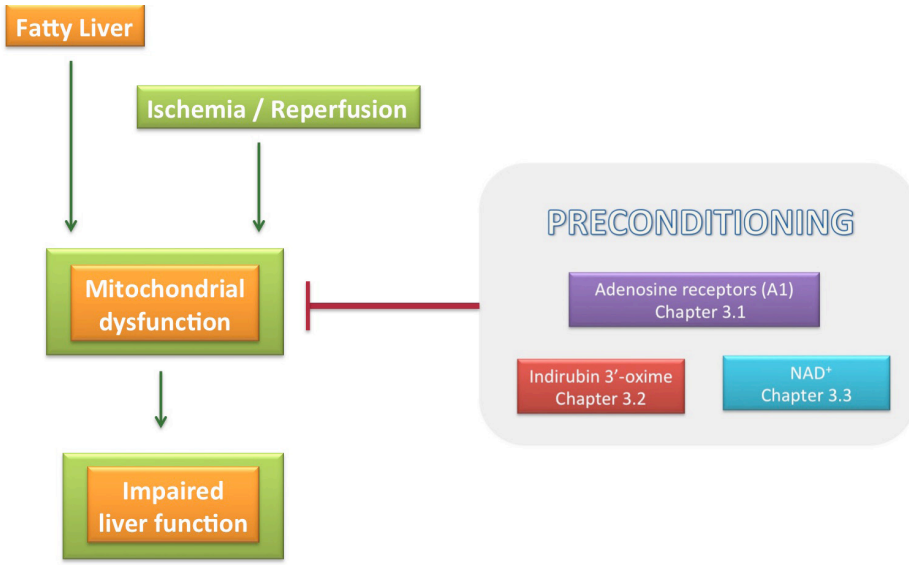
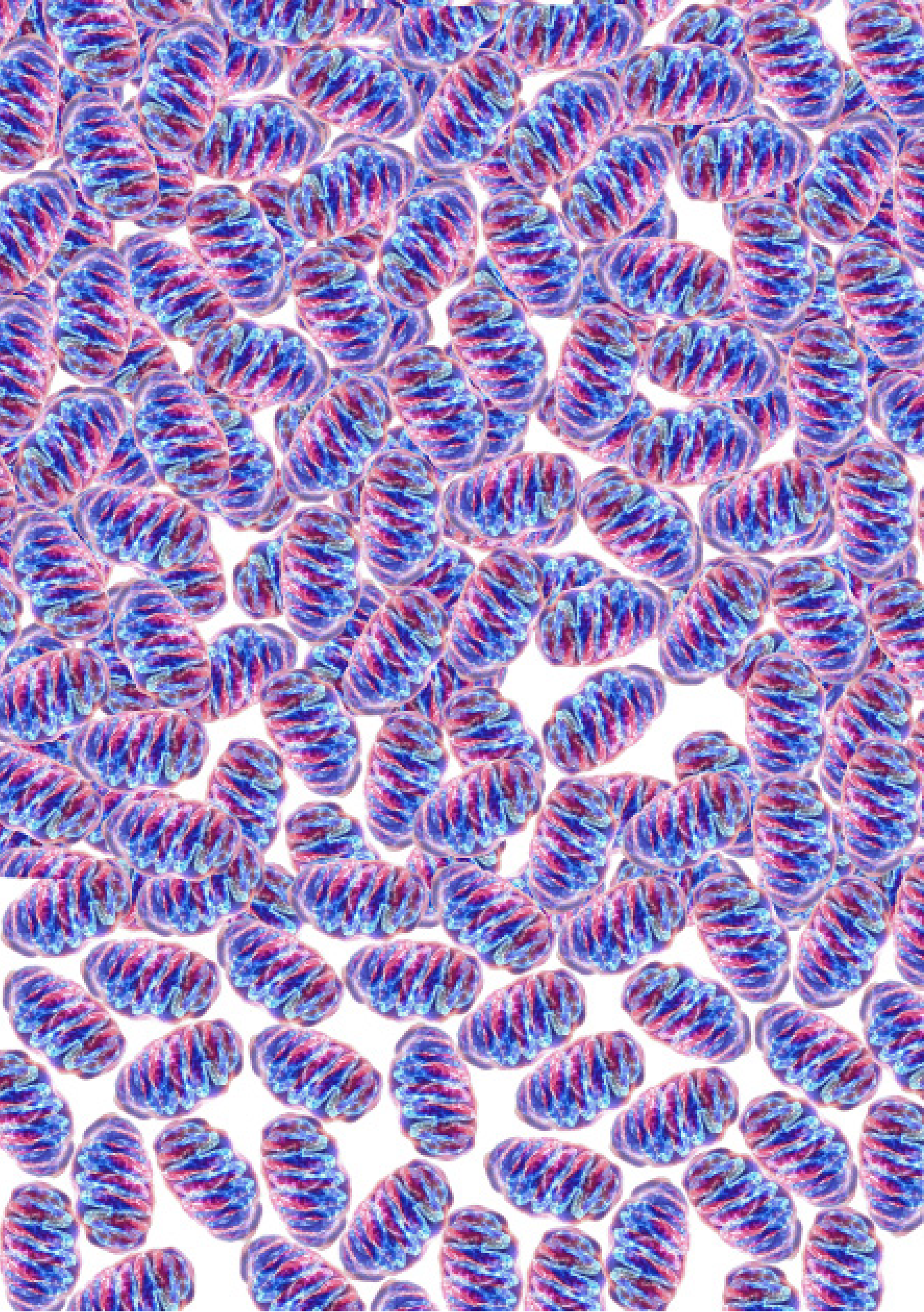
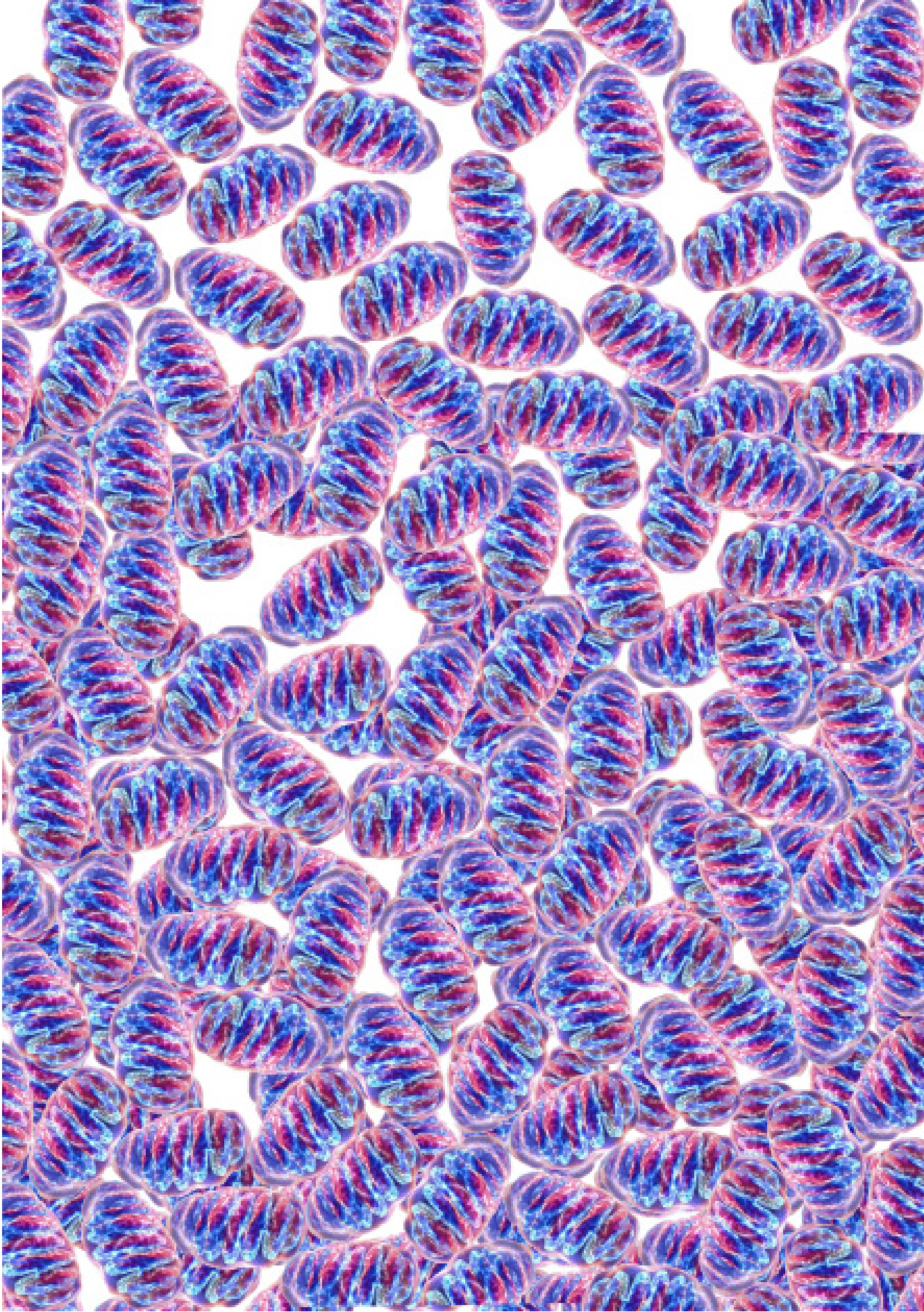



**Figure 1.16 - Schematic representation relating fatty liver with I/R injury .** Possible strategies to protect fatty livers by reducing preconditioning as proposed work.





## 2.1 Materials

Indirubin-3'-oxime, $NAD^+$ and 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) were purchased from Sigma Chemical Co (St. Louis, MO). 8-cyclopentyl-1,3-dipropulxanthine (DPCPX) and 2-chlloro-N6-cyclopentyladenosine (CCPA) and were purchased from Tocris Biosciences (United Kingdom). Indirubin-3'-oxime and $H_2$DCFDA were dissolved in DMSO, all others in saline solution.

ANT crosslinked to agarose beads was obtained from MitoSciences. WesternDot 625 goat anti-rabbit and goat anti-mouse, $Ca^{2+}$-Green, Tau protein and Tau [pS199] phosphoELISA kit and TNF-alpha ELISA kit were purchased from Invitrogen (Invitrogen,UK). SIRT3 direct fluorescent screening assay kit was purchased from Cayman Chemical Company.

The ALT, AST and LDH kits obtained from Hospitex Diagnostics. All other reagents and chemicals used were the highest grade of purity commercially available.

## 2.2 Animals

Seven-weeks-old male Wistar and Zucker fatty rats were obtained from Charles River (France). Upon arrival, animals were allowed 7 days to acclimatize (room temperature was 22-24º C) and housed under controlled light (12h-light cycle) and humidity (moisture 50-60%) conditions with free access to food and water. All animals received care according to institutional guidelines.

## 2.3 Hepatic ischemia/ reperfusion

Animals were weighed and anaesthetized with ketamine (50 mg/kg) and chlorpromazine (50 mg/kg) by intramuscular injection. A model of partial ischemia (70%) was used in order to prevent mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. After a midline laparotomy, the hepatic artery and portal vein to the left and median liver lobes were occluded. Reperfusion was initiated by removal of the clamp.

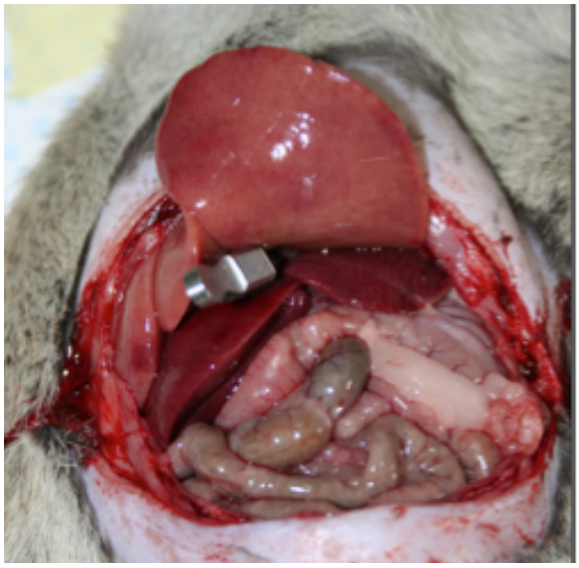


**Figure 2.1- Hepatic ischemia**. Model of partial ischemia (70%): the hepatic artery and portal vein to the left and median liver lobes were occluded.

## 2.4 Experimental Groups:

- **Ischemic preconditioning and DPCPX and CCPA administration**

In preconditioning group (IPC), before I/R, brief (5 minutes) ischemic episodes were applied, and followed by a 10 min period of reperfusion. In the treated groups, DPCPX and CCPA, 0,5mg/Kg compound was administered intraperitonially 2 hours before IPC or IR, respectively. Both control (sham-operated) and I/R (subjected to ischemia/reperfusion without treatment) animals were injected with an equal volume of saline buffer also for 2 hours.

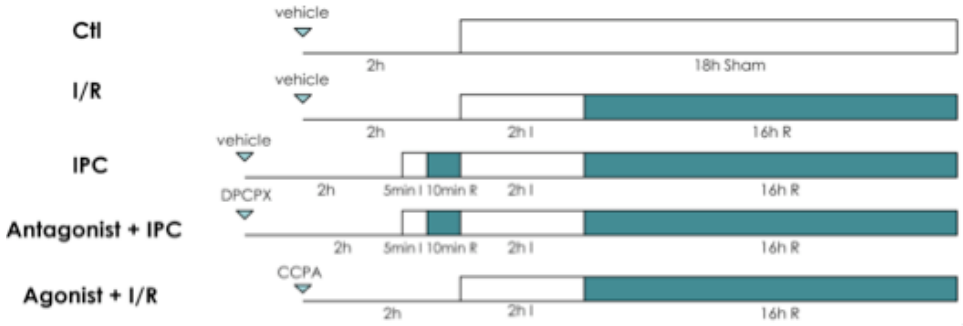



**Figure 2.2 - Schematic representation of the experimental protocols of I/R and DPCPX and CCPA treatment.** In the treated group, antagonist (DPCPX) and agonist (CCPA) were administered intraperitonially 2 hours before ischemic preconditioning (IPC) or ischemia/reperfusion (I/R), respectively. Ctl (sham-operated), I/R and IPC animals were injected with an equal volume of vehicle (saline solution) also for 2h. In preconditioning group (IPC), before I/R, brief (5 minutes) ischemic episodes were applied, and followed by a 10 min period of reperfusion. A model of partial ischemia (70%) was used during 2h. Reperfusion was initiated by removal of the clamp (16h).

- **Indirubin-3'-oxime administration**

In the treated group, 1 μM indirubin-3'-oxime (Ind) was administered in the hepatic artery 30 min before ischemia. Both control (sham-operated) and I/R (subjected to ischemia/reperfusion without Ind treatment) animals were injected with an equal volume of vehicle (DMSO) also for 30 min.

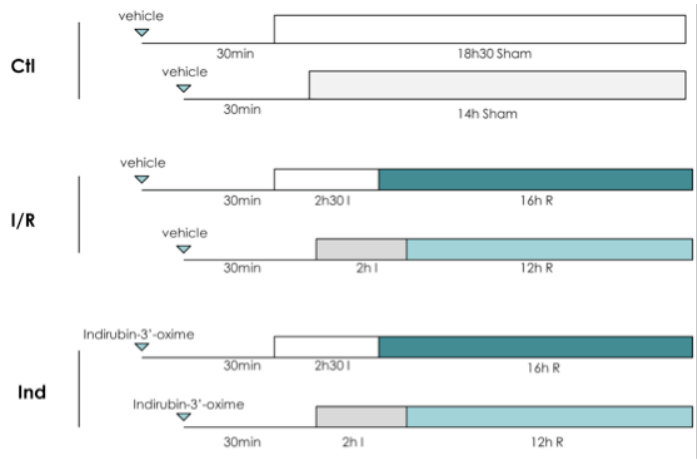


**Figure 2.3 Schematic representation of the experimental protocols of I/R and indirubin-3'-oxime treatment in lean and fatty livers.** In the treated group, 1 μM indirubin-3'-oxime (Ind) was administered in the hepatic artery 30 min before ischemia. Both Ctl (sham-operated) and I/R animals were injected with an equal volume of vehicle (DMSO) also for 30 min. A model of partial ischemia (70%) was used during 2h30 in lean livers and 2h in fatty livers. Reperfusion was initiated by removal of the clamp (12h or 16h).

• **NAD$^+$ administration**

In the treated group, 50mg/Kg NAD+ was administered in the hepatic artery 30 min before ischemia. Both control (sham-operated) and I/R (subjected to ischemia/reperfusion without NAD+ treatment) animals were injected with an equal volume of saline solution also for 30 min before.

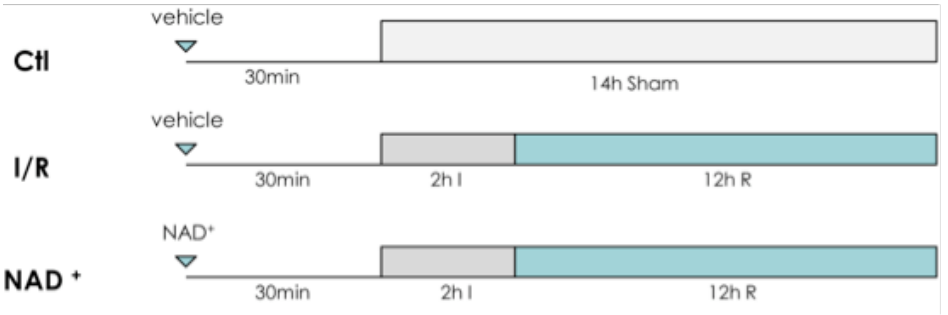



**Figure 2.4- Schematic representation of the experimental protocols of I/R and NAD$^+$ treatment.** In the treated group, NAD$^+$ was administered in the hepatic artery 30 min before ischemia. Both Ctl (sham-operated) and I/R animals were injected with an equal volume of vehicle (saline solution) also for 30 min. A model of partial ischemia (70%) was used during 2h. Reperfusion was initiated by removal of the clamp (12h).

All procedures were conducted according to the guidelines for the care and use of laboratory animals approved by our Institution.

### 2.5 Plasma biochemical determination

Following reperfusion, animals were killed by decapitation. Blood were collected and centrifuged at 5000 rpm for 5 min. Plasma samples were collected and enzymatic determinations of ALT, AST and LDH performed using commercial kits.

### 2.6 TNF-α

Plasma samples were diluted in Incubation Buffer and Tumor Necrosis Factor- α (TNF-α) was determined using commercial kit (Rt TNF-α ELISA, Invitrogen) according to the manufacturer's recommended protocol.

### 2.7 Preparation of liver mitochondria

Mitochondria were isolated from liver rats by conventional methods (Gazzoti et al.,1979), with slight modifications (Palmeira et al., 1994). All isolation material was kept on ice during the isolation procedure. Briefly, animals were and the liver was immediately excised and finely minced in an ice-cold homogenization medium. Homogenization medium contained 250mM sucrose, 10mM HEPES (pH 7.4), 0.5mM EGTA, and 0.1% fat-free bovine serum albumin (BSA). The minced blood-free tissue was then homogenized with a tightly fitted homogenizer (Teflon: glass pestle). The homogenate was then centrifuged at 3000 rpm for 10 min at 4°C. The resulting supernatant was spun at 10 000 rpm for 10 min (4°C), to pellet mitochondria which were resuspended with a paintbrush in a final washing medium (250mM sucrose, 10mM HEPES (pH 7.4)). The resulting supernatant of first centrifuge at 10 000 rpm was used as the cytosolic fraction. The mitochondrial pellet was then washed twice, suspended in the washing medium, and immediately used. Protein content was determined by the biuret method (Gornall et al., 1949), calibrated with BSA.

### 2.8 Measurement of mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was polarographically determinated with a Clark oxygen electrode (Estabrook, 1967) connect to a suitable recorder, through an oxygen electrode control system (YSI model 5300). The electrode was calibrated as previously described (Rickwood et al., 1987). Mitochondria (1 mg) were suspended under constant stirring,

at 25ºC, in 1.3 ml of standard respiratory medium supplemented with 3 µM rotenone. The succinate, as respiratory substrate (5 mM), was added.

State 3 respiration was induced by adding 200 nmol adenosine diphosphate (ADP). The oxygen consumption was also measured in the presence of 1 µM carbonylcyanide-p-trifluoromethoxyphenylhydrazon (FCCP).

The respiratory control ratio (RCR) was calculated taking into account oxygen consumption rates during state 3 (following ADP phosphorylation) and subsequent state 4 respiration. State 3 and state 4 respiration rates are expressed as nmol oxygen per minute per mg protein. The ADP/O ratio was calculated as the nmol of ADP phosphorylated by natom oxygen consumed (Chance and Williams, 1956).

The specific experimental procedures in each section are described in the legends to the figures.

### 2.9 Mitochondrial membrane potential (ΔΨ) measurements

The mitochondrial transmembrane potential, was estimated using an ion- selective electrode to measure the distribution of tetraphenylphosphonium ($TPP^+$), according to previously established methods (Kamo et al., 1979; Palmeira et al., 1994).

$TPP^+$ is a membrane-permeable cation that accumulates electrophoretically in mitochondria upon energization. The reference electrode was Ag/ $AgCl_2$ (model MI 402, Microelectrodes Inc, Bedford, NH). Both electrodes were connected to an adequate potentiometer (Jenway pH meter 3305). The signal was delivered to a Kipp and Zonen recorder via a manufactured potential compensatory box (Madeira, 1975). Reactions were carried out at 25 ºC, in a temperature-controlled water-jacketed chamber with magnetic stirring. Mitochondria (1 mg) were suspended in 1 ml of standard respiratory medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM $KH_2PO_4$, 50 µM EDTA, 5 mM HEPES (pH 7.4), and 3 µM rotenone) supplemented with 3 µl $TPP^+$ and were energized by adding succinate to a final concentration of 5 mM.

The electrode was calibrated with $TPP^+$, assuming Nerstian distribution of the ion across the synthetic membrane. ΔΨ, which is expressed in millivolts (mV), was calculated using the equation $\Delta\Psi = 59 \log (v/V) - 59 \log 10\Delta E/59 - 1)$ (Muratsugu et al., 1977; Kamo et al., 1979). v, V, and ΔE represent the mitochondrial matrix volume, the volume of the incubation medium and the potential deflection, respectively. A matrix volume of 1.1 µl/mg protein was assumed (Masini et al., 1984).

In our experiments, we didn't take into account the "passive" binding of TPP+ to the mitochondrial membrane, since we were interested in relative alterations in the potential rather than absolute values. Consequently, we predict that the obtained ΔΨ values were overestimated.

The specific experimental procedures in each section are described in the legends to the figures.

### 2.10 Titration with carboxyatractyloside (CAT)

Carboxyatractyloside (CAT) is a highly selective inhibitor of cytosolic side-specific ANT that causes stabilization of the c conformation of ANT leading to MPT induction and loss of mitochondrial membrane potential.

The mitochondrial transmembrane potential, was estimated as described in 2.9. Energized mitochondria were titrated with 0,02 nmol carboxyatractyloside (CAT). Successive additions of CAT aliquots before ADP addition, progressively block a higher number of ANT units contributing to the observed decreased number of phosphorylation cycles induced by successive ADP additions.

### 2.11 Determination of ATP content

Adenosine nucleotide extraction was performed as follows. Tissue samples (20 mg) were pulverized with a mortar and pestle in liquid $N_2$ and homogenized in ice-cold 25 µl of KOH buffer (KOH 2.5M, $K_2HPO_4$ 1.5 M), on ice. For mitochondria were added 10ul KO buffer. Samples were vortexed and centrifuged at 14 000 g for 2 min., at 4 ºC. The supernatants were collected and dissolved in 100 µl of $K_2HPO_4$ 1 M. pH was adjusted to 7 and samples were frozen at -80 ºC for posterior use.

Adenosine nucleotides were separated by reverse-phase high performance liquid chromatography as previously described (Stocchi et al., 1985) or were quantified with an ATP bioluminescent assay kit (Sigma-Aldrich) on a Victor 3 plate reader.

### 2.12 Evaluation of reactive oxygen species (ROS) generation

ROS were determined fluorometrically using a Perkin-Elmer VICTOR $^3$ plate-reader fluorometer, with an excitation wavelength 485 nm and an emission wavelength 538 nm, corresponding to the excitation and emission wavelengths of $H_2$DCFDA (Zhou et al.,2001). Isolated mitochondria (1 mg/ml) were suspended in standard respiratory medium

(130 mM sucrose, 50 mM KCl, 5 mM MgCl2, 5 mM $KH_2PO_4$, 50 µM EDTA, 5 mM HEPES (pH 7.4), and 3 µM rotenone) loaded with 50 µM $H_2$DCFDA (in DMSO) for 15 min at 25ºC. After incubation, the samples were spun at 3000 rpm for 3 min and the resultant pellet was suspended in medium. 200 µl of the mitochondrial suspension were loaded into a 96-well plate and the fluorescence monitored. After basal fluorescence measurement, antimycin A was added to all preparation to block complex III of the mitochondrial chain, to induce maximal ROS generation.The results were expressed are expressed as relative fluorescence units (RFUs).

### 2.13 Measurement of the mitochondrial permeability transition (MPT)

Mitochondrial swelling was estimated by changes in light scattering, as monitored spectrophotometrically (Heλiosy spectrophotometer, Thermo ElectronCorporation) at 540 nm (Palmeira and Wallace, 1997). The experiments were started by the addition of mitochondria (1 mg) to reaction medium (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 1mM $KH^2PO^4$, 5 mM succinate and 10 uM EGTA supplemented with 3 µM rotenone and 5 mM succinate) (final volume 2ml). Reactions were carried out at 25ºC and MPT induced with $Ca^{2+}$. The specific experimental procedures in each section are described in the legends to the figures.

### 2.14 Measurement of mitochondrial calcium fluxes

The accumulation and release of calcium by isolated rat liver mitochondria were determined using a calcium-sensitive fluorescent dye, Calcium Green-5 N (Rajdev and Reynolds, 1993). The reactions were carried out at 25ºC, in 2 ml of MPT medium (200 mM sucrose, 10 mM Tris-MOPS (pH 7.4), 1mM $KH_2PO_4$, and 10  EGTA) supplemented with 3 µM rotenone. Free calcium was monitored with 100 nM Calcium Green 5-N. Mitochondria (1 mg) were energized with succinate (5 mM) after the addition of 15 nmol $Ca^{2+}$. Fluorescence was recorded continuously using a Perkin-Elmer VICTOR $^3$, with excitation and emission wavelengths of 485 and 535, respectively. Calcium fluxes are expressed as relative fluorescence units (RFUs).

### 2.15 ATPase activity

ATPase activity of the mitochondrial ATP shynthase complex (or complex V) was determined spectrophotometrically at 660 nm, in

association with ATP hydrolysis (Varela et al., 2008). The reaction was carried out at 37 °C, in 2 ml reaction medium (100 mM NaCl, 25 mM KCl, 5 mM MgCl2, and 50 mM HEPES, pH 7.4). After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 2 mM $Mg^{2+}$ -ATP, in the presence or absence of oligomycin (1 ug /mg protein) protein). After 10 min, adding 1 ml of 40% trichloroacetic acid stopped the reaction. 2 ml of ammonium molybdate plus 2 ml $dH_2O$ were then added to 1 ml of supernatant. ATPase activity was calculated as the difference in total activity and activity in the presence of oligomycin.

### 2.16 Western blotting analysis

Tissue homogenates (10 µg), mitochondrial pellets (25 µg) or cytosolic fractions (25 µg) were lysed in ice-cold lysis buffer supplemented with a cocktail of protease, phosphatise and acetilase inhibitors.

**Table 2.1 List of utilized antibodies for Western blot, source and utilized dilution.**

| Antibody | Specie | Dilution | Supplier | Reference |
|---|---|---|---|---|
| **Acetilated lysine** | rabbit | 1:100 | Cell Signalling | 9441 |
| **Actin** | mouse | 1:5000 | Sigma | A 5441 |
| **Akt** | rabbit | 1.500 | Cell Signaling | 9272 |
| **ANT** | mouse | 1:500 | MitoSciences | MSA02 |
| **Bax** | rabbit | 1:1000 | Cell Signaling | 2772 |
| **Bcl-2** | rabbit | 1:1000 | Cell Signaling | 2870 |
| **Cleaved caspase-3** | rabbit | 1:500 | Cell Signaling | 9664 |
| **Cyp D** | mouse | 1.1000 | MitoSciences | MSA04 |
| **Cytochrome-*c*** | mouse | 1:5000 | BD Biosciences | 556433 |
| **COX IV** | mouse | 1:5000 | MitoScience | MS407 |
| **LC3** | rabbit | 1:1000 | Sigma | L7 543 |
| **,phospho-Ser9-GSK-3β** | rabbit | 1:100 | Cell Signalling | 9272 |
| **phospho-Thr308-Akt** | rabbit | 1:100 | Santa Cruz | SC 16646 |
| **phospho-threonine** | mouse | 1:100 | Qiagen | 37420 |
| **GSK-3β** | rabbit | 1:500 | Cell Signalling | 9315 |
| **HIF-1 α** | rabbit | 1.1000 | Santa Cruz | SC 10790 |
| **HSP70** | mouse | 1:1000 | Santa Cruz | SC 137210 |

Equal amounts of protein were loaded and electrophoresed on SDS-polyacrylamide 15% gel and transferred to a PVDF membrane. Membranes were blocked with 5% nonfat milk and incubated with antibody overnight at 4ºC. Immunodetection was performed with Western-Dot 625 goat anti-rabbit or goat anti-mouse western blot kits. Membranes were imaged using a Gel Doc instrument and the densiometric analysis was performed with the ImageJ software. (Bio-Rad Laboratories, Hercules, CA).

### 2.17 Evaluation of GSK-3β activity

GSK-3β activity was evaluated by promoting the in vitro reaction of phosphorylation of exogenous Tau by GSK-3β present in liver homogenates. To promote the reaction (100 µl), 250 ug of liver homogenates were incubated with 10 ng/ml exogenous Tau and 200 µM ATP for 1 hr at 30ºC. Phosphorylated Tau was detected using a Tau[pS199] phosphoELISA kit. Lithium chloride (10 mM) was used as an inhibitor of GSK-3β. Samples and standard dilutions were read in a Perkin-Elmer VICTOR 3 plate-reader at 450 nm.

### 2.18 Measurement of sirtuin-3 activity

Sirtuin-3 activity was measured in mitochondrial extracts using the SIRT3 direct fluorescent screening assay kit (Cayman Chemical Company). A sirtuin-3 peptide substrate that is acetylated and fluorescently labeled is mixed with the mitochondrial extract (25 µg) at 37º C for 45 min, 25 µl of developer was added and following 30 minutes of incubation. Fluorescence intensity was measured on a Perkin-Elmer VICTOR 3, with excitation and emission wavelengths of 340 and 440, respectively.

### 2.19 Myeloperoxidase (MPO) activity

MPO activity was determined by spectrophotometrically using tetramethylbenzidine (TMB) as substrate. The reaction mixture consisted of 40% phosphate-buffered saline (PBS), 8% DMFA, 0.3 mM H2O2, 80 mM phosphate buffer (pH 5.4), and 1.6 mM TMB in a total volume of 1 ml. The mixture is incubated at 37 °C for 3 min and the reaction is initiated by the addition of 20 µl of homogenate tissue containing 40 µg protein. The reaction was terminated after 3 min by addition of sodium acetate buffer (200 mM, pH 3.0). The rate of MPO-catalyzed oxidation of TMB was

followed by recording the increase of absorbance at 655 nm at 37 °C. We measured the absorbance change per minute, and one enzyme unit was defined as the amount of enzyme producing one absorbance change per minute under assay conditions. Enzyme activity was calculated as units per milligram of protein.

### 2.20 Immunoprecipitation of ANT and detection of cyclophilin D

ANT was immunocaptured from mitochondrial extracts using monoclonal antibodies to ANT crosslinked to agarose beads . Mitochondria (1mg) collected after $Ca^{2+}$ -induced MPT were resuspended in 300 µl of IP buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris, 0.5% Triton, protease Inhibitor cocktail, pH 7.2) and freeze in liquid N2. Samples (250 µl) were incubated overnight at 4ºC with 8 µl of agarose beads. Beads were washed four times with ice-cold buffer. Elution of immunocomplexes with Laemmli sample buffer and heat-denaturation were performed immediately prior to separation on 15% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. The western blots were then probed with antibody against CypD.

### 2.21 Immunoprecipitation of ac-Lys and detection of cyclophilin D

Mitochondria (2mg) were resuspended in a 500 µl IP buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris, 0.5% Triton, protease Inhibitor cocktail, pH 7.2) and lysed by sonication. CypD antibody was used for immunoprecipitation of endogenous CypD. Immunoprecipitated material was washed four times for 15 min each in low stringency lysis buffer, and immune complexes were resuspended in SDS-PAGE buffer. The western blots were developed using antibody against acetylated lysine, then stripped and reprobed with antibody against anti-CypD.

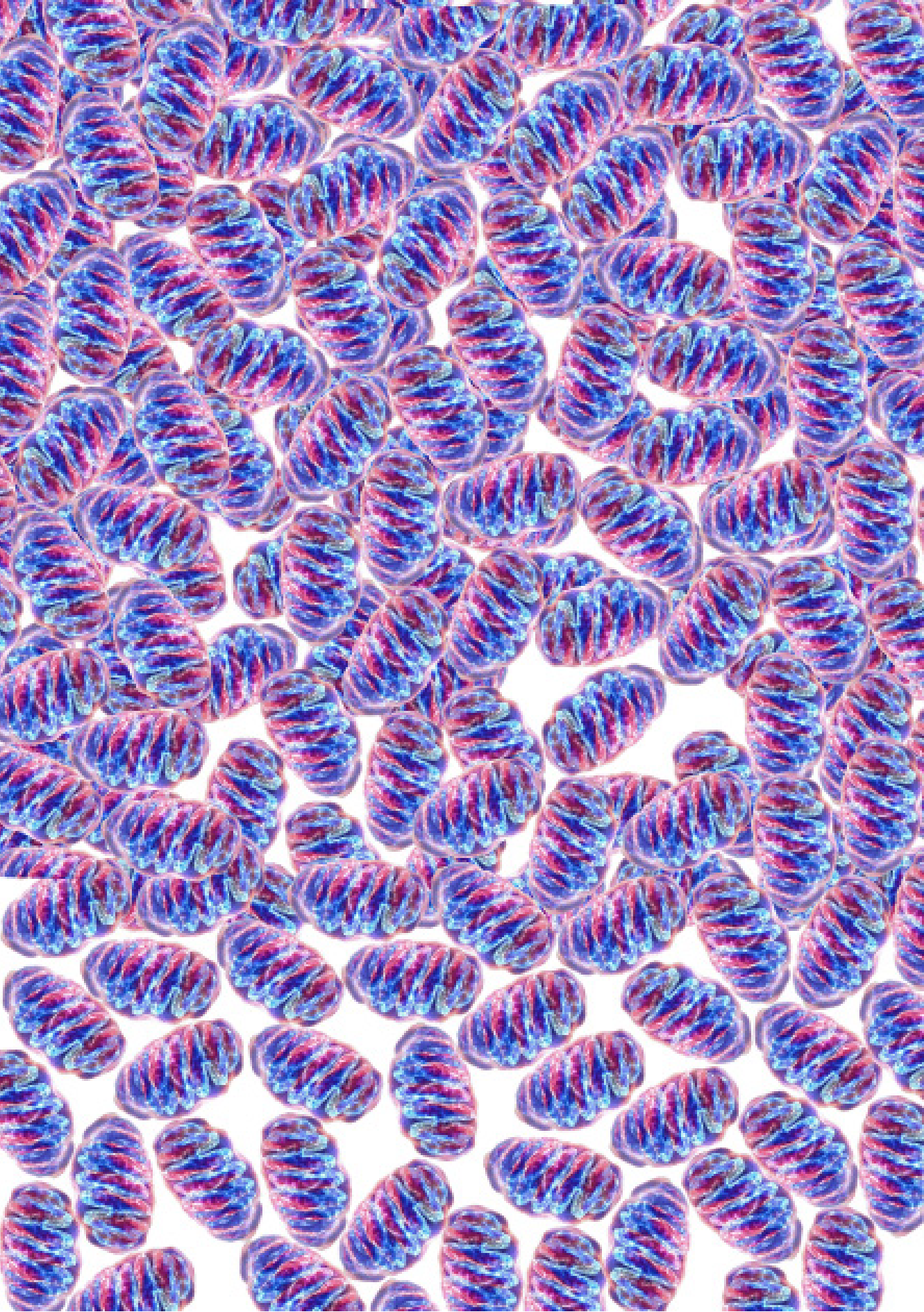
### 2.22 Immunoprecipitation of phospho-Thr and detection of cyclophilin D

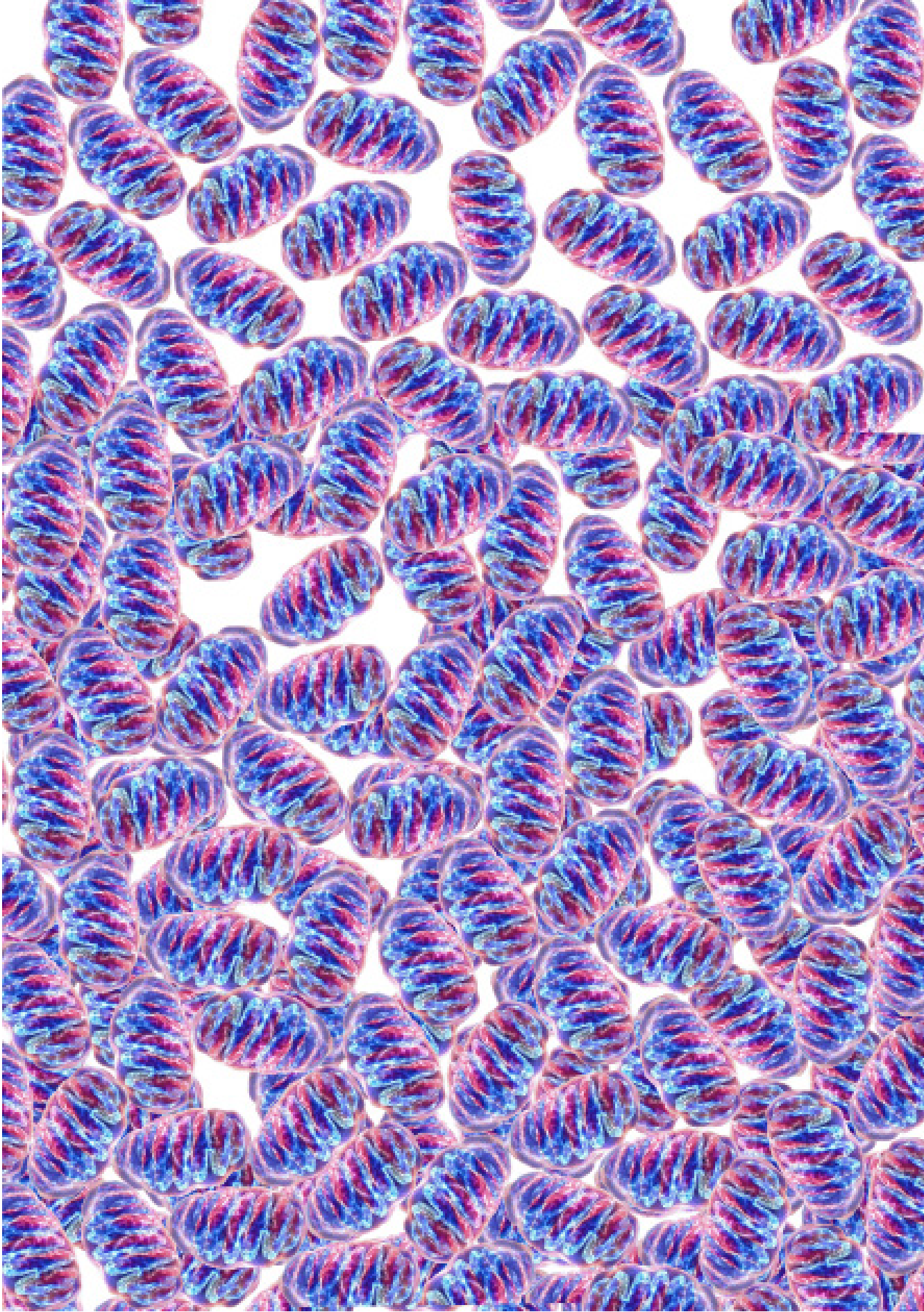
Mitochondria (2mg) were resuspended in a 500 ul IP buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris, 0.5% Triton, protease Inhibitor cocktail, pH 7.2) and lysed by sonication. CypD antibody was used for immunoprecipitation of endogenous CypD. Immunoprecipitated material was washed four times for 15 min each in low stringency lysis buffer, and

The western blots were developed using antibody against phosphorylated threonine, then stripped and reprobed with antibody against anti-CypD .

### 2.23 Statistic analyses

Data are presented as mean ± S.E.M and were obtained from five different experiments. Statistical significance was determined using one-way ANOVA followed by Bonferroni post hoc test. A value of $P<0.05$ was considered as statistically significant.





# 13

## RESULTS: ADENOSINE $A_1$ RECEPTORS IN PRECONDITIONING

## ADENOSINE A1 RECEPTORS AS REGULATORY ELEMENTS IN THE RECOVERY OF MITOCHONDRIAL FUNCTION INDUCED BY HEPATIC PRECONDITIONING


### Abstract

Preservation of energy production in mitochondria is a key determinant for recovery following ischemia/reperfusion.
Activation of adenosine receptors has been shown to be involved in the protective effect of ischemic preconditioning against I/R-induced injury in tissues such as the brain or heart. However, direct evidence that adenosine receptors preserve the efficiency of oxidative phosphorylation in conditions of hepatic I/R is still missing.

In this study, we evaluated the role of adenosine receptors in preconditioning in the liver by comparing the extent of injury upon preconditioning in the absence and in the presence of selective A1 receptor antagonists (DPCPX). We also explored the potential of perfusion with selective A1 receptor agonists (CCPA) to attempt a pharmacological preconditioning in vivo. Wistar rats were subjected to 120 min of 70% warm ischemia and 16 hrs of reperfusion (I/R). In preconditioning group (IPC), before I/R, brief (5 minutes) ischemic episodes were applied, and followed by a 10 min period of reperfusion. In the treated groups, DPCPX and CCPA, 0,5mg/Kg compound was administered intraperitonially 2 hours before IPC or IR, respectively.

PC (ischemic and pharmacological) decreased blood serum alanine aminotransferase , aspartate aminotransferase, lactate dehydrogenase and tumor necrosis factor- α plasma levels, comparatively to I/R livers. Administration of DPCPX abolished IPC effects while activation of A1 receptors by CCPA mimicked the beneficial action of IPC. Furthermore, IPC and CCPA livers displayed improved OXPHOS with higher state 3 rates and RCR, as well as increased ATP content and decreased lag phase. The susceptibility to mitochondrial permeability transition (MPT) and the rate of ROS generation was higher in I/R livers, but prevented by both IPC and CCPA. Titration with carboxyatractyloside (CAT), a selective inhibitor of cytosolic side-specific adenine nucleotide translocase, showed that less CAT was needed to completely abolish repolarization after phosphorylation in I/R livers, but not in IPC or CCPA. DPCPX administration abrogated IPC effects .This impairment in the phosphorylative system was associated with a decrease in the mitochondrial content of GSK-3β phosphorylation at Ser9, the inactive form of GSK-3β. Both IPC and CCPA increased mitochondrial phospho-Ser9-GSK-3β, possibly due to a reduction in the affinity of ANT to CypD , thus suppressing the MPT and decreasing

caspase activation.

These results show that suppression of GSK-3β activity by Akt-mediated Ser9-GSK-3β phosphorylation in the mitochondria is involved in the cytoprotective signaling mediated by receptors activation, preserving OXPHOS efficiency and preventing cell death.

### 3.1.1 Introduction

I/R injury is a common clinical problem responsible, at least in part, for the morbidity associated with liver surgery under total vascular exclusion or after liver transplantation (Tsung et al., 2005). This has motivated a search for new approaches to reduce ischemic injury, propelled by the discovery of ischemic preconditioning. This endogenous adaptive mechanism of protection against a sustained ischemic insult is afforded by exposure to an initial, brief ischemic stimulus followed by a period of reperfusion (Yoshizumi et al., 1998, Koti et al., 2003) or by the administration of chemical agents, which is known as pharmacological preconditioning (Nakayama et al., 1999; Andraus et al., 2010).

PC reduces the release of transaminases (Yoshizumi et al., 1998) , increases rat survival after liver transplantation (Yin et al., 1998), and even improves human hepatic functions after major liver surgery in patients subjected to 30 min of ischemia (Clavien et al., 2000). In livers, IP decreases liver injury after both warm and cold I/R (Arai et al., 1999; Centurion et al., 2007). Several mechanisms have been proposed for protection by IP, including activation of adenosine receptors (Peralta et al., 1999), increased nitric oxide production (Carini et al., 2003), activation of protein kinase C (Downey et al., 2004),HSP up-regulation of heat shock proteins (Ye et al.,2011) and increases of antioxidant capacity. Decrease tolerance to I/R injury is also related with the inability to restore energetic balance following I/R. IPC also prevents I/R-induced mitochondrial dysfunction suggesting that PC protects the integrity of mitochondrial OXPHOS and increases the resistance to mitochondrial permeability transition induction [5]. The precise mechanism of PC-induced hepatoprotection is unknown but it is likely to be a receptor-mediated process, related with preservation of energy metabolism (Lanir et al.,1988; Marni et al., 1988).

Adenosine is a major homeostatic modulator in eukaryotic tissues since it is produced according to tissue stress or workload and protects from subsequent injury by decreasing metabolic rate. In particular, adenosine receptors have been shown to be responsible for the phenomena of PC in tissues such as the brain, heart and liver (Liu et al., 1991,Peralta et

al.,1999; Hu et al., 2012).

Ischemic and pharmacological PC enhances Akt phosphorylation and translocation to the mitochondria (Bijur and Jope, 2003). In the mitochondria, Akt phosphorylates GSK-3β on serine 9 and inactivates it (Jope and Johnson, 2004). The mitochondrion is a platform of cell signaling and decision-maker of cell death, with multiple roles in the development of I/R injury (Di Lisa et al., 2003). Cell viability is compromised by energy deficiency due to impaired OXPHOS, increased generation of ROS, mitochondrial calcium overload and induction of the MPT. In response to cellular stresses, MPT induction abolishes mitochondrial membrane potential and compromises ATP generation. MPT induction can be suppressed by IPC and other interventions that increase phospho-Ser9-GSK-3β (Juhaszova et al., 2004; Miura et al., 2009; Nishihara et al., 2007; Park et al., 2006; Xi et al., 2009). GSK-3β is an important regulator of cell function, including gene expression, cell cycle, survival and apoptosis (Kockeritz et al., 2006). Phospho-Ser9-GSK-3β in mitochondria physically interacts with the ANT upon reperfusion, which inhibits interaction of the ANT with CypD, elevating the threshold for MPT induction.

Mitochondrial function has an essential key role in adenosine receptors-mediated effects, but the link between both is still to be discovered. A1 receptors may also be involved in the maintenance of mitochondrial membrane potential and sensitivity to pro-oxidant stressors, acting as determining mitochondrial vulnerability factors (Alba et al., 2010). The present study was designed to identify mitochondrial downstream targets involved in hepatic PC protection activated by A1 receptors. Analysis of mitochondrial OXPHOS and MPT susceptibility shows that activation of A1 receptors protects the liver by targeting the MPT through inhibition of GSK-3β and modulating mitochondrial bioenergetics.

### 3.1.2 Results

#### 3.1.2.1 Plasma markers of liver injury:

Analysis of serum levels of AST, ALT and LDH showed that warm I/R induced liver injury. IPC and CCPA administration before ischemia were effectives in reducing plasma levels of markers of liver injury. Such as in the I/R group, AST, ALT and LDH levels were increased in DPCPX group (Figure 3.1.1).

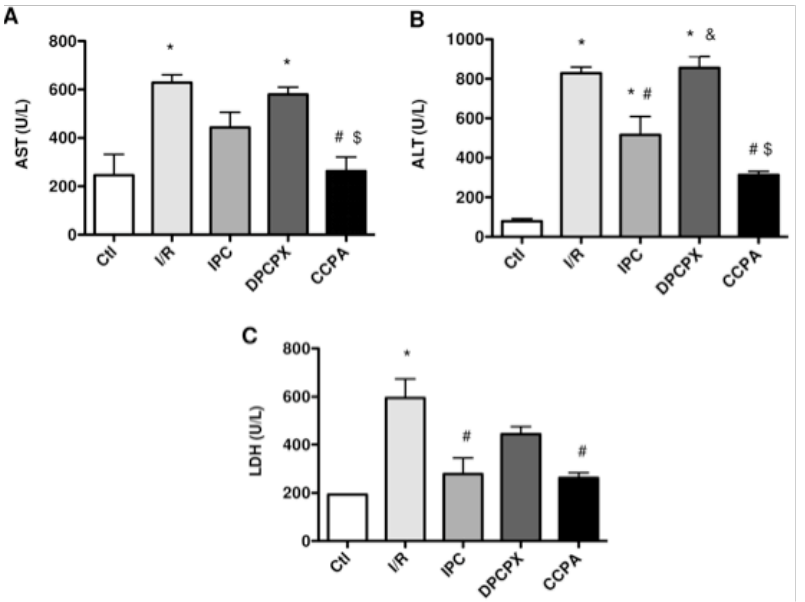



**Figure 3.1.1 The effects of PC on plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH).** Plasma samples were collected and enzimatic determinations of alanine aminotransferase (ALT) (A), aspartate aminotransferase (AST) (B) and lactate dehydrogenase (LDH) (C) performed using commercial kits. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference ($P<0.05$) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

Since TNF-α, a proinflammatory cytokine, has the role to start and perpetuate a later secondary inflammatory phase, causing further hepatic damage, TNF-α concentration in plasma was determined. Figure 3.1.2 shows that it was significantly higher in the I/R group than the other conditions.

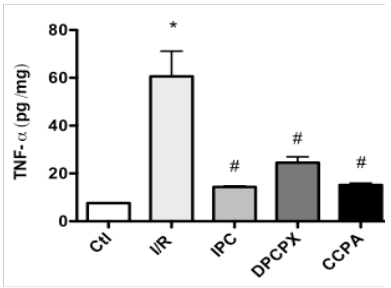


**Figure 3.1.2 The esffects of PC on TNF- α levels**. Plasma samples were collected and TNF- α was measure using commercial kits. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference (P<0.05) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

### 3.1.2.2 HSP70, Bcl-2 and Cleaved caspase-3 content:

In I/R animals, there was a higher content in cleaved caspase-3, as evaluated by western blotting in hepatic tissue homogenates, as well as a decrease in the content of the anti-apoptotic Bcl-2. IPC and CCPA livers showed a higher content in Bcl-2 and decreased cleaved caspase-3 in contrast to I/R animals.

Up-regulation of HSPs has been proposed as a mechanism for PC protection (Ye et al., 2011). Specifically, HSP70 as an anti-apoptotic function associated with mitochondrial stabilization and decreased caspase-3 activity. HSP70 content was increased by IPC, while decreased by I/R. PC by CCPA administration did not alter HSP70 content (Figure 3.1.3).

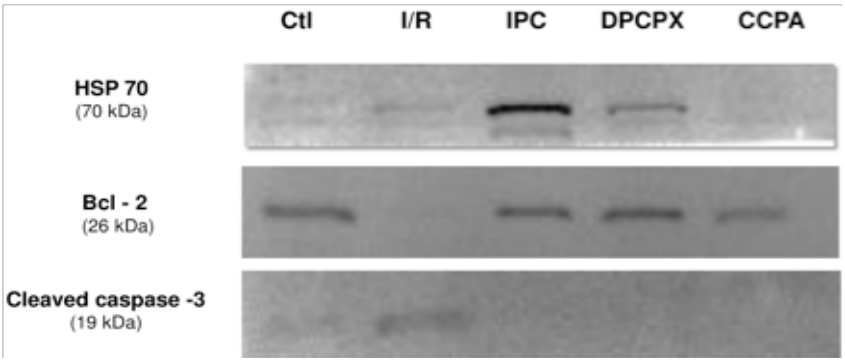



**Figure 3.1.3 HSP70 , Bcl-2 and cleaved caspase-3 content were evaluated by western blotting**. A representative blot from six independent experiments is shown.

### 3.1.2.3 Mitochondrial membrane potential (ΨΔ) and oxygen consumption:

Comparatively to mitochondria isolated from I/R animals, ΔΨ was significantly increased in preconditioned livers (IPC and CCPA). ΔΨ developed by mitochondria isolated from DPCPX livers was similar to that from I/R liver (Figure 3.1.4 A).

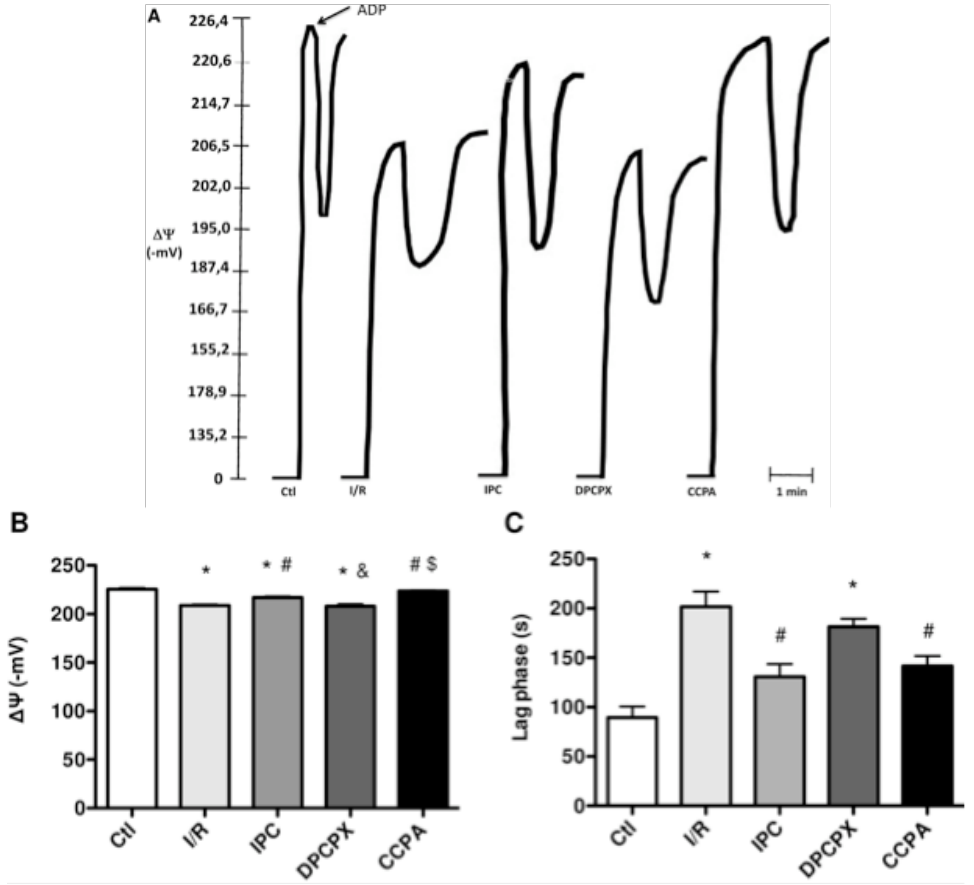



**Figure 3.1.4 The effects of PC on mitochondrial transmembrane potential (ΔΨ) in succinate-energized isolated liver mitochondria**. ΔΨ was measured with a TPP+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 3 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference ($P<0.05$) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

DPCPX also decreased the capacity to establish ΔΨ after repolarization, conversely, CCPA increased mitochondrial ΔΨ after repolarization.

The lag phase (time necessary for ADP phosphorylation) that precedes repolarization, was significantly increased by I/R comparatively to control, and preconditioning (IPC and CCPA) (Figure 3.1.4 B). This suggested that preconditioning was able to preserve the efficiency of mitochondrial phosphorylation. In the presence of DPCPX the effect of preconditioning was blocked, with the lag phase increased relatively to PC.

State 3 respiration (Figure 3.1.5 A) and the respiratory control ratio (RCR) (Figure 3.1.5 B) were significantly decreased in mitochondria isolated from livers subjected to I/R, when compared to the control. Preconditioning (IPC and CCPA) prevented I/R deleterious action on mitochondrial respiration, increasing respiratory state 3 to a value similar to the Ctl group.

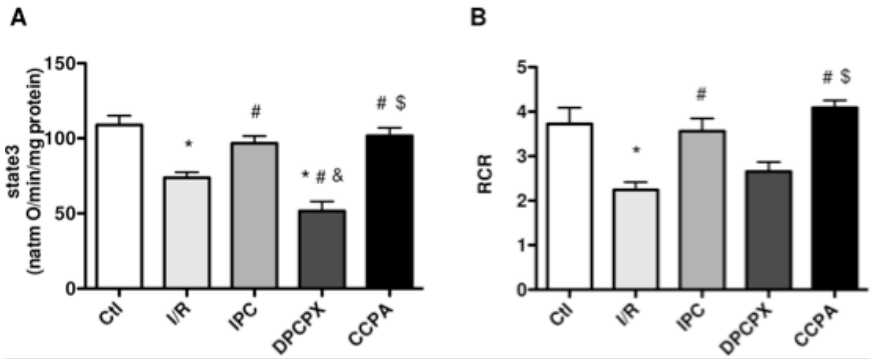



**Fig. 3.1.5 The effects of PC on state 3 (A) respiration and respiratory control ratio (RCR) (B).** Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode. Mitochondria (1mg) were suspended under constant stirring, at 25ºC, in 1.4 ml of standard respiratory medium and energized by adding succinate to a final concentration of 5mM, 2 μM rotenone were previously added. State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 μg/mg protein) and 1 μM FCCP. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference (P<0.05) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

### 3.1.2.4 Adenine nucleotides content :

To further determine whether preconditioning is able to preserve mitochondrial ATP production upon I/R, endogenous ATP content was evaluated in isolated mitochondria (Figure 3.1.6 A) , energized mitochondria (Figure 3.1.6 B) and tissue (Figure 3.1.6 C). As shown in figure, ATP content was decreased in I/R and DPCPX animals associated with the observed decreased phosphorylation efficiency. PC and CCPA administration before ischemia were able to significantly preserve mitochondrial energy level.

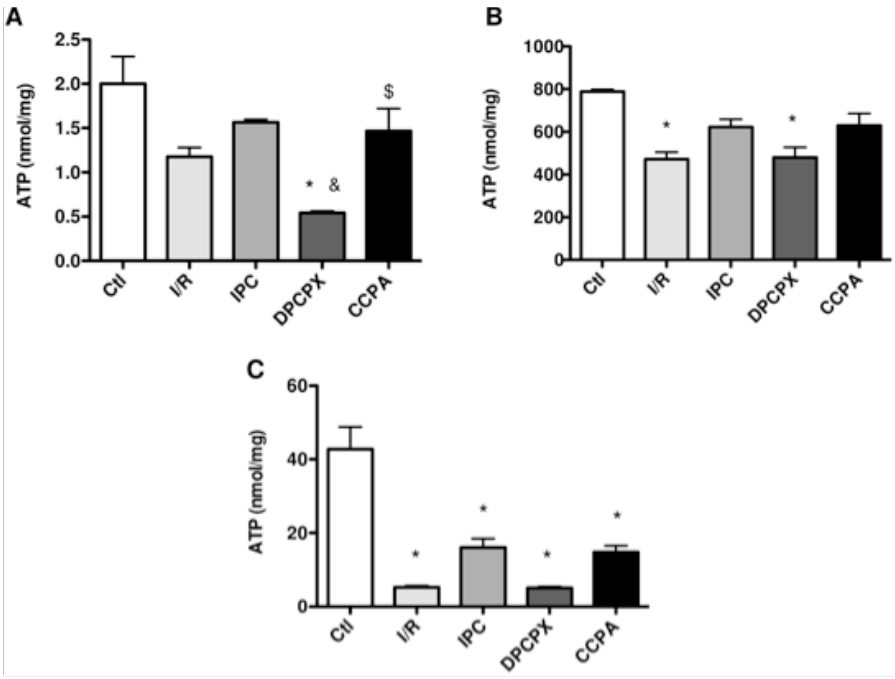



**Figure 3.1.6 The effects of PC on ATP content in isolated mitochondria (A), energized mitochondria (B) and tissue (C).** ATP content was evaluated by bioluminescent assay kit. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference ($P<0.05$) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

### 3.1.2.5 Generation of reactive oxygen species (ROS):

A pathological increase on the endogenous production of ROS after I/R has been associated with decreased capacity for cell survival. In the presence of antimycin A, an inhibitor of the electron transport chain,

mitochondria isolated from I/R livers exhibited increased ROS generation, when compared to the control group. The generation of reactive oxygen species (ROS) was decreased in mitochondria isolated from PC and CCPA livers, demonstrating that preconditioning is able to control mitochondrial ROS generation induced by I/R and thus prevent the deleterious cycle of ROS-induced ROS generation (Figure 3.1.7).

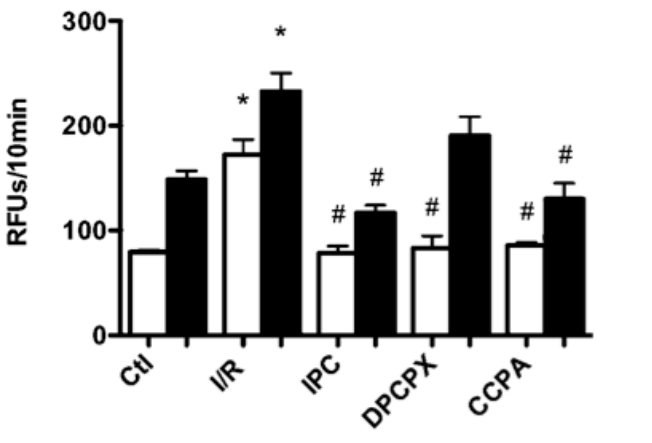
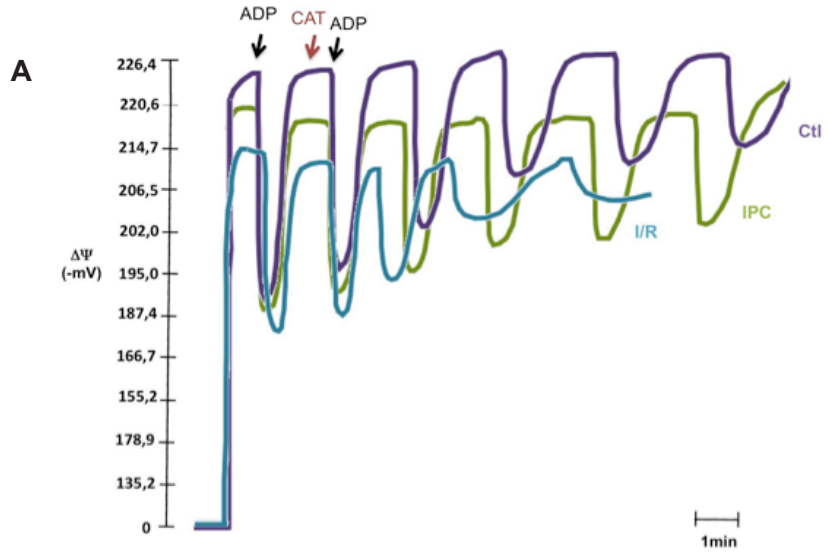


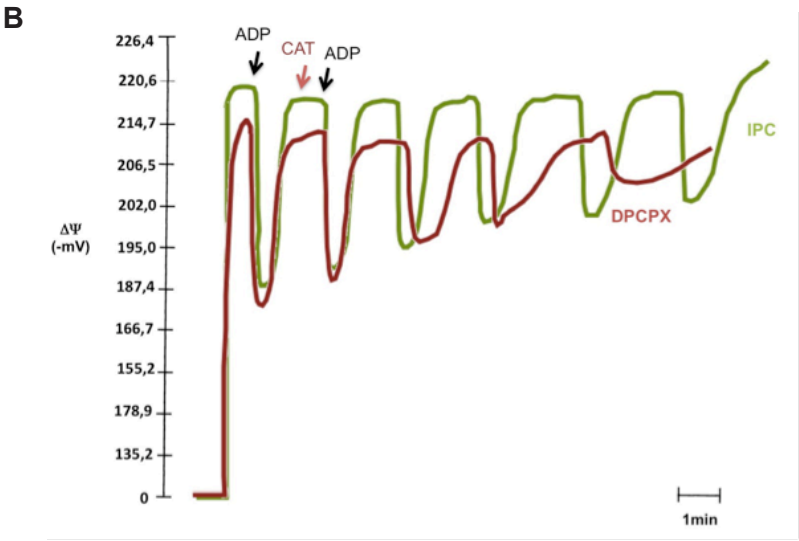

**Figure 3.1.7 The effects of PC on reactive oxygen species (ROS) generation.** ROS were estimated fluorometrically using the probe $H_2$DFDA (485 nm, 538 nm). After basal fluorescence (white) measurement, antimycin A was added to all preparation (black) to block complex III of the mitochondrial chain, to induce maximal ROS generation. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference ($P<0.05$) versus Ctl, # indicates statistically significant difference versus I/R, & indicates statistically significant difference versus IPC, $ indicates statistically significant difference versus DPCPX.

### 3.1.2.6 Interference with ADP-induced depolarization:

The adenine nucleotide translocase (ANT) facilitates the one-for-one exchange of extramitochondrial ADP for intramitochondrial ATP during oxidative phosphorylation. Assuming that ADP/ATP transport is the rate-limiting step in OXPHOS, depression of the ANT activity would impair the energy-producing capacity of the mitochondria. The observed increase in the lag phase induced by I/R could thus be related with an impaired activity of the ANT. In view of this, energized mitochondria were titrated with carboxyatractyloside (CAT). CAT is a highly selective inhibitor of cytosolic side-specific ANT that causes stabilization of the c conformation of ANT and blocks the exchange of matrix ATP and

cytoplasmic ADP. Matrix ADP is an important modulator of pore opening and acts by decreasing the sensitivity of the calcium trigger site to [$Ca^{2+}$]; therefore CAT is also a mitochondrial permeability transition (MPT) inducer. Measuring mitochondrial membrane potential after ADP addition showed that, in mitochondria subjected to I/R, less CAT was needed to completely abolish repolarization after phosphorylation (Figure 3.1.8), indicating that I/R mitochondria already had ANT inhibition and thus an impaired phosphorylative system. PC (IPC and CCPA) prevented this effect of I/R.



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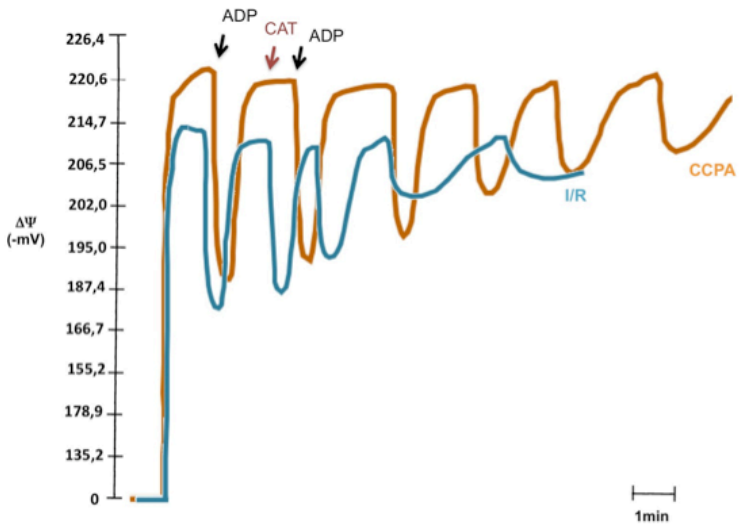



**Figure 3.1.8 Interference with ADP-induced depolarization.** ΔΨ was measured with a TPP+-selective electrode. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Energized mitochondria were titrated with carboxyatractyloside (CAT). Successive additions of CAT aliquots before ADP addition, progressively block a higher number of ANT units contributing to the observed decreased number of phosphorylation cycles induced by successive ADP additions, as well as to the observed increasing in lag phase corresponding to successive phosphorylation cycles. A) Ctl,violet; IR,blue; IPC green; B) IPC green, DPCPX,red; C) IR,blue CCPA, orange. The traces are representative of experiments performed with six independent mitochondrial preparations.

### 3.1.2.7 Mitochondrial permeability transition (MPT):

In isolated mitochondria, the ability to tolerate a calcium challenge is an indicator of the susceptibility to the MPT. Thus, as mitochondria possess a finite capacity for accumulating calcium before undergoing the MPT, calcium-induced mitochondrial swelling was evaluated.

Mitochondria isolated from I/R animals were more susceptible to undergo mitochondrial swelling induced by calcium, comparatively to control (Figure 3.1.9). In vitro pre-treatment of mitochondria isolated from I/R livers with the MPT inhibitor cyclosporine A (CyA), prevented mitochondrial swelling induced by calcium, demonstrating that the swelling was caused by MPT induction. IPC and CCPA increase the threshold to MPT induction. Acute treatment of livers, before preconditioning, with DPCPX increases

the susceptible to undergo mitochondrial swelling induced by calcium, blocking IPC protective action.

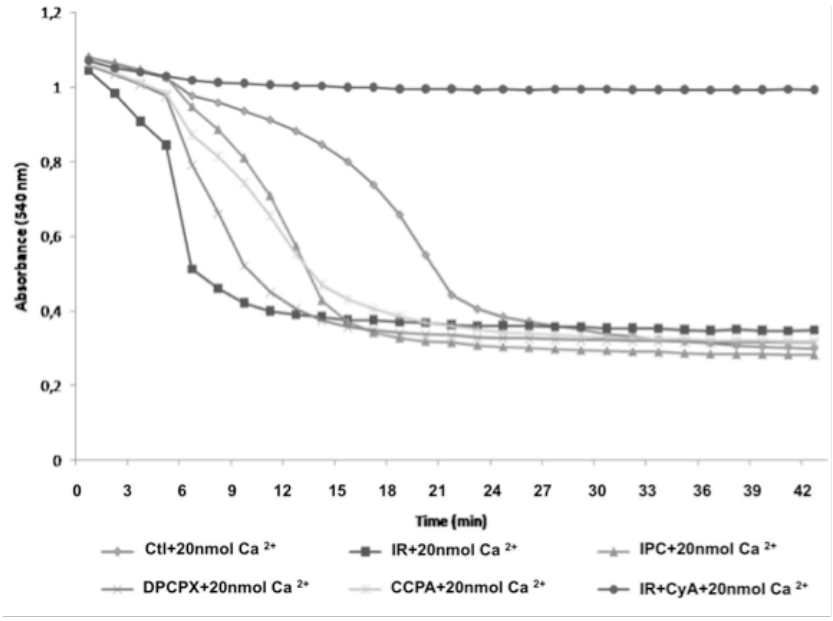



**Figure 3.1.9 The effects of PC on the susceptibility to the induction of mitochondrial permeability transition (MPT).** Mitochondrial swelling was spectrophotometrically monitored at 540 nm. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μM rotenone and 5 mM succinate. MPT was induced with 20 nmol CaCl2. Cyclosporin A (CyA )(1 μM) , was added to the reaction medium prior to calcium addition. The traces are representative of experiments performed with six independent mitochondrial preparations

### 3.1.2.8 Mitochondrial calcium flux

Since mitochondria isolated from PC livers decreased the susceptibility to calcium –induced swelling, mitochondrial calcium fluxes were evaluated.

Mitochondria isolated from control animals were able to accumulate the added calcium after energization with succinate and retained it during the entire time of the assay (Figure 3.1.10). Conversely, mitochondria isolated from animals subjected to I/R, displayed an immediate release of calcium into the media assay. Pre-treatment with CyA prevented calcium release, implying that increased calcium efflux from mitochondrial isolated from I/R animals was the result of MPT induction. PC and treatment with

CCPA before ischemia prevented mitochondrial calcium efflux after calcium challenge. On the other hand, in DPCPX mitochondria the accumulated calcium was early released, demonstrating the increased susceptibility to a calcium challenge.

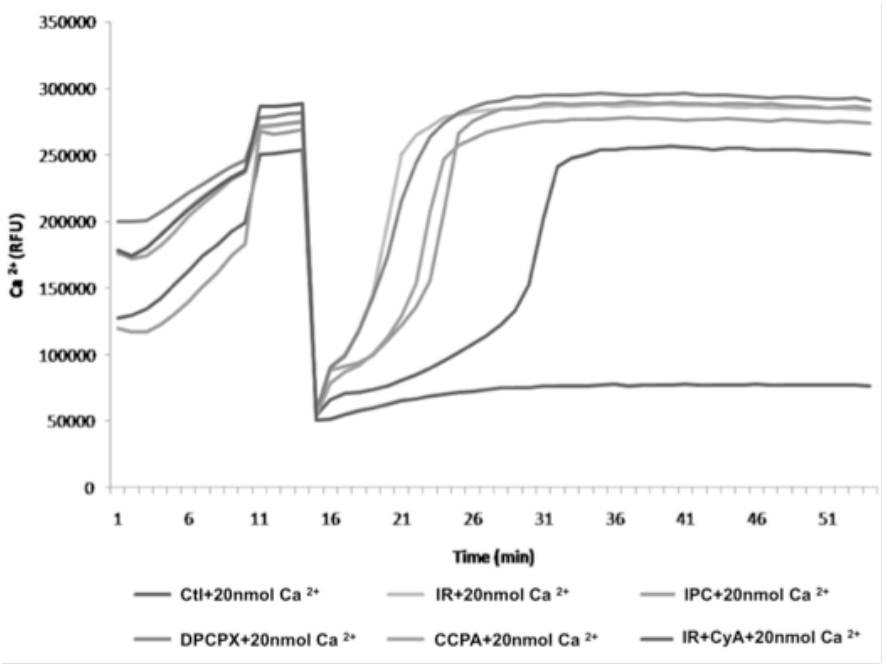



**Figure 3.1.10 The effects of PC on mitochondrial calcium fluxes.** Calcium fluxes were fluorometrically monitored using the probe Calcium Green (485 nm, 535 nm). Mitochondria (1mg) were incubated in 2 ml of standard incubation medium prior to calcium addition (20 nmol). Energization was achieved with succinate 5 mM. Calcium fluxes, expressed as relative fluorescence units (RFU). Cyclosporin A (CyA) 1μM was added to the reaction prior to calcium addition. The traces are representative of experiments performed with six independent mitochondrial preparations.

### 3.1.2.9 Phosphorylation of Akt and GSK-3β status

Activation of $A_1$ receptors has been shown to protect liver from I/R injury via pathways involving Akt activation (Park et al., 2010). Taking into account that activated Akt decreases GSK-3β activity, which is related with modulation of the MPT ,we determined if PC protective effect against hepatic I/R injury involves the activation of the Akt / GSK-3β signaling pathway to inhibit mitochondria-mediated I/R injury.

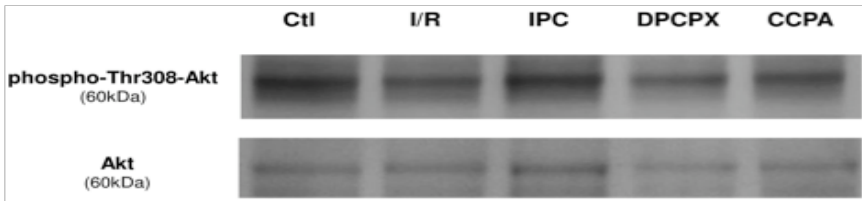



**Figure 3.1.11 Phospho-Thr308-Akt and Akt content were evaluated by western blotting.** A representative blot from six independent experiments is shown.

On western blot analysis, compared with control, I/R decreased the ratios of phospho-Thr308-Akt/Akt (Figure 3.1.11) and mitochondrial phospho-Ser9-GSK-3β /GSK-3β (Figure 3.1.12), indicating decreased Akt and increased GSK-3β activities. IPC and CCPA blocked this effect of I/R, suggesting the involvement of the Akt/ GSK-3β pathway on the improvement of mitochondrial function by IPC. Thus, PC maintains the phosphorylated inactive GSK-3β that regulates the threshold for MPT induction.

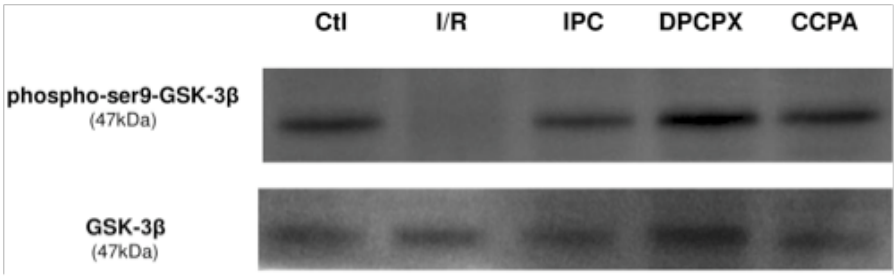



**Figure 3.1.12 Phospho-Ser9-GSK-3β and GSK-3β content in isolated mitochondria were evaluated by western blotting**. A representative blot from six independent experiments is shown.

### 3.1.3 Discussion

Accumulated evidence suggest that $A_1$ adenosine receptors ($A_1R$) play a role in the protection from I/R injury in several organs such as, heart (Koeppen et al., 2009; Yang et al., 2009 a,b), lung (Wilson et al., 2009; Schepp and Reutershan, 2008), and brain (Ilie et al., 2009; Stone et al., 2009). Since IPC has been demonstrated to preserve mitochondrial efficiency following I/R, this study examined if A1R modulation during PC induces mitochondrial adaptation and could protect the liver from I/R injury. This study demonstrated that A1R agonist (CCPA) reproduced the beneficial effects of PC, an effect abolished by the antagonist (DPCPX), indicating the importance of this receptors subtype in preconditioning. Moreover, A1R protective effects involved preservation of mitochondrial OXPHOS efficiency.

Since ATP content declines substantially during I/R, which is associated with hepatocellular injury and higher mortality, prevention of defective energy production plays an important role in the resistance to I/R injury. Associated with alterations in pH, $Na^+$, and oxidative phosphorylation, I/R triggers a cascade of events that involve increased ROS generation and loss of $Ca^{2+}$ homeostasis. This culminates in MPT induction and cell death. Therefore, modulation of mitochondria has emerged as a critical survival strategy in the prevention against I/R injury. IPC-triggered signal transduction appears to directly preserve several cell functions including intracellular energy state, pH, and redox system (Rehman et al., 2008). Our data show that preconditioning (ischemic and pharmacological) was capable to decrease plasmatic ALT, AST and LDH activities, indicating a clear hepatoprotective effect against warm I/R injury. Preconditioning (ischemic- PC and pharmacological - CCPA) reduced TNF-α concentration, while blocking $A_1R$ aggravated the release of this cytokine. The decrease in TNF-α by both types of could be associated with decreased ROS generation in IPC and CCPA conditions .

PC action was associated with increased performance of mitochondrial function, as shown by the preservation of ATP content. Oxidative phosphorylation efficiency was impaired in mitochondria isolated from I/R and DPCPX livers, but PC and administration of CCPA was able to maintain $\Delta\Psi$, state 3 respiration, RCR and the lag phase to a level compared to the control. CCPA reproduced the beneficial effects of PC and DPCPX suppress them. The increased lag phase may be caused by disruption of mitochondrial ATP/ADP exchange mediated by the ANT. The drop in cytosolic ATP levels caused by impaired mitochondrial ATP/

ADP exchange could stimulate metabolic pathways related to cytosolic acidification in the aerobic cell and initiate apoptosis in these cells (Vander Heiden et al., 1999). The involvement of the ANT in the inhibition of mitochondrial phosphorylation was supported by the fact that less CAT was needed to abolish ADP-induced depolarization, indicating that the ANT was rate-limiting phosphorylation in I/R conditions and implying that I/R was enhancing the "c" conformation of the ANT. In energized mitochondria, stabilization of the "c" conformation of the ANT (as it is by CAT) sensitizes the MPT to calcium. Also, oxidative stress activates the MPT by inhibiting ADP binding to the ANT and enhancing CypD-ANT interaction, decreasing the sensitivity of the calcium trigger site to [$Ca^{2+}$]. Thus, inhibition of ATP/ADP translocation sensitizes mitochondria to MPT.

Mitochondria isolated from I/R animals were highly susceptible to undergo mitochondrial swelling induced by calcium, but PC (ischemic and pharmacological) was able to restore the capacity of mitochondria to accumulate calcium, without inducing the MPT. The population undergoing the MPT was increased in mitochondria from I/R livers, as demonstrated by the inhibition of mitochondrial swelling and calcium efflux in the presence of CyA. The MPT leads to decreased mitochondrial membrane potential, causing failure of oxidative phosphorylation and necrotic cell death. The MPT also causes release of cytochrome c from mitochondria, triggering apoptosis. By preventing MPT induction, PC prevented caspase-3 activation after warm hepatic I/R. This was also associated with increased Bcl-2 content, which was not abolished by A1R antagonist, although induced by A1R agonist. This may be caused by increased HSP70 levels induced by IPC (not prevented by DPCPX) but not by pharmacological preconditioning. HSP70 has multiple anti-apoptotic mechanisms that involve mitochondrial protection; it functions both upstream and downstream from the caspase cascades and in a caspase-independent manner via direct association with apoptosis-inducing factor (AIF) (Nylandsted et al., 2000; Garrido et al., 2003; Lee et al., 2005).

HSP70 upregulates Bcl-2 and interferes with apaf-1 function, thereby preventing the formation of the apoptosome, and also block Bax translocation to the mitochondria (Stankiewicz et al., 2005). In fact, up-regulation of HSP's has been associated with maintenance of mitochondrial integrity and function during I/R. Early on, hypothermic adaptation in a perfused rabbit heart model has been shown to involve preservation of HSP response and signaling for mitochondrial biogenesis (Ning et al., 1998). Deletion of HSP70 exacerbates I/R-induced cardiac apoptosis, mediated by AIF activation (Choudhury et al., 2011). In rat neonatal

myocytes, overexpression of HSP60 and HSP10 protect cells against apoptosis induced by I/R (Lin et al., 2011). Overexpression of HSP70 is also associated with a reduction of cytochrome c release from the mitochondria in focal ischemia (Tsuchiya et al., 2003), and postconditioning increases the level of HSP70 in the cortex during cerebral I/R (Stroke et al., 2008). Also up-regulation of HSP's facilitates protein import into mitochondria, such as mitochondrial Mn-SOD, decreasing ROS production and preventing mitochondrial dysfunction (Rehman et al., 2008). Recently, Yang and colleagues showed that activation of adenosine receptors induces translocation of PKCε to the mitochondria, and this process is regulated by HSP90-dependent mitochondrial import mechanism (Yang et al., 2012).
Although HSP´s overexpression block MP pore conductance due to HSP binding to protein aggregates, formed by aggregation of damaged, misfolded integral membrane proteins in association with CypD under MPT-inducing conditions (He et al., 2002; He et al., 2003). Modulation of MPT sensitivity by IPC may also be explained by modulation of CypD-ANT binding via Akt/GSK-3β pathway. It has been proposed that IPC increases the levels of mitochondrial phospho-Ser9-GSK-3β and that phospho-Ser9-GSK-3β physically interacts with the ANT, which inhibits interaction of the ANT with CypD (Nishiara et al., 2007) .This may alter mitochondrial function in a way that makes the MPT less likely to form, under oxidant stress and high calcium concentrations, conditions that are associated with I/R. Both IPC and CCPA increase the levels of active Akt and phospho-Ser9-GSK-3β, which may modulate MPT induction.

In conclusion, this study demonstrates that $A_1$R agonist CCPA reproduced the beneficial effects of PC, an effect abolished by the antagonist DPCPX. This involves preservation of OXPHOs efficiency and increase resistance to MPT onset. Both IPC and pharmacological preconditioning may modulate the MPT in a GSK-3β-dependent manner. The increase in HSP70 induced by IPC may further protect mitochondria and prevent the MPT, thus maintaining restoring the ability to generate ATP and recover from I/R insult.

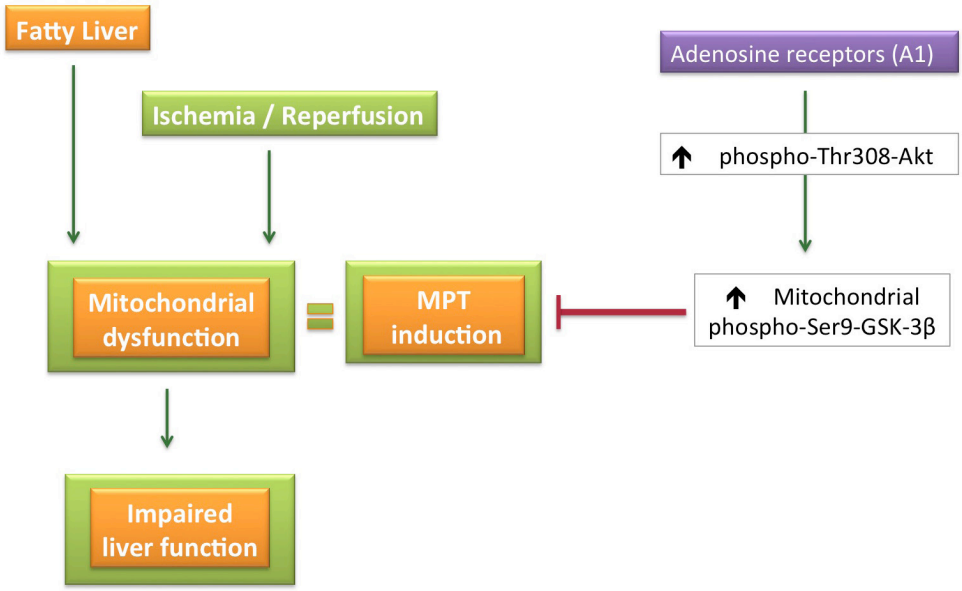



**Figure 3.1.13 – Mytochondrial dysfunction increases the susceptibility of fatty livers to I/R injury.** Preconditioning via modulation of adenosine receptores prevents MPT induction.

# 3.2 RESULTS: INDIRUBIN-3'-OXIME PREVENTS I/R DAMAGE

## INDIRUBIN-3'-OXIME PREVENTS I/R DAMAGE BY INHIBITING GSK-3β AND MITOCHONDRIAL PERMEABILITY TRANSITION


### Abstract

Higher susceptibility to the mitochondrial permeability transition and decreased phosphorylative capacity are causes for impaired hepatic function in fatty livers exposed to ischemia/reperfusion I/R. Thus, preserving mitochondrial function increases the success rate following surgery of fatty livers. In view of this, we tested if an acute treatment with indirubin-3'-oxime, an inhibitor of glycogen synthase kinase 3β, prevents mitochondrial dysfunction caused by I/R in lean and fatty livers. For that we investigated the regulation of MPT by GSK-3β and its relation to mitochondrial damage.

Wistar rats were subjected to 150 min and Zucker fatty rats were subjected to 120 min of 70% warm ischemia and 16 and 12 hrs of reperfusion (I/R) respectively. In the treated group (Ind) 1 μM indirubin-3'-oxime was administered in the hepatic artery 30 min before ischemia. Acute treatment of livers before ischemia, with indirubin-3'-oxime, decreased serum markers of injury and also preserved mitochondrial cytochrome c content, comparatively to I/R livers. Indirubin-3'-oxime also protected mitochondria from calcium-induced MPT, prevented the impairment in oxidative phosphorylation as well as decreased the formation of ROS.

Evaluation of GSK-3β status in both liver homogenates and isolated mitochondria, showed that treatment with indirubin-3′-oxime preserves the phosphorylation of GSK-3β at Ser9 which prevents the increase in phosphorylated and active CypD, induced by I/R. This in turn, was associated with suppression of the ANT-CypD interaction, therefore increasing the threshold for MPT induction and preserving cytochrome c content, preserving fatty liver from I/R damage. These findings indicate that, although fatty livers exhibit increased susceptibility to mitochondrial damage, indirubin-3′-oxime could protect fatty livers against injury after I/R.

### 3.2.1 Introduction

Accumulation of fat in the liver increases the risk of damage following I/R, thus bearing additional risks of primary nonfunction subsequent to liver surgery or transplantation (Fukumori et al., 1997; Selzner et al., 2001). The increased incidence of this pathology demands new and effective strategies that preserve fatty liver function when subjected to I/R.

Different hypothesis, have been proposed for the increased susceptibility of fatty organs to ischemic injury (Selzner et al., 2000; Teramoto et al., 1993; Selzner et al., 2006). Under I/R conditions fatty livers are ATP-depleted, and the predominant hepatocellular fate is shifted from apoptosis to necrosis, strongly implicating inappropriate energy homeostasis as the primary cause of liver sensitivity (Caraceni et al., 2005; Vendemiale et al., 2001). Fatty degeneration, which induces a series of alterations in mitochondria, renders these organelles intrinsically more susceptible to I/R injury (Caraceni et al., 2005; Vendemiale et al., 2001; Honda et al., 2005; Rolo et al., 2009).

Impairment of oxidative phosphorylation and induction of the mitochondrial permeability transition (MPT) are critical determinants for mitochondrial dysfunction induced by prolonged periods of ischemia followed by reperfusion (Halestrap et al., 2004; Juhaszova et al., 2008). Inhibition of the MPT by preconditioning and postconditioning has been proposed to mediate the protective effect of these interventions against I/R injury, highlighting the significance of this mitochondrial event as a therapeutic target (Rolo et al., 2009; Varela et al., 2010; Park et al., 2006; Hausenloy et al., 2004; Murphy et al., 2007).

Cyclophilin D (CypD) plays a critical role in MPT opening and consequent cell death. It is predominantly localized in the mitochondrial matrix and when it binds to the adenine nucleotide translocator (ANT) increases calcium sensitivity, promoting mitochondrial dysfunction (Zorov et al., 2009). Xi et al suggested that reperfusion injury may be prevented by targeting the MPT through translocation of glycogen synthase kinase 3β (GSK-3β) from cytosol to mitochondria, which may ultimately interact with CypD thus modulating the MPT (Xi et al., 2009). Studies have shown that GSK-3β inhibition by phosphorylation plays a central role in the beneficial action of ischemic and pharmacological preconditioning, involving modulation of the MPT (Varela et al., 2010; Juhaszova et al 2004; Nishihara et al., 2007; Miura et al., 2009). With GSK-3β inhibited, the GSK3β-mediated phosphorylation of CypD is prevented and the consequent MPT, assuring normalization of intracellular ATP content (Zorov et al., 2009). In

situations of I/R, GSK3β promotes the systemic inflammatory response, increases the proinflammatory release of cytokines and induces apoptosis (Ren et al., 2011).

Indirubin 3'-oxime (Ind), a chemical agent of the indirubin family, inhibits GSK-3β by competing with ATP for binding to the catalytic site (Zorov et al., 2009). A previous work by Barillas and co-workers has shown that reperfusion of isolated hypertrophied hearts with lithium or indirubin-3'-monoxime-5' iodo prevents the decrease in contractile function induced by I/R (Xi et al., 2009). While inhibition of GSK-3β by indirubin-3'-monoxime,5-iodo has been shown to protect the heart from I/R injury, it has not been examined if this cardioprotection involves increased tolerance to MPT induction. In addition, it has not been yet evaluated if GSK-3β inhibition by phosphorylation-independent mechanisms results in adaptation of the liver to withstand I/R injury. It should be taken into consideration that incubation of isolated rat liver with indirubin 3'-oxime prevents MPT induction but impairs oxidative phosphorylation, therefore decreasing the ability to generate ATP (Jope et al., 2004).

In this study, we propose to evaluate the effects of indirubin-3'-oxime given before ischemia on the mitochondrial function of livers subjected to warm ischemia/reperfusion. We plan to address if indirubin-3'-oxime protects the liver by increasing the threshold for MPT induction through inhibition of GSK-3β.

### 3.2.2 Results

#### 3.2.2.1 Indirubin-3'-oxime prevents hepatic I/R damage in lean livers

##### 3.2.2.1.1 Plasma markers of liver injury:

To determine if indirubin-3'-oxime given before ischemia protects liver from I/R injury, serum levels of AST and ALT were evaluated. As shown in Figure 3.2.1, warm I/R significantly increased plasma AST and ALT levels relative to the control group. Administration of indirubin-3 -oxime (Ind before ischemia (30 min), was effective in reducing the increase in plasma levels of AST and ALT caused by I/R.

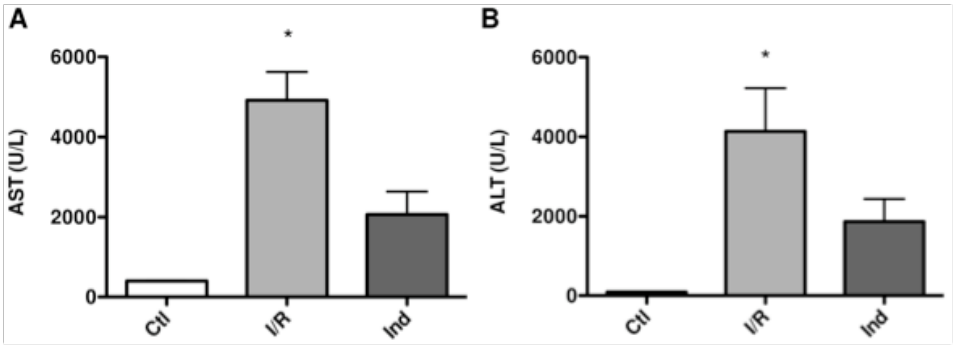



**Figure 3.2.1 The effects of indirubin-3'-oxime (Ind) administered before ischemia on plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)**. Plasma samples were collected and enzimatic determinations of ALT (A) and AST (B) performed using commercial kits. Data are means ± SEM of experiments performed with six animals/group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

### 3.2.2.1.2 Mitochondrial membrane potential (ΔΨ) and oxygen consumption:

Taking into account the fundamental role of mitochondrial membrane potential for the phenomenon of oxidative phosphorylation, ΔΨ was evaluated in succinate-energized mitochondria. Although not statistically significant, mitochondria isolated from livers subjected to I/R exhibited decreased mitochondrial membrane potential, when compared to mitochondria from both control and Ind animals (Figure 3.2.2 A). The lag phase (time necessary for ADP phosphorylation) was significantly increased in animals subjected to I/R, comparatively to control (Figure 3.2.2 B). Treatment with Ind before ischemia markedly reduced the lag phase to a level compared with control animals suggesting that Ind administration is able to preserve the efficiency of mitochondrial phosphorylation.

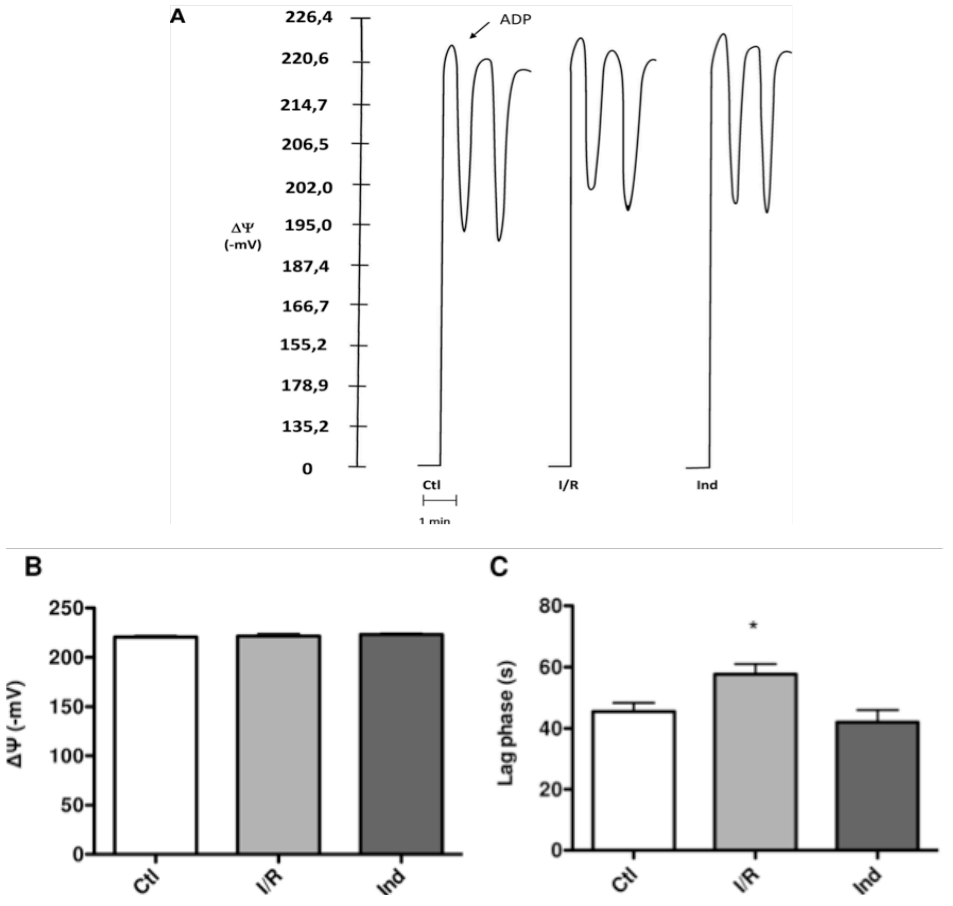



**Figure 3.2.2 The effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial membrane potential (A,B) and lag phase (A,C).** ΔΨ was measured with a TPP+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 3 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data are means ± SEM of experiments performed with six animals/ group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

Mitochondrial state 3 respiration (ADP-induced oxygen consumption) and the respiratory control ratio (RCR, ratio between mitochondrial state 3 and rate of oxygen consumption after ADP

phosphorylation) were significantly decreased in mitochondria isolated from livers subjected to I/R, when compared to mitochondria from both control and Ind-treated mitochondria (Figure 3.2.3 A and B). Uncoupled respiration (maximal rate of oxygen consumption induced by FCCP) was identical between the three experimental groups.

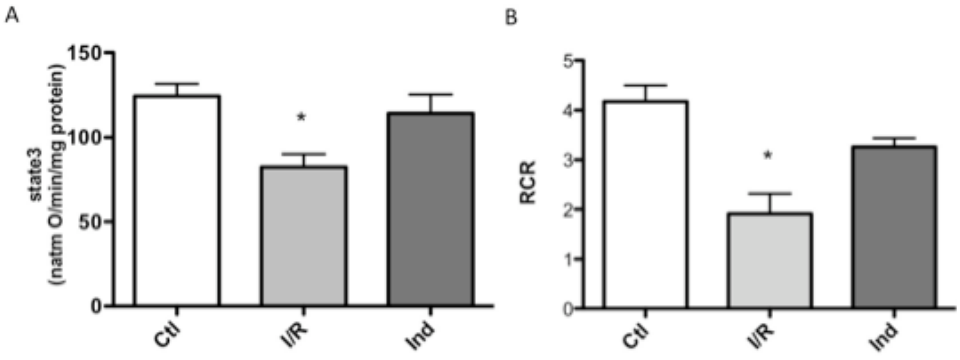



**Figure 3.2.3 The effects of indirubin-3'-oxime (Ind) administered before ischemia on state 3 (A) respiration and respiratory control ratio (RCR) (B).** Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode. Mitochondria (1mg) were suspended under constant stirring, at 25ºC, in 1.4 mlof standard respiratory medium and energized by adding succinate to a final concentration of 5mM, 2 µM rotenone were previously added. State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 µg/mg protein) and 1 µM FCCP. Data are means ± SEM of experiments performed with six animals/group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

### 3.2.2.1.3 Adenine nucleotides content:

To further determine whether indirubin-3-oxime treatment is able to preserve mitochondrial ATP production upon I/R, endogenous ATP content was evaluated in isolated mitochondria. As shown in Figure 3.2.4, ATP content was decreased in I/R animals associated with the observed decreased phosphorylation efficiency. Ind administration before ischemia was able to significantly preserve mitochondrial energy level.

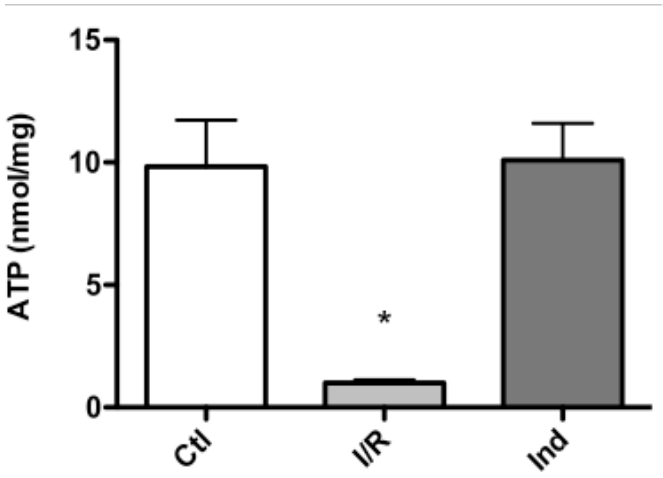



**Figure 3.2.4- The effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial endogenous ATP content**. ATP content was determined by HPLC. Data are means ± SEM of experiments performed with six animals/group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

### 3.2.2.1.4 Generation of reactive oxygen species (ROS):

A pathological increase on the endogenous production of ROS after I/R has been associated with decreased capacity for cell survival. In the presence of antimycin A, an inhibitor of the electron transport chain, succinate-energized mitochondria isolated from I/R livers exhibited increased ROS generation, when compared to the control group (Figure 3.2.5).

As shown in the figure, mitochondria isolated from the livers treated with Ind revealed decreased ROS generation, indicating that Ind is able to control mitochondrial ROS generation induced by I/R and thus prevent the deleterious cycle of ROS-induced ROS generation.

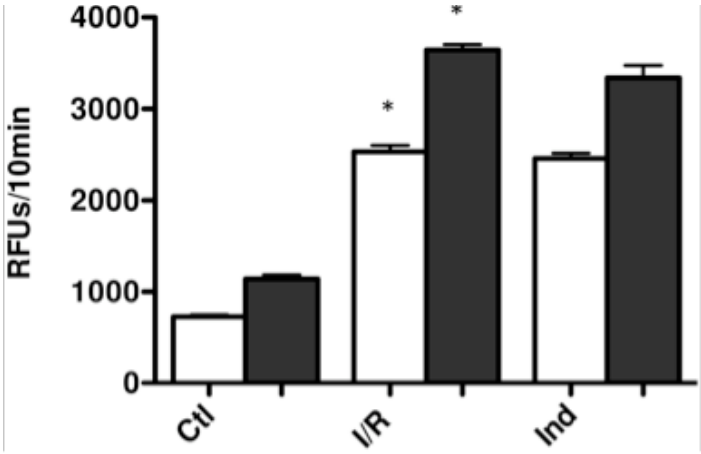



**Figure 3.2.5 The effects of indirubin-3'-oxime (Ind) administered before ischemia on reactive oxygen species (ROS) generation.** ROS were estimated fluorometrically using the probe $H_2$DFDA (485 nm, 538 nm). After basal fluorescence (white) measurement, antimycin A was added to all preparation (black) to block complex III of the mitochondrial chain, to induce maximal ROS generation. Data are means ± S.E.M of experiments performed with six animals/group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

### 3.2.2.1.5 Mitochondrial permeability transition:

Since induction of the mitochondrial permeability transition is a critical determinant of I/R injury, we evaluated whether treatment of livers submitted to I/R with indirubin-3-oxime protects from MPT induction. In isolated mitochondria, the ability to tolerate a calcium challenge is an indicator of the susceptibility to the MPT since mitochondria possess a finite capacity for accumulating calcium before undergoing the MPT. Calcium-induced mitochondrial swelling was measured as a decrease in absorbance at 540 nm. Prior to calcium addition, mitochondria isolated from I/R animals exhibited decreased absorbance at 540 nm compared with control and Ind, indicating swelling of the mitochondrial population (Figure 3.2.6). Additionally, mitochondria isolated from I/R animals were more susceptible to undergo mitochondrial swelling induced by calcium, comparatively to both control and Ind treatment. In fact, Ind restored the

capacity of mitochondria isolated from livers subjected to I/R to accumulate calcium, without inducing the MPT. In vitro pre-treatment of mitochondria isolated from I/R livers with the MPT inhibitor cyclosporine A (CyA), prevented mitochondrial swelling induced by calcium, demonstrating that the swelling was caused by MPT induction.

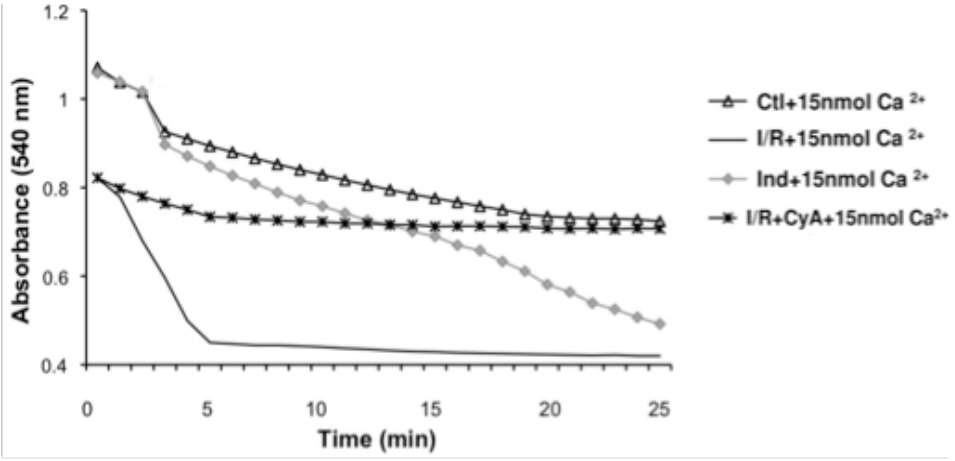



**Figure 3.2.6 - The effects of indirubin-3'-oxime (Ind) administered before ischemia on the susceptibility to the induction of mitochondrial permeability transition (MPT)**. Mitochondrial swelling was spectrophotometrically monitored at 540 nm. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μM rotenone and 5 mM succinate. MPT was induced with 20 nmol CaCl2. Cyclosporin A CyA (1 μM) was added to the reaction medium prior to calcium addition. The traces are representative of experiments performed with six independent mitochondrial preparations.

### 3.2.2.1.6 Mitochondrial calcium fluxes:

Since mitochondria isolated from I/R livers pre-treated with indirubin-3-oxime decreased the susceptibility to calcium-induced swelling, mitochondrial calcium fluxes were evaluated. Mitochondria isolated from control animals were able to accumulate the added calcium after energization with succinate and retained it during the entire time

of the assay (Figure 3.2.7). Conversely, mitochondria isolated from animals subjected to I/R, displayed an immediate release of calcium into the media assay before the calcium challenge. This indicated that I/R caused a disturbance on mitochondrial calcium homeostasis leading to increased calcium efflux. Since succinate addition lead to a decrease in fluorescence, mitochondria isolated from I/R livers showed no alterations in the ability for calcium uptake from the media. However, the accumulated calcium was early released, demonstrating the increased susceptibility to a calcium challenge. Pre-treatment with CyA prevented calcium release, implying that increased calcium efflux from mitochondrial isolated from I/R animals was the result of MPT induction. Treatment with indirubin-3-oxime before ischemia prevented mitochondrial calcium efflux before and after a calcium challenge.

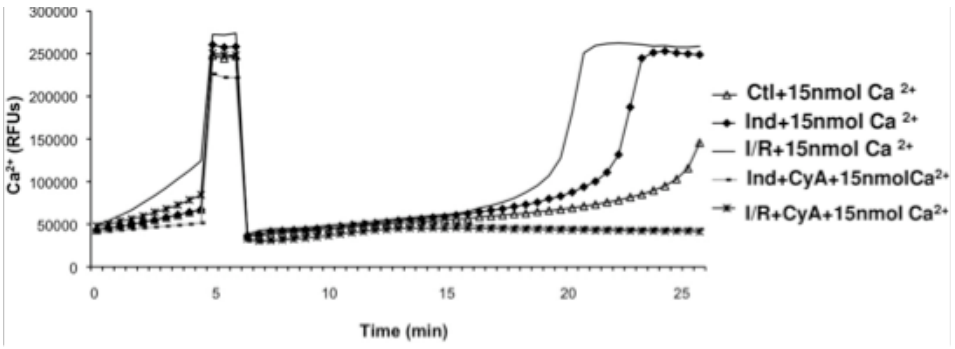



**Figure 3.2.7 The effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial calcium fluxes.** Calcium fluxes were fluorometrically monitored using the probe Calcium Green (485 nm, 535 nm). Mitochondria (1mg) were incubated in 2 ml of standard incubation medium prior to calcium addition (20 nmol). Energization was achieved with succinate 5 mM. Calcium fluxes, expressed as relative fluorescence units (RFU). Cyclosporin A (CyA) 1μM was added to the reaction prior to calcium addition. The traces are representative of experiments performed with six independent mitochondrial preparations.

### 3.2.2.1.7 Cytochrome *c* and Bcl-2 content and Bax translocation:

Because treatment with indirubin-3'-oxime prevented the increased calcium efflux observed in mitochondria isolated from animals subjected to hepatic I/R, endogenous cytochrome c content was evaluated. As shown in Figure 3.2.8, mitochondria from livers treated with Ind exhibited preserved

cytochrome c content to a level compared to the control. However, in the I/R group there was a significant decrease in mitochondrial cytochrome *c*. Since cytochrome *c* release from the mitochondria is an important downstream occurrence following Bax translocation to the mitochondria, mitochondrial Bax content was evaluated. In this model, Bax translocation to the mitochondria occurs during I/R and is prevented by Ind treatment. Additionally, due to the survival function of Bcl-2, by preventing apoptosis induction, we also evaluated Bcl-2 content. Mitochondria from livers treated with Ind before I/R showed a higher content in Bcl-2 in contrast to mitochondria from I/R animals.

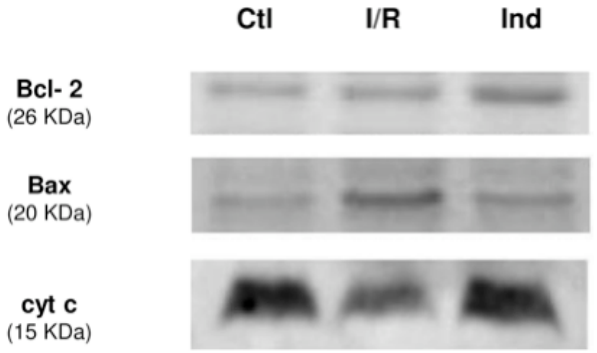



**Figure 3.2.8 Bcl-2, Bax and Cyt *c* content were evaluated by western blotting**. A representative blot from six independent experiments is shown.

### 3.2.2.1.8 GSK-3β content and activity:

Since indirubin-3-oxime is an inhibitor of GSK-3β, we evaluated if administration of Ind before ischemia was associated with decreased hepatic activity of GSK-3β after an episode of warm I/ R. As shown in Figure 3.2.9, GSK-3β activity was increased in livers from animals subjected to I/R but treatment with Ind inhibited the activity of this kinase.

Accordingly, in I/R animals, there was a decrease in the content of GSK-3β phosphorylation at Ser9, as evaluated by western blotting in hepatic tissue homogenates (Figure 3.2.10 A).

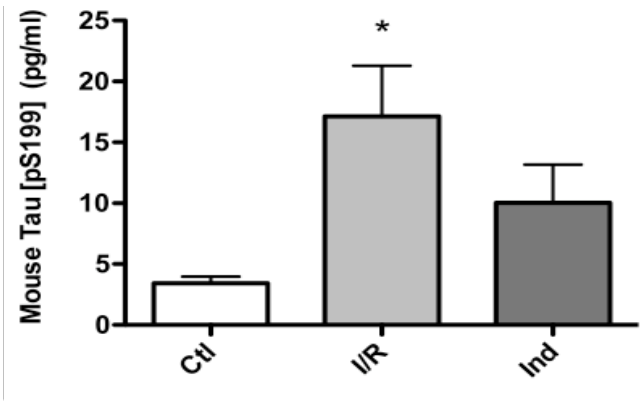



**Figure 3.2.9 The effects of indirubin-30-oxime (Ind) administered before ischemia on GSK-3β activity on liver homogenates.** Tau phosphorylation by GSK-3β was detected using a Tau(pS199) phosphoELISA kit. Data are means ± SEM of experiments performed with six animals/group. * Indicates statistically significant difference versus Ctl and Ind groups ($P < 0.05$).

Treatment with Ind before ischemia, maintained the content of phosphor-Ser9-GSK-3β to a level compared to the control. Because Ind was able to prevent increased mitochondrial calcium efflux and decreased cytochrome c content induced by I/R, and the threshold for MPT induction in myocardial I/R injury has been shown to be elevated by increased phosphorylation of GSK-3β at Ser9, we evaluated mitochondrial GSK-3β content.

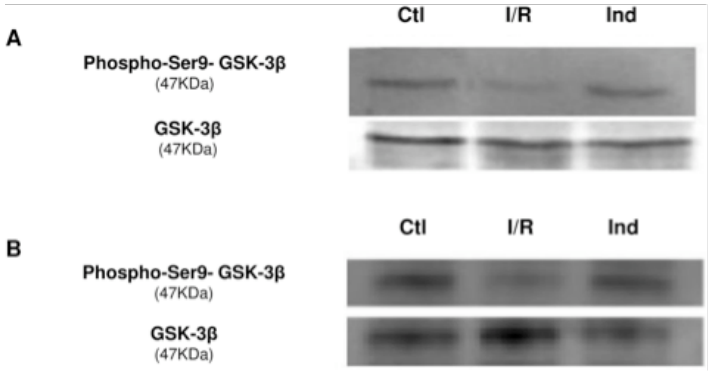



**Figure 3.2.10 Phospho-Ser9-GSK-3β and GSK-3β content in liver homogenates (A) and isolated mitochondria (B) were evaluated by western blotting**. A representative blot from six independent experiments is shown.

I/R induced the translocation of GSK-3β to the mitochondria and a reduction in the content of phospho-Ser9-GSK-3β (Figure 3.2.10 B). GSK-3β inhibition by Ind was able to maintain the mitochondrial con- tent of total GSK-3β and phospho-Ser9- GSK-3β at to a level compared to the control, indicating that Ind treatment maintains the phosphorylated GSK-3β that regulates the threshold for MPT induction.

This work shown that indirubin 3'-oxime prevents mitochondrial dysfunction in lean livers subjected to warm I/R. Since fatty livers exhibit mitochondrial dysfunction before I/R, with this study we propose to evaluate if pre-treatment with indirubin 3'-oxime in conditions of hepatic I/R in fatty livers, protects the liver by maintaining mitochondrial calcium homeostasis, thus preserving mitochondrial function and hepatic energetic balance.

### 3.2.2.2 Indirubin-3'-oxime prevents hepatic I/R damage in fatty livers

#### 3.2.2.2.1 Plasma markers of liver injury:

To determine if indirubin-3'-oxime given before ischemia protects liver from I/R injury, serum levels of AST and ALT were evaluated. As shown in Figure 3.2.11, warm I/R significantly increased plasma AST and ALT levels relative to the control group. Ind administration before ischemia was effective in reducing plasma levels of AST and ALT.

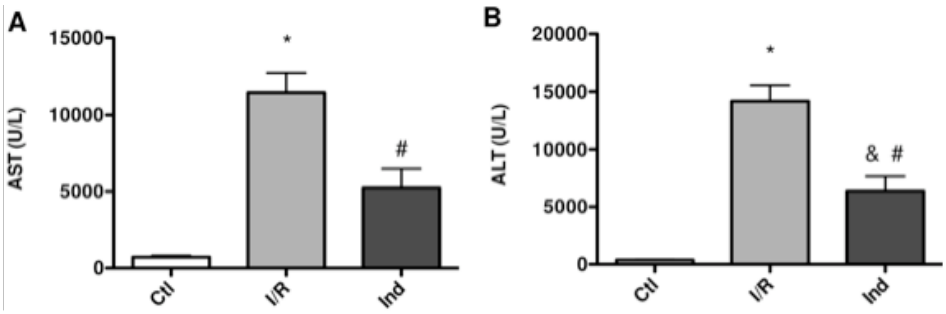



**Figure 3.2.11 Fatty livers and effects of indirubin-3'-oxime (Ind) administered before ischemia on plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)**. Plasma samples were collected and enzimatic determinations performed using commercial kits. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind.

### 3.2.2.2.2 Mitochondrial membrane potential (ΨΔ) and oxygen consumption:

Mitochondria isolated from livers subjected to I/R exhibited decreased mitochondrial membrane potential, when compared to mitochondria from both control and Ind animals (Figure 3.2.12 A).

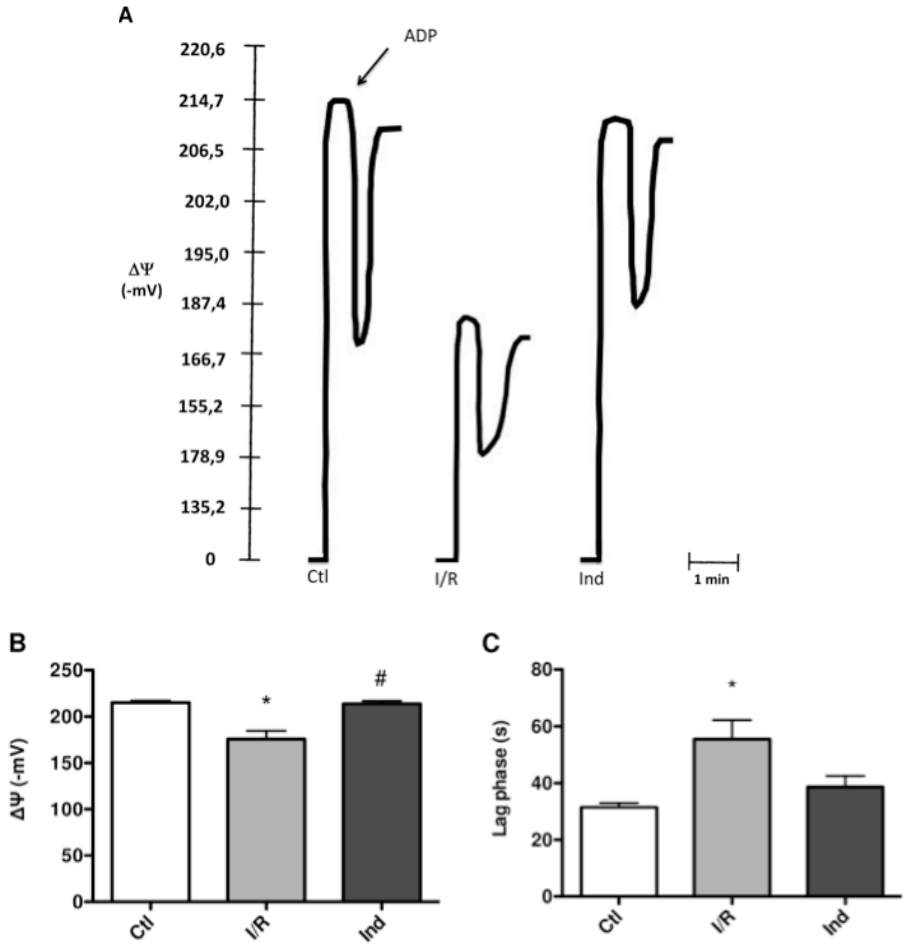



**Figure 3.2.12 Fatty livers and the effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial membrane potential (A,B) and lag phase (A,C)**. ΔΨ was measured with a TPP+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 3 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind.

The lag phase (time necessary for ADP phosphorylation) was significantly increased in animals subjected to I/R, comparatively to control (Figure 3.2.12 B). Treatment with Ind before ischemia markedly reduced the lag phase to a level similar to control animals, suggesting that Ind administration is able to preserve the efficiency of mitochondrial phosphorylation.

Mitochondrial state 3 respiration and the respiratory control ratio (RCR) were decreased in mitochondria isolated from livers subjected to I/R, when compared to mitochondria from both control and Ind-treated mitochondria (Figures 3.2.13 A and B). Uncoupled respiration was identical between the three experimental groups. This suggests that Ind treatment preserves the electrochemical gradient necessary for ATP synthesis but has no effect on the rate of electron flux through the electron transport chain.

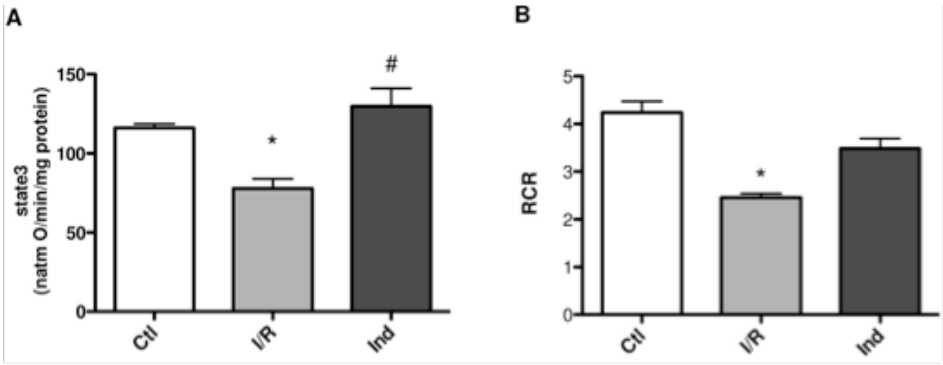



**Figure 3.2.13 Fatty livers and effects of indirubin-3'-oxime (Ind) administered before ischemia on state 3 (A) respiration and respiratory control ratio (RCR) (B)**. Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode. Mitochondria (1mg) were suspended under constant stirring, at 25ºC, in 1.4 ml of standard respiratory medium and energized by adding succinate to a final concentration of 5mM, 2 µM rotenone were previously added. State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 µg/mg protein) and 1 µM FCCP. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind.

### 3.2.2.2.3 ATPase activity

The decreased performance of phosphorylation in I/R fatty livers, as reflected by an increased lag phase, suggested alterations in the F1F0-ATPsynthase, a key component of the phosphorylative system.
ATPase activity was decreased in mitochondria from I/R animals, comparatively to control. Ind preserved ATPase activity similarly to the observed in control group (Figure 3.2.14).

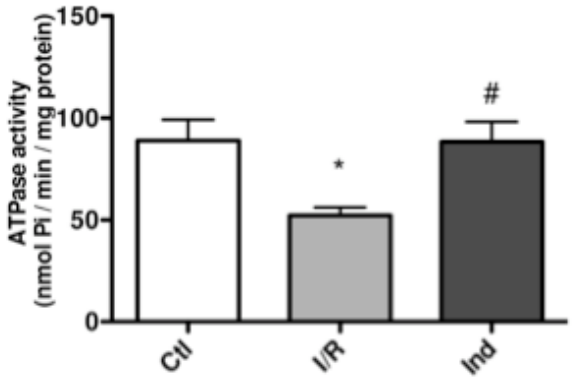



**Figure 3.2.14 Fatty livers an the effects of indirubin-3'-oxime (Ind) administered before ischemia on ATPase activity.** ATPase activity was determined spectrophotometrically at 660 nm and was calculated as the difference in total activity and activity in the presence of oligomycin. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind.

### 3.2.2.2.4 Adenine nucleotides content:

To further determine whether Ind treatment is able to preserve mitochondrial ATP production upon I/R, endogenous ATP content was evaluated in isolated mitochondria.

As shown in Figure 3.2.15 , ATP content was decreased in I/R animals associated with the observed decreased phosphorylation efficiency. Ind administration before ischemia was able to significantly preserve mitochondrial energy level.

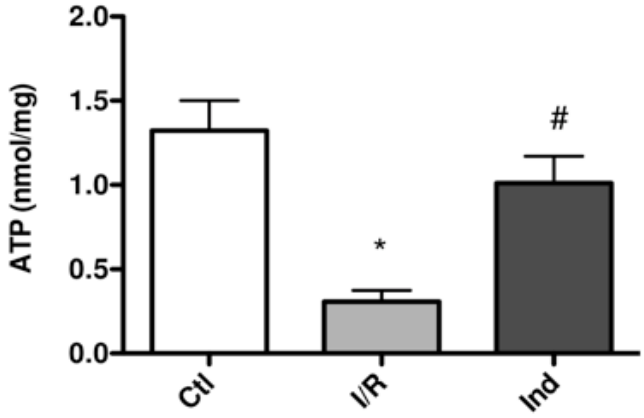



**Figure 3.2.15 The fatty livers and the effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial endogenous ATP content.** ATP content was evaluated by a bioluminescent assay kit. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind .

### 3.2.2.2.5 Generation of reactive oxygen species (ROS)

In the presence of antimycin A, mitochondria isolated from I/R livers exhibited increased ROS generation, when compared to the control group (Figure 3.2.16). Mitochondria isolated from the livers treated with Ind revealed decreased ROS generation, indicating that Ind is able to control mitochondrial ROS generation induced by I/R and thus prevent the deleterious cycle of ROS-induced ROS generation.

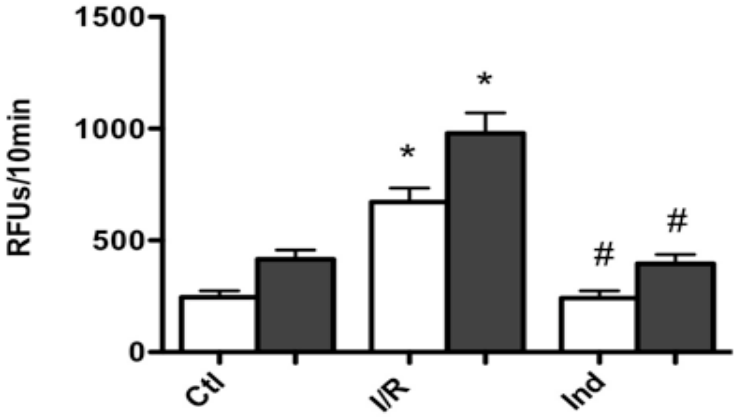


**Figure 3.2.16 The effects of indirubin-3'-oxime (Ind) administered before ischemia in fatty livers on reactive oxygen species (ROS) generation.** ROS were estimated fluorometrically using the probe CM-H2DFDA (485 nm, 538 nm). After basal fluorescence (white) measurement, antimycin A was added to all preparation (black) to block complex III of the mitochondrial chain, to induce maximal ROS generation. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind.

### 3.2.2.2.6 Mitochondrial permeability transition

In isolated mitochondria, the ability to tolerate a calcium challenge is an indicator of the susceptibility to the MPT, since mitochondria possess a finite capacity for accumulating calcium before undergoing the MPT.

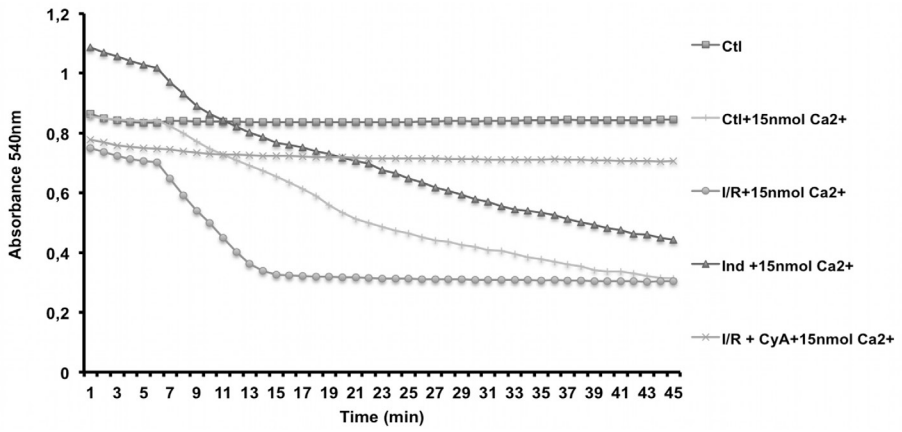



**Figure 3.2.17 Fatty livers and the effects of indirubin-3'-oxime (Ind) administered before ischemia in fatty livers on the susceptibility to the induction of mitochondrial permeability transition (MPT).** Mitochondrial swelling was spectrophotometrically monitored at 540 nm. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μM rotenone and 5 mM succinate. MPT was induced with 15 nmol $CaCl_2$. Cyclosporin A (CyA) (1 μM), was added to the reaction medium prior to calcium addition. The traces are representative of experiments performed with four independent mitochondrial preparations.

Prior to calcium addition, mitochondria isolated from I/R animals exhibited decreased absorbance at 540 nm compared with control and Ind, indicating swelling of the mitochondrial population. Additionally,

mitochondria isolated from I/R animals were more susceptible to undergo mitochondrial swelling induced by calcium, comparatively to both control and Ind treatment (Figure 3.2.17). Ind restored the capacity of mitochondria to accumulate calcium, without inducing the MPT. In vitro pre-treatment of mitochondria isolated from I/R livers with the MPT inhibitor cyclosporine A (CyA), prevented mitochondrial swelling induced by calcium, demonstrating that the swelling was caused by MPT induction.

### 3.2.2.2.7 Mitochondrial calcium fluxes:

Mitochondria isolated from control animals were able to accumulate the added calcium after energization with succinate and retained it during the entire time of the assay (Figure 3.2.18).

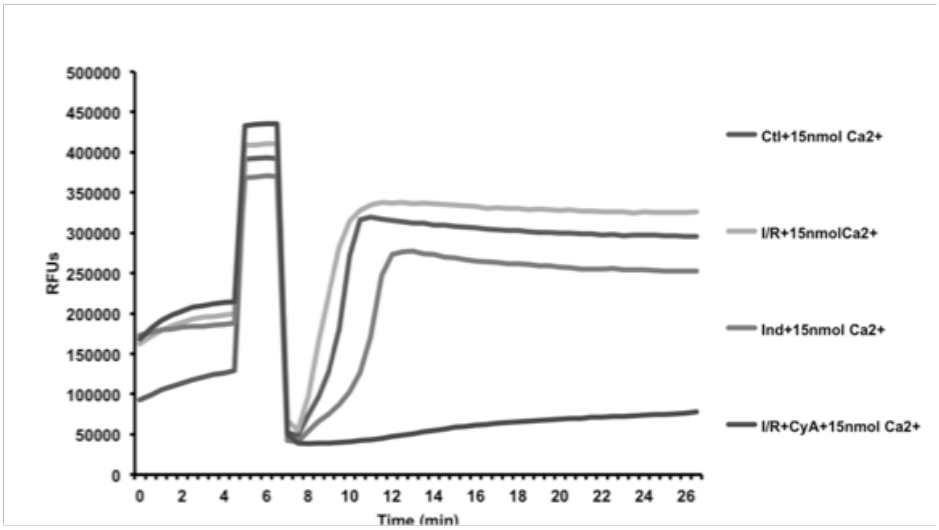



**Figure 3.2.18 Fatty livers and the effects of indirubin-3'-oxime (Ind) administered before ischemia on mitochondrial calcium fluxes.** Calcium fluxes were fluorometrically monitored using the probe Calcium Green (485 nm, 535 nm). Mitochondria (1mg) were incubated in 2 ml of standard incubation medium prior to calcium addition (15 nmol). Energization was achieved with succinate 5 mM. Calcium fluxes, expressed as relative fluorescence units (RFU) ,were recorded for an additional 25min. Cyclosporin A (CyA) 1μM was added to the reaction prior to calcium addition. The traces are representative of experiments performed with four independent mitochondrial preparations.

Conversely, mitochondria isolated from animals subjected to I/R, displayed an immediate release of calcium into the media assay before the calcium challenge. Since succinate addition lead to a decrease in fluorescence, mitochondria isolated from I/R livers showed no alterations in the ability for calcium uptake from the medium. However, the accumulated calcium was early released, demonstrating the increased susceptibility to a calcium challenge. Pre-treatment with CyA prevented calcium release, implying that increased calcium efflux from mitochondrial isolated from I/R animals was the result of MPT induction. Treatment with indirubin-3'-oxime before ischemia prevented mitochondrial calcium efflux before and after a calcium challenge.

**3.2.2.2.8 Cytochrome *c* , cleaved caspase-3 and Bcl-2 content :**

Because treatment with indirubin-3'-oxime prevented the increased calcium efflux observed in mitochondria isolated from animals subjected to hepatic I/R, endogenous cytochrome *c* content was evaluated. As shown in Figure 3.2.19, mitochondria from livers treated with Ind exhibited preserved cytochrome c content to a level compared to the control. However, in the I/R group there was a significant decrease in mitochondrial cytochrome *c*. In I/R animals, there was a higher content in cleaved caspase-3 comparatively to control and Ind treatment, as evaluated by western blotting in hepatic tissue homogenates.

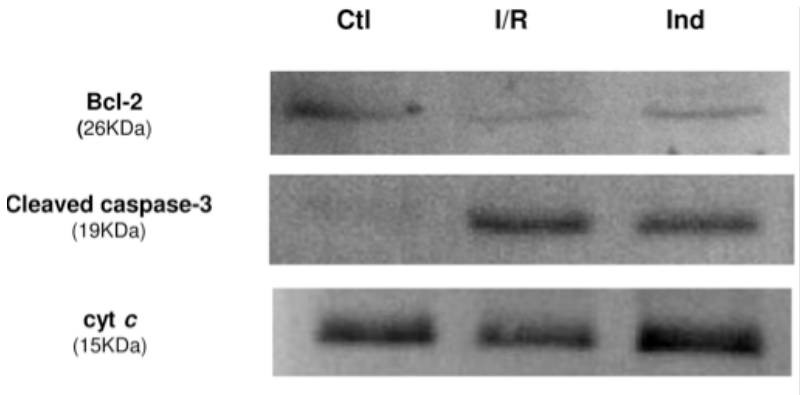



**Figure 3.2.19 Bcl-2 , cleaved caspase-3 and cytochrome *c* content was evaluated by western blotting**. A representative blot from four independent experiments is shown.

Additionally, due to the survival function of Bcl-2, by preventing apoptosis induction, we also evaluated Bcl-2 content. Livers treated with Ind before I/R showed a higher content in Bcl-2 in contrast to mitochondria from I/R animals.

### 3.2.2.2.9 GSK-3β content and its relationship with acetylation and binding of cyclophilin-D to ANT:

Since indirubin-3'-oxime is an inhibitor of GSK-3β, we evaluated if administration of Ind before ischemia was associated with decreased hepatic activity of GSK-3β after an episode of warm I/R. In I/R liver homogenates, there was a decrease in the content of GSK-3β phosphorylation at Ser9, the inactive form of GSK-3β (Figure 3.2.20 A ).

Treatment with Ind before ischemia, maintained the content of phospho- Ser9-GSK-3β to a level compared to the control. Because Ind was able to maintain the threshold for MPT induction and prevented cytochrome c release by I/R, we evaluated if alterations on mitochondrial GSK-3β content were involved in the effects of In on mitochondria. I/R induced the translocation of GSK-3β to the mitochondria and a reduction in the content of phospho-Ser9- GSK-3β (Figure 3.2.20 B).

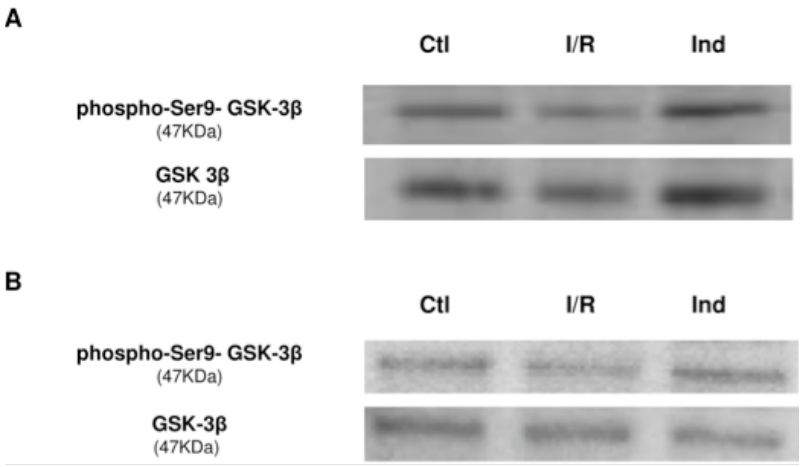



**Figure 3.2.20 Phospho-Ser9-GSK-3β and GSK-3β content in liver homogenates and isolated mitochondria were evaluated by western blotting.** A representative blot from four independent experiments is shown.

GSK-3β inhibition by Ind was able to maintain the mitochondrial content of total GSK-3β and phosphorylated GSK-3β at Ser9 to a level compared to the control.

Since treatment with indirubin decreases the susceptibility to the MPT, phosphorylation and the binding of CypD to ANT modulates MPT induction, we evaluated the association between these mitochondrial proteins. As shown in Figure 3.2.21, mitochondria isolated from liver subjected to I/R displayed increased levels of phosphorylated CypD.

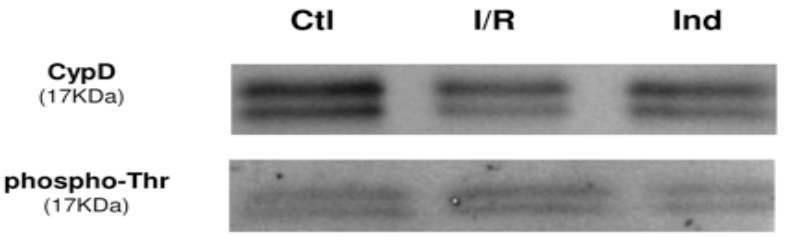



**Figure 3.2.21 Phosphorylated CypD in mitochondria isolated from livers pre treated with Ind**. CypD was immunoprecipitated from isolated mitochondria. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Western blots were then probed with antibodies against CypD. To access CypD phosphorylation, the CypD blots were stripped and then re-probed with antibody against phosphorylated threonine (phospho-Thr).

When phosphorylated in Thr residue, CypD is active, causing its association to the ANT, which was evaluated by immunoprecipitation. As shown in Figure 3.2.22, I/R promoted an increase in the level of CypD that was co-immunoprecipitated with ANT. In contrast, treatment with Ind before ischemia prevented phosphorylation of CypD causing its dissociation from the ANT.

These data show that I/R promotes an enhancement of CypD phosphorylation, resulting in an increase in CypD associated with the ANT and consequently MPT induction. This phenomenon was reversed in mitochondria isolated from I/R livers pre-treated with indirubin.

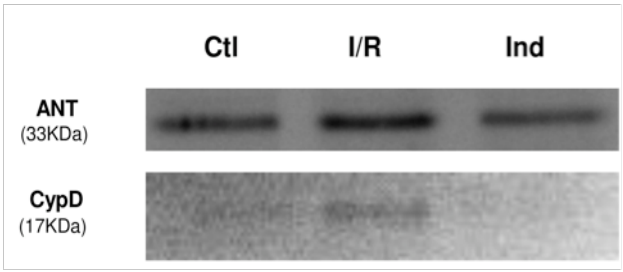



**Figure 3.2.22 CypD-ANT interaction in mitochondria isolated from livers pre treated with indirubin-3'-oxime (Ind).** ANT was immunoprecipitated from isolated mitochondria. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Western blots were then probed with antibodies against CypD or ANT.

### 3.2.2.2.10 HIF-1α content and TNF-α levels:

The hypoxia inducible factor-1α (HIF-1α) mediates the expression of cytokines, for example tumor necrosis factor-α (TNF-α). As shown in Figure 3.2.23, the content of HIF-1α is increased in I/R comparatively to Ctrl and Ind livers.

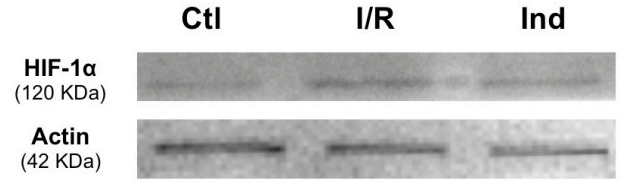



**Figure 3.2.23 HIF-1α content in liver homogenates was evaluated by western blotting**. A representative blot from four independent experiments is shown.

Kupffer cell activation results in production of several proinflammatory cytokines, such as TNF-α. This molecule has the role to start and perpetuate a later secondary inflammatory phase, which causes more damages to the liver. The TNF-α concentration in tissue was significantly higher in the I/R group than both control and Ind (Figure 3.2.24).

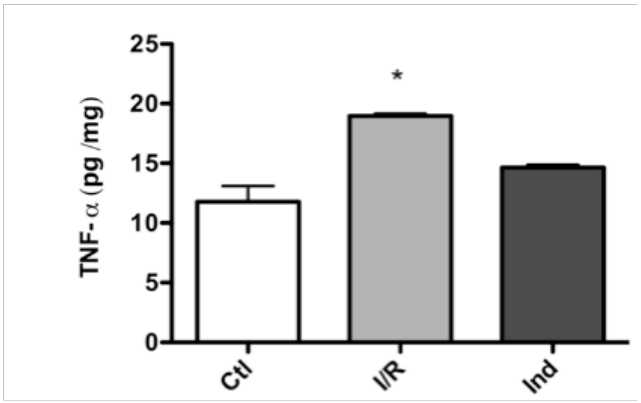



**Figure 3.2.24 Fatty livers and effects of indirubin-3'-oxime (Ind) administered before ischemia on TNF-α levels.** TNF-α levels in tissue was measure using commercial kits. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind .

### 3.2.2.2.11 MPO activity

Neutrophil recruitment in liver was assessed by biochemical analysis (MPO activity). MPO activity was low and similar between control and In-treated groups. In animals that just underwent hepatic I/R, MPO activity was significantly increased, indicating relevant neutrophil migration into liver tissue (Figure 3.2.25).

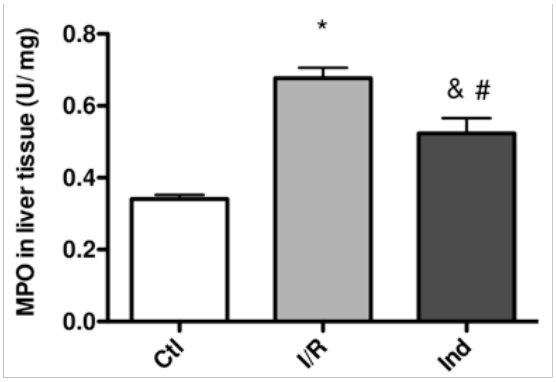



**Figure 3.2.25 Fatty livers and the effects of indirubin-3'-oxime (Ind) administered before ischemia on MPO activity.** MPO activity was evaluated in tissue homogenates by spectrophotometrically at 655 nm. Data are means ± S.E.M of experiments performed with four animals/group. *Ctl versus I/R; # I/R versus Ind; & Ctl versus Ind .

### 3.2.3 Discussion

Clinical data has shown that fatty livers are more susceptible to I/R injury, leading to diminish liver function (Selzner et al., 2000; Teramoto et al., 1993; Selzner et al., 2006; Caraceni et al., 2005; Vendemiale et al., 2001; Honda et al., 2005; Rolo et al., 2009). Regarding this, we have aimed to improve fatty condition to produce better outcomes following I/R.

Fatty livers intracellular accumulation of nonesterified fatty acids that increases mitochondrial uncoupling and inhibit gluconeogenesis, which leads to impaired ATP synthesis. Previous works has demonstrated that in fatty livers, IPC preserves ATP content during ischemia and restores intrahepatic ATP after reperfusion (Rolo et al., 2009).

Accumulating evidence suggests that decreased activity of GSK-3β caused by phosphorylation at Ser9 (Dajani et al., 2001), converges the signalling protective pathways by which ischemic and pharmacological preconditioning are effective in myocardial protection against ischemia/reperfusion injury (Juhaszova et al., 2004; Miura et al., 2009; Nishihara et al., 2007; Park et al., 2006; Xi et al., 2009). The end effector is modulation of the mitochondrial permeability transition, with an increased threshold for MPT induction in conditions of GSK-3β inhibition (Juhaszova et al., 2004). This study demonstrates that livers treated with indirubin-3'-oxime, an inhibitor of GSK-3β (Leclerc et al., 2001) are resistant to I/R injury, suggesting that indirubin-30-oxime has great therapeutic potential in the clinical setting of hepatic ischemia/reperfusion.

Induction of the MPT is an important cause for impairment of mitochondrial function after ischemia/reperfusion injury. Induction of the MPT after reperfusion mediates cell death by uncoupling oxidative phosphorylation, inducing mitochondrial swelling and collapsing mitochondrial membrane potential, thus blocking cellular ATP formation (Halestrap et al., 2004; Hausenloy et al., 2004; Juhaszova et al., 2008; Park et al., 2006). The essential role of the MPT in mediating protection against myocardial I/R injury has been demonstrated in hearts deficient in cyclophilin D, a component of the MPT. In these animals, ischemic and pharmacological preconditioning and postconditioning are not able to protect from I/R injury (Lim et al., 2007). GSK-3β inhibition as a successful therapeutic strategy to prevent impairment of hepatic mitochondrial function and preservation of cellular ATP content after I/R damage has not yet been evaluated.

We observed that indirubin-3'-oxime administered 30 min prior

to ischemia in lean and fatty livers was able to preserve ATP content. Pretreatment with indirubin-3'-oxime also decreased plasmatic ALT and AST activities, indicating a relevant hepatoprotective effect against warm I/R injury. This hepatoprotective effect and preservation of ATP content were associated with increased performance of mitochondrial function.

Decreased ΔΨ and oxygen consumption and also decreased efficiency of the phosphorylation system, caused by depletion of the ANT, are more consequences of fat accumulation in the liver. Direct measurement of mitochondrial membrane potential in isolated mitochondria shows that ΔΨ was decreased in livers from I/R animals but indirubin-3'-oxime preserved mitochondrial membrane potential. In mitochondria isolated from I/R livers, oxidative phosphorylation efficiency was impaired, but administration of indirubin-3'-oxime was able to maintain state 3 respiration, RCR and the lag phase to a level compared to the control.

The decrease in ATPase activity in I/R fatty livers supports the loss of mitochondrial phosphorylative efficiency, which was maintained by pre-treatment with indirubin-3'-oxime. Preservation of mitochondrial bioenergetics and cellular ATP by treatment with indirubin-3'-oxime is also due to protection against MPT induction (Varela et al., 2010).

Mitochondria isolated from I/R animals were highly susceptible to undergo mitochondrial swelling induced by calcium. Treatment with indirubin-3'-oxime was able to restore the capacity of mitochondria to accumulate calcium, without inducing the MPT. The population undergoing the MPT was increased in mitochondria from I/R livers, as demonstrated by the inhibition of mitochondrial swelling and calcium efflux in the presence of cyclosporine A.

Furthermore, mitochondrial swelling caused loss of the integrity of the mitochondrial outer membrane that lead to cytochrome *c* release into the cytosol, which is a cause for apoptotic cell death after I/R (Juhaszova et al., 2008; Murphy and Steenbergen, 2007). By preventing MPT induction, indirubin-3'-oxime preserved cytochrome *c* content and prevented caspase-3 activation after warm hepatic I/R. This was associated with decreased mitochondrial ROS production. Since oxidative stress favors MPT induction, the decrease in ROS generation by indirubine-3'-oxime also decreases the probability for this event to occur.

I/R has been shown to induce cell death by inducing Bax translocation to the mitochondria (Anderson et al., 2004). The Bcl-2 proteins are a family of proteins involved in the apoptotic pathway of cell death. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl-2 proteins. Bax translocation from

the cytosol to the mitochondrial membrane is a critical step in apoptosis induction. Through interactions with pore proteins on the mitochondrial membrane, Bax increases mitochondrial membrane permeability and leads to cytochrome c release. The preservation of cytochrome *c* by Ind was associated with decreased Bax content in isolated mitochondria, as well as increased content of Bcl-2. Bcl-2 has been shown to prevent mitochondrial dysfunction associated with apoptosis by preventing the decrease in mitochondrial membrane potential observed in conditions of hypoxia (Shimizu et al., 1998).

Although GSK-3β mainly localizes in the cytosol its activity of GSK-3β is higher in mitochondria (Bijur et al., 2003). According to diverse studies, GSK-3β inhibition (increased mitochondrial content in phospho-Ser9-GSK-3β) converge the signalling protective pathways by which ischemic and pharmacological preconditioning are effective in protection against ischemia/reperfusion injury (Varela et al., 2010; Park et al., 2006; Xi et al., 2009; Juhaszova et al., 2004; Nishihara et al., 2007; Miura et al., 2009). The study in lean livers showed that I/R induces translocation of GSK-3β from the cytosol to the mitochondria, increasing mitochondrial GSK-3β activity (Varela et al., 2010). On the other hand, treatment of livers before ischemia with indirubin-3'-oxime preserves the level of the inactivated form (phospho-Ser9-GSK-3β) in the mitochondrial pool of GSK-3β. We conclude that the effects of indirubin-3'-oxime on mitochondrial function in conditions of hepatic I/R are mediated by GSK-3β inhibition.

Interactions between GSK-3β and components of the MPT have been indicated as the mechanism by which I/R increases the susceptibility to MPT induction (Varela et al., 2010; Xi et al., 2009; Das et al., 2009). Rasola and colleagues proposed a model in which inhibition of GSK-3β activity, decreases its association with CypD leading to MPT desensitization, whereas the Ser/Thr phosphorylation of CypD by GSK-3β favours pore opening (Rasola et al., 2010). When phosphorylated, CypD binds to the ANT and increases sensitivity to calcium promoting MPT induction and thus, cell death after I/R. I/R promotes an enhancement of CypD phosphorylation, resulting in increased CypD associated with the ANT and consequently MPT induction (Zorov et al., 2009; Bao et al., 2012). So, as for a mechanism of phospho-GSK-3β-mediated protection by indirubin-3'-oxime, we demonstrated that by maintaining the levels of inhibited mitochondrial GSK-3β, indirubin-3'-oxime inhibits the interaction between CypD–ANT. In organs such as the heart, perfusion with GSK-3β inhibitors have been shown to reduce cell death induced by I/R, when added before ischemia or at the start of reperfusion (Tong et al., 2002;

This study shows that indirubin-3'-oxime acts as pharmacological preconditioning, modulating mitochondrial function and energy metabolism caused in conditions of I/R. Indirubin-3'-oxime preserves oxidative phosphorylation and decreases the probability for MPT induction, by inhibiting GSK-3β and the interaction CypD-ANT.

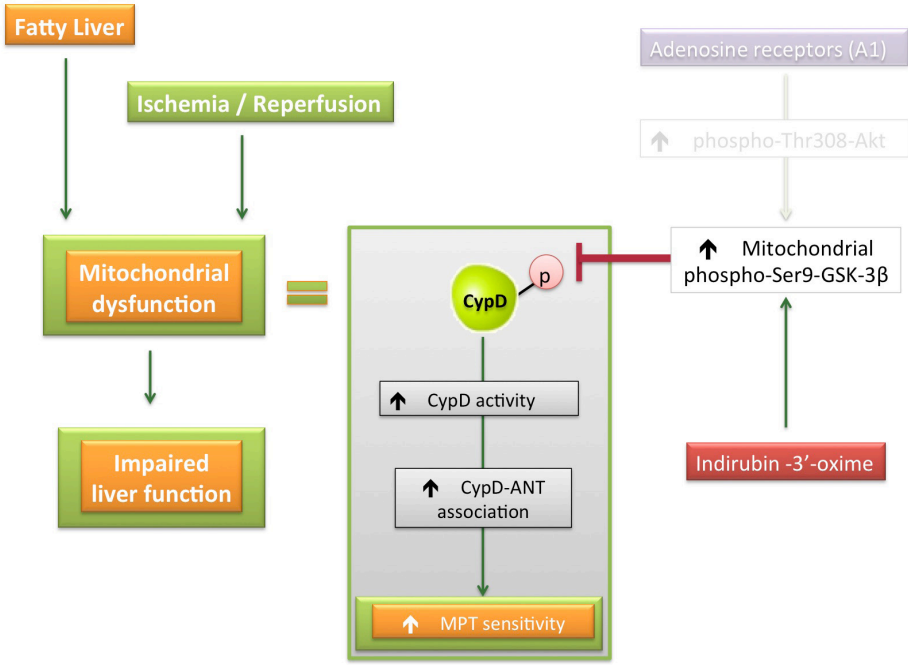



**Figure 3.2.26 – Mitochondrial dysfunction increase the susceptibility of fatty livers to I/R injury.** Inhibition of GSK-3β by indirubin-3'-oxime decreases CypD binding ANT, preventing MPT induction.

# 3.3

## RESULTS: MODULATION OF THE MPT BY SIRT3-CYPD-ANT AXIS

Regulation of mitochondrial function in ischemia/reperfusion: looking for therapeutic strategies in fatty livers

## $NAD^+$ PREVENTS I/R DAMAGE IN FATTY LIVERS: MODULATION OF THE MPT BY THE SIRT3 CYPD-ANT AXIS


### Abstract

In this study, we wanted to establish if an acute treatment with $NAD^+$, a sirtuin cofactor, prevents mitochondrial dysfunction associated with warm I/R injury in fatty livers. Zucker fatty rats were subjected to 120 min of 70% warm ischemia and 12 h of reperfusion. In the treated group ($NAD^+$) $NAD^+$ was administered in the hepatic artery 30 min before ischemia. Acute treatment of livers before ischemia, decreased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) levels and also preserved mitochondrial cytochrome c content, comparatively to I/R livers. $NAD^+$ also prevented calcium-induced mitochondrial permeability transition (MPT), the decline in mitochondrial respiratory state 3 and ATP content caused by I/R. The generation of reactive oxygen species (ROS) was also decreased in mitochondria isolated from I/R livers pre-treated with $NAD^+$. Data shows that deacetylation of cyclophilin D (CypD) at Lys residue causes dissociation from the adenine nucleotide translocator (ANT), contributing to an increase in MPT threshold in $NAD^+$-treated animals. This is due to a stimulation of the activity of mitochondrial SIRT3 that reduces the content in acetylated CypD in mitochondria from control and $NAD^+$ livers, compared to the I/R group. These observations point CypD as a new protective target against I/R injury in fatty livers.

Pre-treatment with $NAD^+$ protects the fatty liver by maintaining mitochondrial calcium homeostasis, thus preserving mitochondrial function and hepatic energetic balance


### 3.3.1 Introduction

Prevention of MPT induction by preconditioning and postconditioning has been proposed to mediate the protective effect of these interventions against I/R injury (Halestrap et al., 2004; Hausenloy et al., 2004; Park et al., 2006; Lemasters, 2007; Murphy et al., 2007; Rolo et al., 2009; Varela et al., 2010). The therapeutic potential of maintaining the MPT in a closed state is highlighted by the resistance of Cyclophilin D (CypD) knockout mice (Ppif-/-) to a variety of diseases (Baines et al., 2005). Binding of CypD to the adenine nucleotide translocator (ANT) increases sensitivity to $Ca^{2+}$, promoting mitochondrial dysfunction and cell death after I/R (Zorov

et al., 2009).

Recently, Zheng and colleagues suggested that $NAD^+$ prevents MPT induction and decreases brain ischemic damage (Zheng et al., 2012). $NAD^+$ plays an important role in energy metabolism and mitochondrial functions, but also, gene expression, calcium homeostasis and immunological functions (Ying, 2008). $NAD^+$ is required as a co-substrate for the activity of sirtuins, a family of protein deacetylases with beneficial effects on several human diseases (Ma et al., 2012). Recent reports have indicated that exogenous $NAD^+$ prevents neurons from degeneration and I/R-induced cell death as well as cardiac hypertrophic damage, by activating SIRT3 (Ying et al., 2007; Pillai et al., 2010). SIRT3 activity correlates with the level of mitochondrial protein acetylation (Schwer et al., 2008). Studies have established a relationship between SIRT3 and CypD: SIRT3 deacetylates and inactivates CypD causing its dissociation from the ANT and increasing the threshold to the MPT (Shulga et al., 2010; Hafner et al., 2010). Fatty liver is associated with decreased $NAD^+$ levels and decreased SIRT3 activity, mitochondrial protein hyperacetylation and reduced mitochondrial function (Hirschey et al., 2010; Kendrick et al., 2011; Teodoro et al., 2006). Fatty liver is also associated with down-regulation of nicotinamide phosphoribosyltransferase that is necessary for $NAD^+$ synthesis (Dahl et al., 2010). Therefore, fatty livers exhibit a background that increases the susceptibility to undergo the MPT, diminishing the possibilities of organ recovery after I/R injury. There is an urgent need for strategies against I/R injury in fatty livers, due to the increased prevalence of this kind of livers mainly related to the overall increase of obesity and alcohol consumption in all industrialized countries (Feldstein, 2010).

We propose that the impairment in SIRT3 activity in fatty livers and its ability to suppress MPT formation increases the sensitivity of fatty livers to I/R injury. The consequent mitochondrial damage will lead to accumulation of dysfunctional mitochondria inducing further cellular injury due to mitochondrial ROS formation, calcium overload and futile ATP consumption. Treatment of fatty livers with NAD+ before I/R, is expected to increase sirtuin activity, decrease CypD acetylation and its association with the MPT, therefore preventing I/R damage.

### 3.3.2 Results

#### 3.3.2.1 Plasma markers of liver injury:

To determine if $NAD^+$ given before ischemia protects liver from IR injury, serum levels of LDH, AST and ALT were evaluated. As shown in Figure 3.3.1, warm I/R significantly increased plasma LDH, AST and ALT levels relative to the control group. NAD+ administration before ischemia was effective in reducing plasma levels of LDH, AST and ALT.

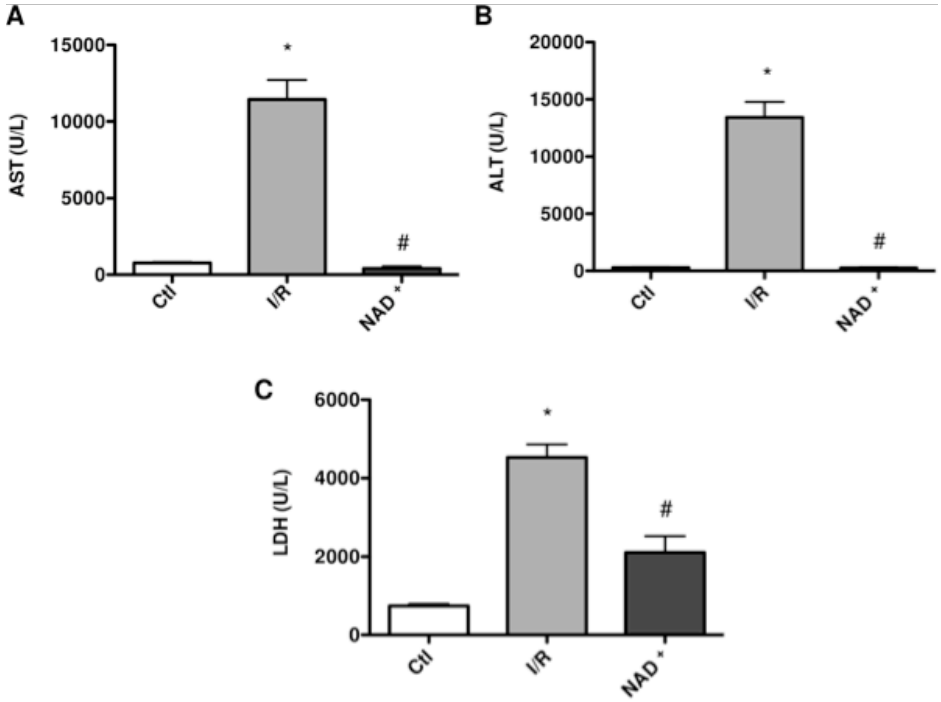



**Figure 3.3.1 The effects of $NAD^+$ administered before ischemia on plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)**. Plasma samples were collected and enzimatic determinations of ALT (A), AST (B) and LDH (C) performed using commercial kits. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus $NAD^+$; & Ctl versus $NAD^+$($P<0.05$).

### 3.3.2.2 HIF-1α content and TNF-α and MPO activity :

As shown in Figure 3.3.2, I/R induced an increase in hepatic HIF-1α (a master regulator of cell response to hypoxia), comparatively to Ctl and NAD+ livers. The hypoxia inducible factor-1α (HIF-1α) mediate the expression of cytokines, for example tumor necrosis factor-α (TNF-α). TNF-α plasmatic concentration was also significantly higher in the I/R group than in the Ctl and NAD+ groups (Figure 3.3.3 A ).

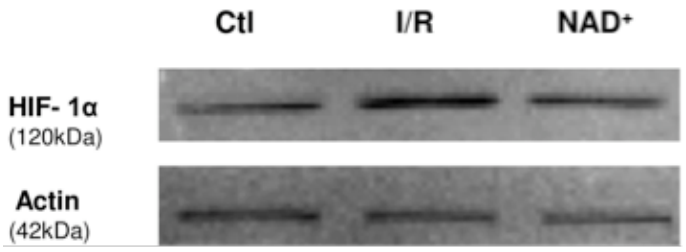



**Figure 3.3.2 HIF-1α content in liver homogenates was evaluated by western blotting**. A representative blot from four independent experiments is shown.

TNF-α release leads to neutrophil activation and accumulation. In animals that just underwent hepatic I/R, MPO activity was significantly increased, indicating the presence of relevant neutrophil migration into liver tissue (Figure 3.3.3 B). This was prevented by NAD+ treatment.

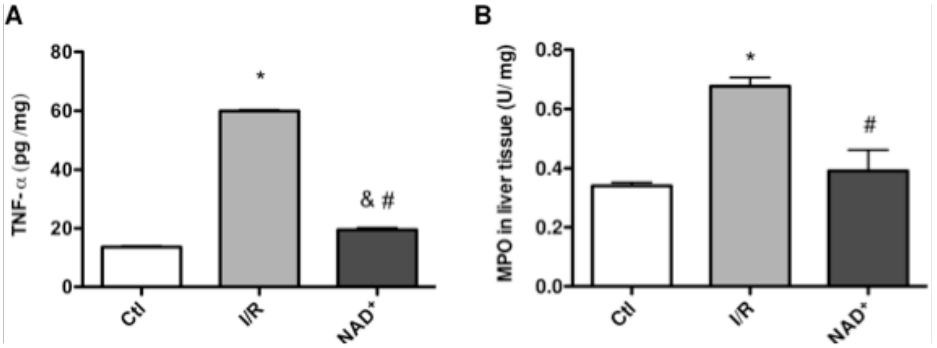



**Figure 3.3.3 The effects of NAD+ administered before ischemia on TNF-α levels (A) and MPO activity (B).** TNF-α levels in plasma were measure using commercial kits. MPO activity was evaluated in tissue homogenates by spectrophotometrically at 655 nm. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus NAD+; & Ctl versus NAD+(P<0.05).

### 3.3.2.3 Mitochondrial membrane potential (ΨΔ) and oxygen consumption:

To address if protection afforded by $NAD^+$ involved mitochondria, we evaluated several functional mitochondrial parameters.

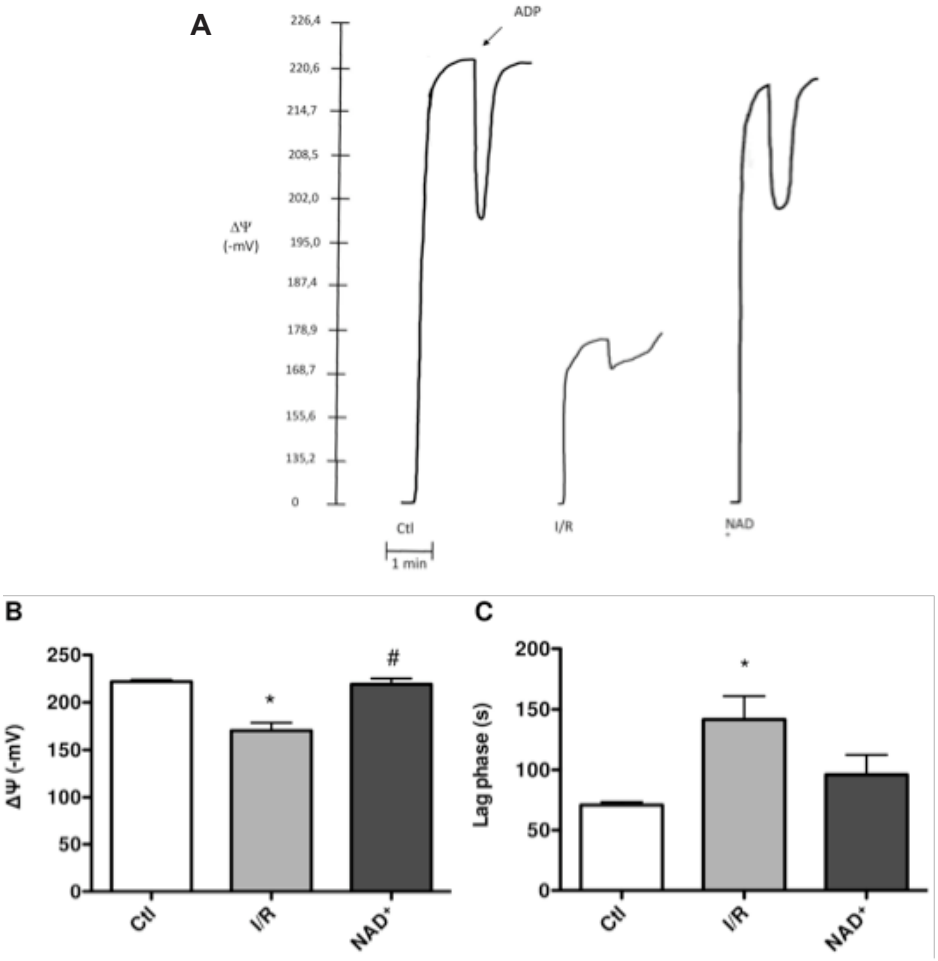



**Figure 3.3.4- The effects of $NAD^+$ administered before ischemia on mitochondrial membrane potential (A,B) and lag phase (A,C)**. ΔΨ was measured with a TPP+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 3 μM rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus $NAD^+$; & Ctl versus $NAD^+$($P<0.05$).

Mitochondria isolated from livers subjected to I/R exhibited decreased mitochondrial membrane potential (Figure 3.3.4 A,B), state 3 respiration and RCR (Figure 3.3.5 A,B) , when compared to mitochondria from both control and NAD animals.

The lag phase (time necessary for ADP phosphorylation) was significantly increased in animals subjected to I/R, comparatively to control. Treatment with $NAD^+$ before ischemia markedly reduced the lag phase to a level compared with control animals, showing that $NAD^+$ administration is able to preserve the efficiency of mitochondrial phosphorylation.

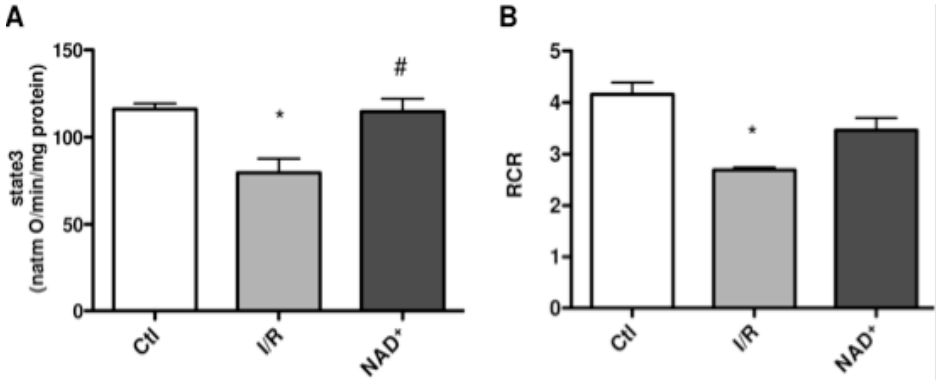



**Figure 3.3.5 The effects of NAD + administered before ischemia on state 3 (A) respiration and respiratory control ratio (RCR) (B)**. Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode. Mitochondria (1mg) were suspended under constant stirring, at 25ºC, in 1.4 ml of standard respiratory medium and energized by adding succinate to a final concentration of 5mM, 2 μM rotenone were previously added. State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 μg/mg protein) and 1 μM FCCP. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus $NAD^+$; & Ctl versus $NAD^+$($P<0.05$).

### 3.3.2.4 Adenine nucleotides content

To further determine whether $NAD^+$ treatment is able to preserve mitochondrial ATP production upon I/R, endogenous ATP content was evaluated in tissue and in isolated mitochondria. As shown in Figure 3.3.6, ATP content decreased in I/R animals associated with the observed decreased phosphorylation efficiency. $NAD^+$ administration before ischemia was able to significantly preserve mitochondrial energy level.

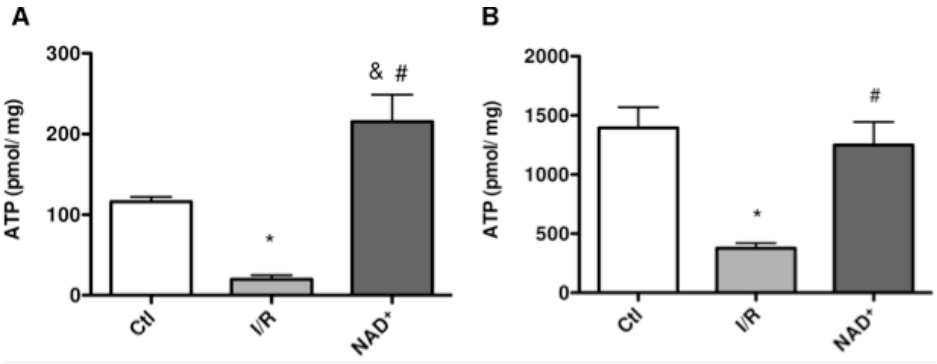



**Figure 3.3.6 The effects of NAD⁺ administered before ischemia on ATP content tissue (A) and mitochondria (B)**. ATP content was evaluated by a bioluminescent assay kit. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus NAD⁺; & Ctl versus NAD⁺(P<0.05).

### 3.3.2.5 ATPase activity

The decreased performance of phosphorylation in I/R fatty livers, suggested alterations in the F1F0-ATPsynthase, a key component of the phosphorylative system. ATPase activity was decreased in mitochondria from I/R animals, comparatively to control. NAD⁺ preserved ATPase activity similarly to the observed in control group (Figure 3.3.7).

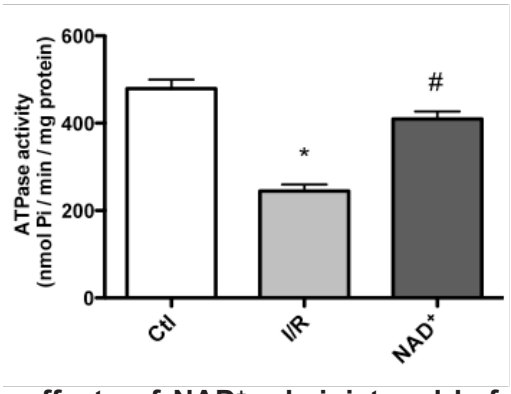



**Figure 3.3.7 The effects of NAD⁺ administered before ischemia on ATPase activity**. ATPase activity was determined spectrophotometrically at 660 nm and was calculated as the difference in total activity and activity in the presence of oligomycin. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus NAD⁺; & Ctl versus NAD⁺(P<0.05).

### 3.3.2.6 Generation of reactive oxygen species (ROS)

A pathological increase on the endogenous production of ROS after I/R has been associated with decreased capacity for cell survival. $NAD^+$-treatment prevented the damage to the electron transport chain induced by I/R, as shown by the decrease in ROS generation in $NAD^+$ animals (Figure 3.3.8).

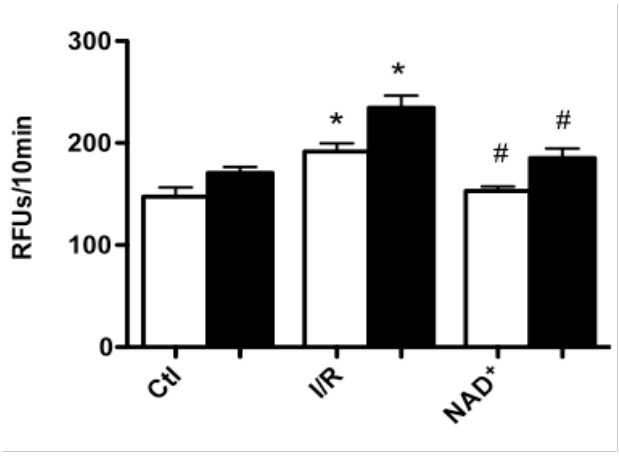



**Figure 3.3.8 The effects of $NAD^+$ administered before ischemia on reactive oxygen species (ROS) generation**. ROS were estimated fluorometrically using the probe CM-H2DFDA (485 nm, 538 nm). After basal fluorescence (white) measurement, antimycin A was added to all preparation (black) to block complex III of the mitochondrial chain, to induce maximal ROS generation. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus $NAD^+$; & Ctl versus $NAD^+$($P<0.05$).

### 3.3.2.7 Mitochondrial permeability transition:

Since induction of the MPT is a critical determinant of mitochondrial performance and I/R injury, we evaluated whether treatment of livers submitted to I/R with $NAD^+$, protects from MPT induction. Prior to $Ca^{2+}$ addition, mitochondria isolated from I/R animals exhibited decreased absorbance at 540 nm compared with control and treated group, indicating swelling of the mitochondrial population. Additionally, mitochondria from I/R animals were more susceptible to $Ca^{2+}$-induced mitochondrial swelling, comparatively to both control and $NAD^+$ treatment. In vitro pre-treatment of

mitochondria isolated from I/R livers with the MPT inhibitor CyA, prevented mitochondrial swelling induced by calcium, demonstrating that the swelling was due to MPT induction (Figure 3.3.8).

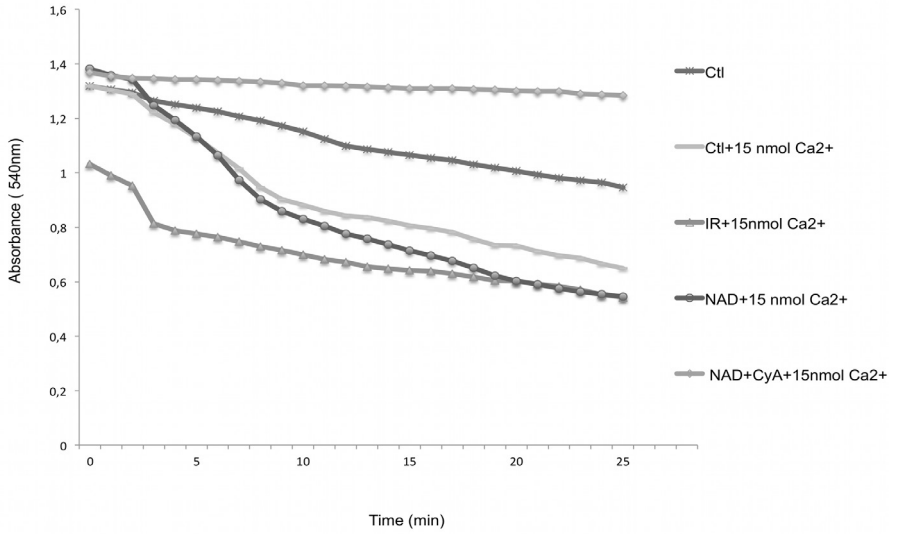



**Figure 3.3.8 The effects of $NAD^+$ administered before ischemia on the susceptibility to the induction of mitochondrial permeability transition (MPT).** Mitochondrial swelling was spectrophotometrically monitored at 540 nm. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μM rotenone and 5 mM succinate. MPT was induced with 15 nmol $CaCl_2$. Cyclosporin A (CyA) (1 μM) was added to the reaction medium prior to calcium addition. The traces are representative of experiments performed with four independent mitochondrial preparations.

### 3.3.2.8 Mitochondrial calcium fluxes

As mitochondria isolated from I/R livers pre-treated with $NAD^+$ decreased the susceptibility to calcium-induced swelling, mitochondrial calcium fluxes were evaluated. Mitochondria isolated from control animals were able to accumulate the added calcium after energization with succinate and retained it during the entire time of the assay (Figure 3.3.9). On the contrary, mitochondria isolated from animals subjected to I/R, displayed an immediate release of calcium into the media assay before the calcium challenge. Mitochondria isolated from I/R livers showed no alterations in the ability for calcium uptake from the medium. However, the accumulated calcium was early released, demonstrating the increased susceptibility to a calcium challenge. Pre-treatment with CyA prevented calcium release, implying that increased calcium efflux from mitochondrial isolated from I/R

animals was the result of MPT induction. Administration of NAD+ before ischemia prevented mitochondrial calcium efflux before and after a calcium challenge.

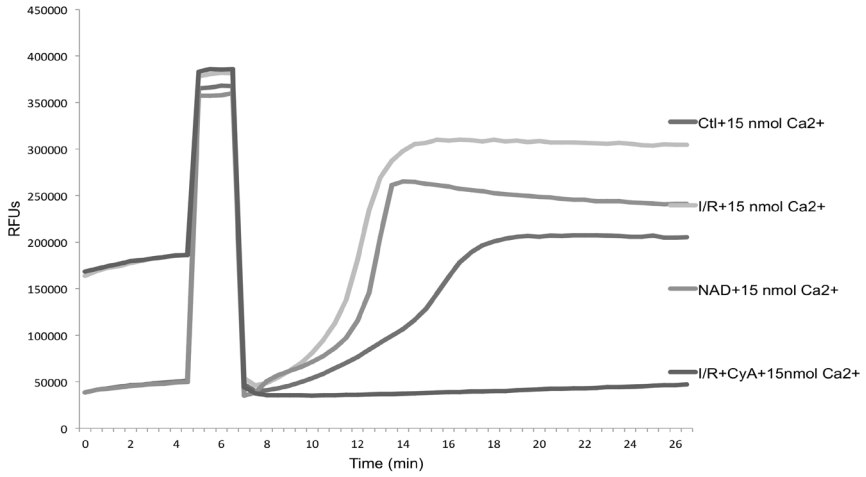



**Figure 3.3.9 The effects of NAD$^+$ administered before ischemia on mitochondrial calcium fluxes.** Calcium fluxes were fluorometrically monitored using the probe Calcium Green (485 nm, 535 nm). Mitochondria (1mg) were incubated in 2 ml of standard incubation medium prior to calcium addition (15 nmol). Energization was achieved with succinate 5 mM. Calcium fluxes, expressed as relative fluorescence units (RFU) ,were recorded for an additional 25min. Cyclosporin A (CyA) 1μM was added to the reaction prior to calcium addition. The traces are representative of experiments performed with four independent mitochondrial preparations.

### 3.3.2.9 Sirtuin-3 content and activity and their relationship with acetylation and binding of CypD to ANT:

Since the MPT is induced by I/R, and NAD$^+$ treatment decreases the susceptibility to MPT, acetylation and binding of CypD to ANT were evaluated. As shown in Figure 3.3.10 A, in I/R, mitochondria displayed increased levels of acetylated CypD. When acetylated in Lys residue, CypD is active, causing its association to the ANT. As so, NAD$^+$ addition before I/R reduced CypD co-immunoprecipitated with the ANT (Figure 3.3.10 B), probably due to increased deacetylation of CypD by SIRT3.

Although SIRT3 content was unchanged (Figure 3.3.11 A), SIRT3 activity was decreased in liver samples from I/R and increased in the NAD$^+$ group (Figure 3.3.11 B).

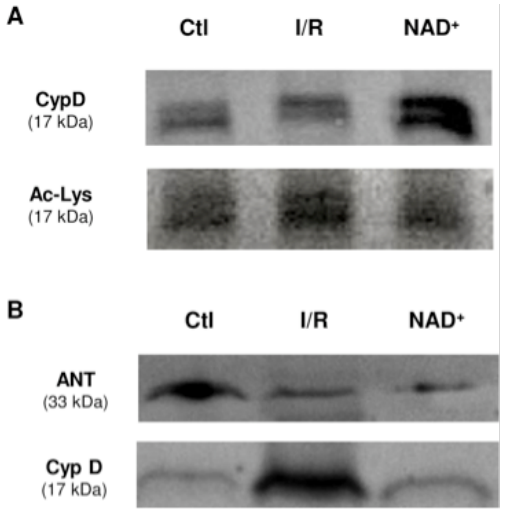



**Figure 3.3.10 Acetylated CypD causing its association to the ANT in mitochondria isolated from I/R livers.** CypD was immunoprecipitated from isolated mitochondria. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Western blots were then probed with antibodies against Ac-Lys, to access CypD acetylation. The blots were stripped and then re-probed with antibody against CypD (A).

ANT was immunoprecipitated from isolated mitochondria. The immunoprecipitates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Western blots were then probed with antibodies against CypD or ANT (B).

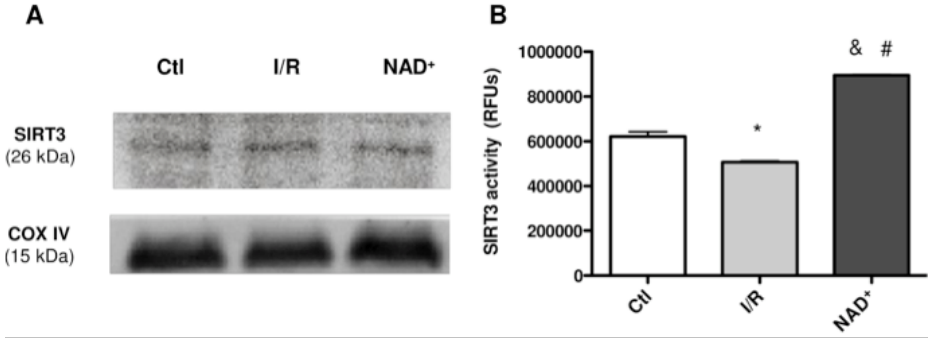



**Figure 3.3.11 The effects of NAD$^+$ administered before ischemia on SIRT3 content and activity.** A representative blot from four independent experiments is shown. SIRT3 activity was measure using commercial kit. Data are means ± S.E.M of experiments performed with four animals/group. Statistically significant difference: *Ctl versus I/R; # I/R versus NAD$^+$; & Ctl versus NAD$^+$($P<0.05$).

### 3.3.2.10 Cytochrome *c*, caspase-3 and Bcl-2 content :

The release of cytochrome *c* due to MPT induction, leads to caspase activation and development of the apoptotic process. As shown in Figure 3.3.12, $NAD^+$ preserved mitochondrial cytochrome *c* content to a level compared to the control. Additionally, I/R animals, exhibited higher content in cleaved caspase-3 comparatively to control and $NAD^+$ treatment (Figure 3.3.12). Additionally, due to the survival function of Bcl-2, by preventing apoptosis induction, we also evaluated Bcl-2 content. Livers injected with NAD+ also showed a higher content in anti-apoptotic Bcl-2, in contrast to I/R animals (Figure 3.3.12).

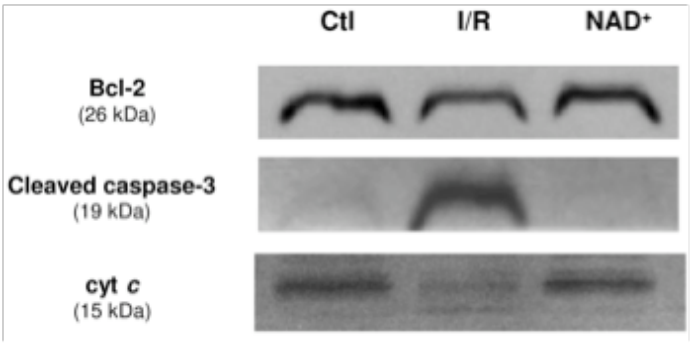



**Figure 3.3.12 Bcl-2, cleaved caspase-3 and cyt *c* content were evaluated by western blotting.** A representative blot from four independent experiments is shown.

### 3.3.2.11 LC3 content

Since MPT induction triggers autophagy, the levels of an autophagy marker, microtubule- associated protein 1 light chain 3 (LC3), were determined. LC3-I conversion to LC3-II was increased in I/R indicating autophagy induction. $NAD^+$ treatment prevented the increase in the LC3-II levels, suggesting that $NAD^+$ administration prevents I/R-induced autophagy (Figure 3.3.13).

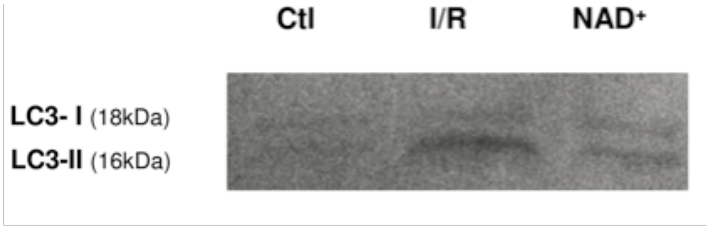



**Figure 3.3.13 LC3 content was evaluated by western blotting**. A representative blot from four independent experiments is shown.

### 3.3. Discussion

This study shows that treatment of fatty livers with $NAD^+$ is a strategy with therapeutic potential in the clinical setting of I/R. We demonstrated for the first time that adding $NAD^+$ to fatty livers prior to I/R significantly increases SIRT3 activity, leading to decreased CypD acetylation and association with the ANT. This increases the resistance to the MPT and prevents I/R injury.

Hepatic I/R is an important cause of organ damage. It triggers a series of adverse events that culminate in cell death. There are currently no drugs for clinical use to reduce reperfusion injury, although the pathways implicated in I/R injury are subject of intense research. I/R in fatty livers was associated with increased hepatocellular injury characterized by an increase in serum transaminase levels and markers of apoptotic cell death. Activation of inflammatory pathways during I/R is due to increased production of cytokines, such as TNF-α, a cell death activator and MPT inducer (Tafani et al., 2000). Both TNF-α and MPO activity, a marker of neutrophil infiltration, as well as HIF-1α, were increased in I/R conditions. Administration of NAD+ 30 min prior to ischemia was effective in decreasing plasmatic ALT, AST and LDH activities, indicating a clear hepatoprotective effect in a situation of warm I/R injury. $NAD^+$ treatment also inhibited the release of TNF-α and the increase in MPO activity.

The pathogenesis of I/R injury involves numerous mechanisms, including alterations in mitochondrial performance. Associated with alterations in pH, $Na^+$, and oxidative phosphorylation, I/R triggers a cascade of events that involve increased ROS generation and loss of $Ca^{2+}$ homeostasis. This culminates in MPT induction and cell death. Therefore, modulation of mitochondria has emerged as a critical survival strategy in the prevention against I/R injury. In fact, protection against I/R injury by

preconditioning implicates a diverse array of signaling cascades, many of which converge at the mitochondrion (Varela et al., 2011; Banga et al., 2005; Juhaszova et al., 2008; Halestrap et al.,2004; Hausenloy et al., 2004; Park et al., 2006; Lemasters, 2007; Murphy et al., 2007; Rolo et al., 2009; Varela et al., 2010).

Fatty livers display a defective oxidative phosphorylation system and decreased capacity to accumulate and retain $Ca^{2+}$, resulting in a decreased tolerance to $Ca^{2+}$ insult (Teodoro et al., 2006).

This inability to handle a pathological $Ca^{2+}$ insult may predispose fatty livers to I/R injury. In the present study, fatty livers subjected to warm I/R exhibited marked biochemical changes in mitochondrial performance, including increasing ROS generation, defective oxidative phosphorylation and increased susceptibility to MPT induction. Mitochondrial swelling caused loss of the integrity of the mitochondrial outer membrane that lead to cytochrome c release into the cytosol, which is a cause for apoptotic cell death after I/R (Halestrap et al., 2004; Murphy et al., 2007). Treatment with $NAD^+$ was able to prevent MPT induction and thus maintain cytochrome c content and prevent caspase-3 activation after warm hepatic I/R. The preservation of cytochrome c within mitochondria by $NAD^+$ was associated with increased content of Bcl-2. Bcl-2 has been shown to prevent mitochondrial dysfunction associated with apoptosis by preventing the decrease in mitochondrial membrane potential observed in conditions of hypoxia (Shimizu et al., 1998). MPT mediates not only apoptosis but also, under certain conditions, autophagy (Lemasters, 2007). Autophagy is a catabolic process for degradation of cytoplasmic contents by using lysosomal machinery. Recently, Zhenga and colleagues suggested an important link between $NAD^+$ and autophagy in ischemic brains of rodents (Zhenga et al., 2012). Our data shows that $NAD^+$ administration can attenuate autophagy induction in the ischemic livers.

The essential role of the MPT in mediating protection against I/R injury has been demonstrated in hearts deficient in CypD, where ischemic and pharmacological preconditioning and postconditioning do not have any protective action against I/R (Baines et al., 2005; Zorov et al., 2009; Schinzel et al., 2005). The mechanism by which CypD promotes induction of the MPT is probably related with mediated alterations in the conformation of mitochondrial inner membrane proteins, thus promoting formation and MPT induction (Hafner et al., 2010). In this work, treatment with $NAD^+$ inhibited ROS formation and decreased the association between CypD and the ANT. Interactions between MPT-regulatory factors are under strict regulation. When acetylated in the Lys residue, CypD is active and

facilitates a conformational change in the ANT, promoting the MPT (Hafner et al., 2010). CypD deacetylation is mediated by SIRT3. SIRT3 localized to the mitochondria plays a role in various mitochondrial functions, such as regulation of energy homeostasis and apoptosis (Hirschey et al., 2010; Kendrick et al., 2011). Recent studies have suggested the protective role of SIRT3 in cells under stress condition, involving the MPT (Hafner et al., 2010; Shulga et al., 2010). In this study, we demonstrated SIRT3-CypD-MPT as a preventive axis in fatty livers against I/R injury.

We observed that the increase in CypD acetylation in I/R group was accompanied by an inhibition of SIRT3 activity. Conversely, NAD+ treatment increased SIRT3 activity and decreased CypD acetylation. This may be explained by the fact that obesity is associated with increased acetylation of mitochondrial proteins (Kendrick et al., 2011; Choudhury et al., 2010), caused by a decrease in SIRT3 activity (Dahl et al., 2010). Adding $NAD^+$ before ischemia increases SIRT3 activity and thereby prevents against I/R injury.

Besides its involvement in MPT regulation, SIRT3 has been reported as regulatory of electron transfer chain components: complex I, II, III (Cimen et al., 2010; Kim et al., 2006; Bao et al., 2010). In studies with SIRT3 deficiency or knockdown there is increased complex acetylation and consequent decreased activity, suggesting that SIRT3 regulates many aspects of mitochondrial oxidative phosphorylation. Several groups have also reported that SIRT3 plays a major role in the suppression of intracellular ROS generation (Bao et al., 2010; Kong et al., 2010; Qiu et al., 2010; Someya et al., 2010), which justifies the decreased ROS generation in NAD+-treated animals. As so, our data demonstrating that oxidative phosphorylation efficiency was impaired in mitochondria isolated from I/R livers comes as no surprise, as is the fact that administration of NAD+ was able to recover state 3 respiration and RCR to control levels. The decrease in ATPase activity in I/R livers reveals the loss of mitochondrial phosphorylative efficiency.

In conclusion, treatment with $NAD^+$ before ischemia is protective against injury in a model of hepatic warm I/R. $NAD^+$ modulates mitochondrial function, preserves oxidative phosphorylation and decreases the probability of MPT induction. Obesity decreases the cellular $NAD^+$/NADH ratio, thereby contributing to a decrease in SIRT3 activity that increases the acetylation of mitochondrial activities, decreasing mitochondrial efficiency. $NAD^+$ increases SIRT3 activity and decreases CypD acetylation, activity and binding of CypD to ANT, increasing the threshold to MPT induction.

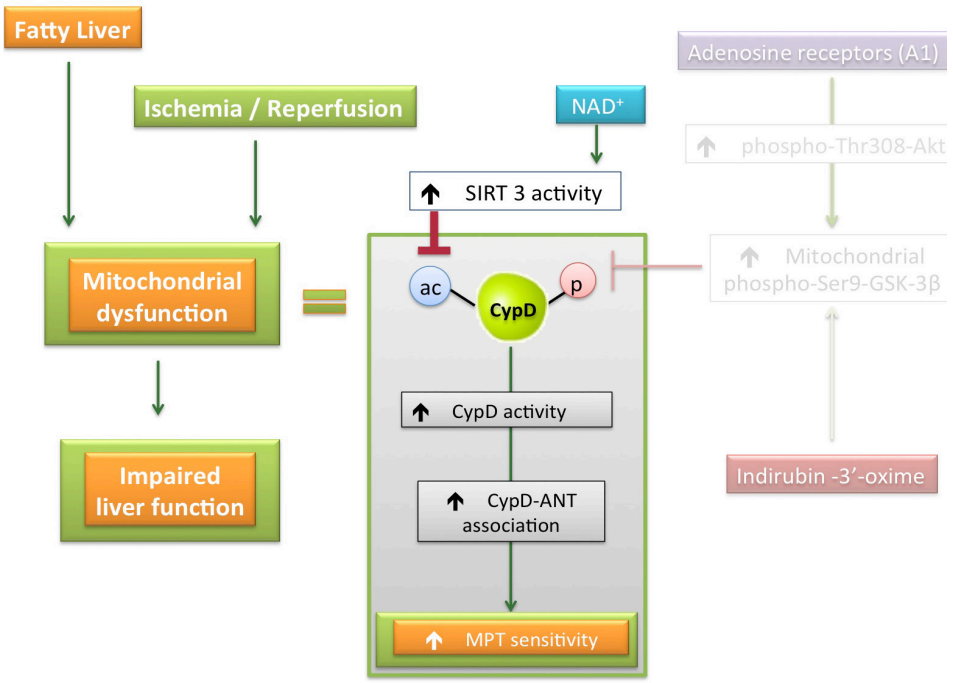
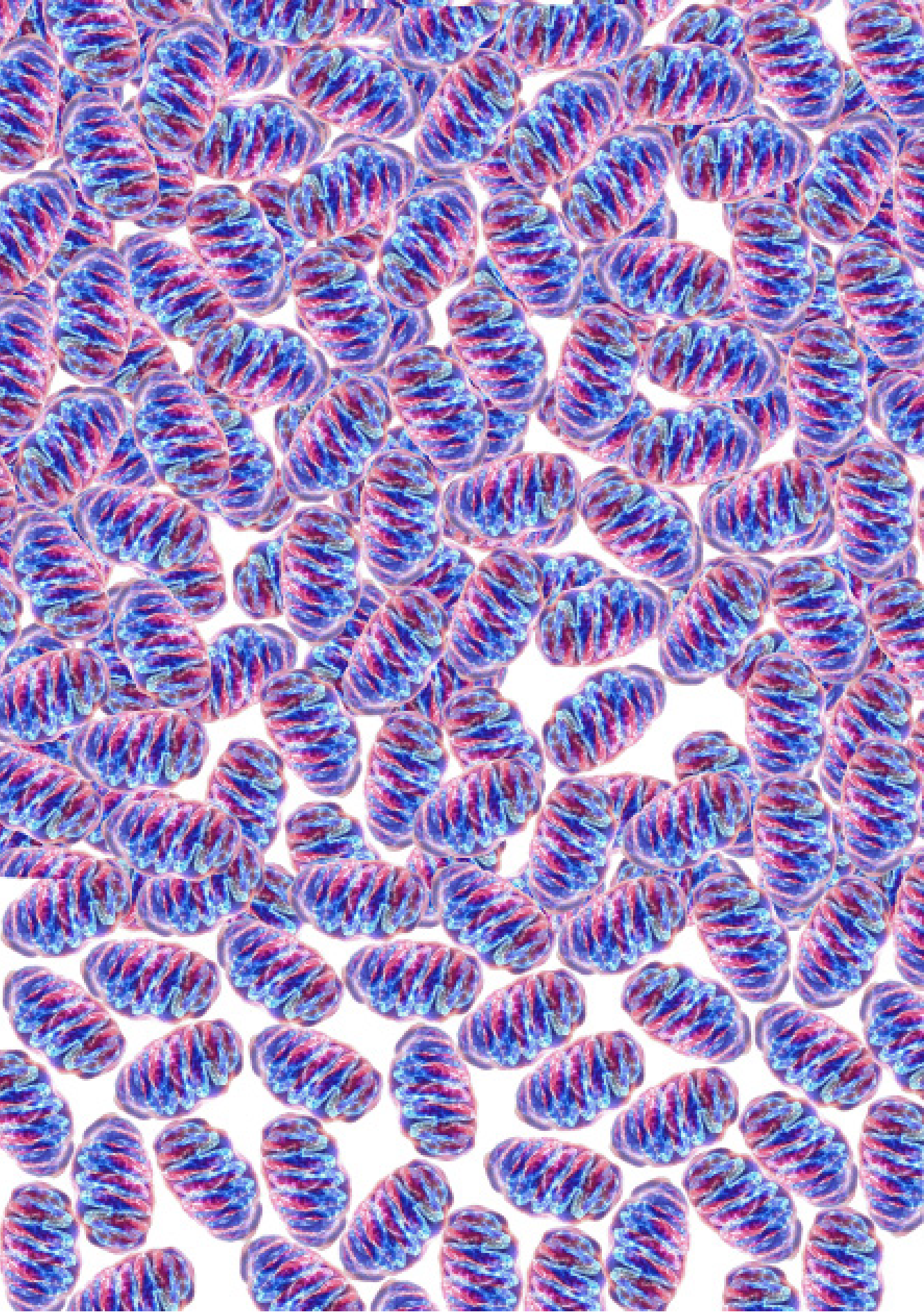
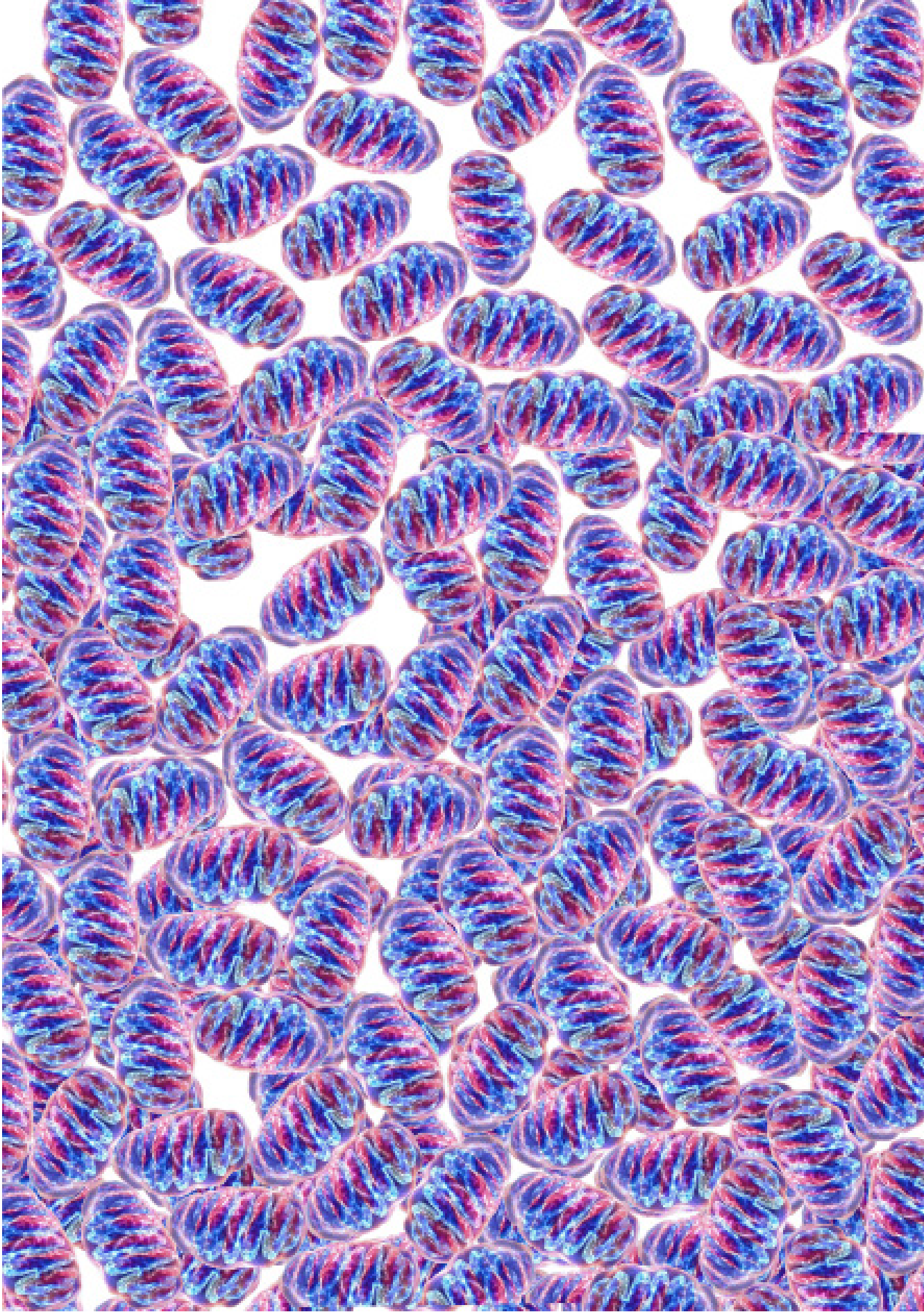



**Figure 3.3.14 – Mitochondrial dysfunction increases the susceptibility of fatty livers to I/R injury.** NAD + administration increase SIRT3 activity , leading to decreased binding of CypD to the ANT, preventing MPT induction.





# 4

# GENERAL DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) is a common clinical condition with histological features that resemble those of alcohol-induced liver injury, but occurs in patients who do not drink an excessive amount of alcohol. It is now considered to be the hepatic representation of the metabolic syndrome, since it is often associated with obesity, insulin resistance, dyslipidemia and hypertension (Levene et al., 2012). The increased incidence of NAFLD and the shortage of liver donors, has led to the acceptance of steatotic livers for transplantation, although steatosis is an important risk factor, associated with decreased recovery of function (McCormack et al., 2011). Similarly, liver resection in patients with steatosis is associated with higher risk of postoperative mortality when compared with patients with non-fatty livers. Such low tolerance to acute injury is probably related with an underlying state of mitochondrial dysfunction induced by fat accumulation (Teodoro et al., 2006; Teodoro et al., 2008). Dysfunctional mitochondria exhibit impaired OXPHOS capacity and increased rates of ROS generation that enhance the susceptibility of hepatocytes to cell death by necrosis, via ATP depletion, or by apoptosis, via release of cytochrome c after mitochondrial swelling. Therefore, in the context of organ transplantation or hepatic surgery, patients with fatty livers are at a higher risk of diminished liver function caused by I/R, a major and unavoidable clinical problem, whether donor livers are lean or steatotic. Fatty hepatocytes have reduced tolerance to ischemic injury with a predominant necrotic form of cell death (Selzner et al., 2000). In particular, I/R injury induces a dramatic increase in mitochondrial permeability, thereby instigating a chain of events that leads to both apoptotic and necrotic death (Varela et al., 2011). The increase in ROS generation induced by I/R further damages lipids, proteins and nucleic acids, perpetuating mitochondrial dysfunction and decreasing hepatocyte viability, which is aggravated by increased release of pro-inflammatory cytokines .Induction of the MPT is now known to mediate the lethal permeability changes that initiate mitochondrial-driven death in I/R injury.In fact, one of the major differences between fatty and lean livers is the higher susceptibility to the MPT following I/R (Rolo et al., 2009). I/R injury is associated with increases in MPT pore activators (calcium, ROS, Pi) and reductions in MPT pore inhibitors (ATP/ADP). While the MPT pore is believed to be quiescent during ischemia, inhibited by low pH, the restoration of pH coupled with the rapid elevation in mitochondrial calcium and ROS leads to MPT induction upon reperfusion. This causes cell death by uncoupling oxidative phosphorylation, inducing

mitochondrial swelling and collapsing mitochondrial membrane potential, thus blocking cellular ATP formation. Such impaired mitochondria have the potential to further damage the population of healthy mitochondria, by exposure to accelerated ROS generation and increased calcium concentrations, creating an environment of dysfunctional mitochondria that decreases the possibility of recovering organ function following I/R. Therefore, the MPT represents an obvious therapeutic target for inhibition of hepatocyte mortality and treatment of ischemia-reperfusion.

Over the past decade, intensive mitochondrial research has shown that mitochondria are not only the major suppliers of energy to a cell, but are also pivotal in the cell's decision to live or die. Maintaining a healthy population of mitochondria is essential to the well being of cells. Indeed, it is now well recognized that besides its crucial role in the pathogenesis of several diseases including I/R injury, protective pathways converging on mitochondria may preserve their function and drastically reduce the lethal I/R injury .This type of protection can be obtained with brief intermittent ischemia (IPC) or with pharmacological agents such as adenosine receptors, kinases and MPT modulation. In this regard, this work aimed to ameliorate the fatty condition to produce better outcomes following I/R, by identification of mitochondrial-dependent protective mechanisms.

First, it was crucial to define the functional mitochondrial mechanisms of IPC to allow drug targeting in a pharmacological treatment in fatty livers as a strategy to reduce I/R injury. By comparing IPC and pharmacological PC (modulation of $A_1R$), (chapter 3.1) we observed that both strategies improved OXPHOS capacity, maintaining ATP content, decreasing ROS generation and caspase-3 activation, as well as the release of markers of hepatocellular damage into the plasma. Selective blocking of $A_1R$ with DPCPX abolished the protection achieved by IPC, while the $A_1R$ agonist CCPA reproduced its beneficial effects. The preservation of phosphorylative efficiency by PC was associated with an increased concentration dependence of the effect of CAT on ADP-induced depolarization in energized mitochondria, comparatively to the I/R condition. This indicates inhibition of ADP transport by I/R that is prevented by PC. It has been previously shown that PC also preserves the ATPsynthase activity, which further contributes to an improved phosphorylative system (Rolo et al., 2009). CAT induces MPT induction by facilitating changes in the conformational state of the ANT, stabilizing the "c" conformation where the nucleotide binding site faces the cytoplasmic side of the membrane (Zoratti and Szabó, 1995). The calcium-dependency of the MPT suggested that there might be a calcium trigger site exposed on the ANT associated

with the “c” conformation (Halestrap and Brenner, 2003). The requirement for the ANT in MPT induction was questioned when liver mitochondria of mice genetically modified to lack the two major isoforms of ANT (ANT1 and ANT2) still exhibited CyA-sensitive MPT (Kokoszka et al, 2004). However, ANT-deficient mitochondria require much higher calcium concentrations to induce MPT, suggesting a regulatory role for the ANT in MPT induction, sensitizing to adenine nucleotides and ANT ligands (Kokoszka et al., 2004; Baines and Molkentin, 2009). Both ischemic and pharmacological PC increased the threshold for MPT induction, suggesting that the observed inhibition of the ANT could be somehow related with prevention of the MPT by PC. In view of this, upstream players, activated by PC that could be related with MPT modulation were studied. Heat shock proteins (HSP) are a family of chaperone proteins induced by hyperthermia, oxidative stress, I/R that inhibit cell death and prevent cytochrome c release by mitochondria, and preserve its integrity and function (Kregel, 2002; Polla et al., 1996). HSP upregulation has been proposed as a mediator of preconditioning beneficial action (Kim et al., 2006). Direct regulation of pore conductance has been proposed for HSP modulation of MPT induction (He and Lemasters, 2002). This model proposed that stress induces misfolding of mitochondrial membrane proteins that cluster to form aqueous pores that are permeable to ions and molecules up to a molecular mass of about 1500 Da. Chaperone proteins and CypD bind to the pore complex to shut off conductance. However, increased $Ca^{2+}$ causes CypD to perturb the MPT pore complex to an open conductance state, an effect that is antagonized by CyA. As the number of misfolded protein clusters exceeds the number of chaperones available to block MPT conductance, unregulated MPT induction occurs. IPC induced the increase of HSP70 that could explain the resistance to MPT induction. However, pharmacological preconditioning by CCPA, although preventing calcium-induced MPT did not upregulate HSP70, suggesting that other mechanisms of regulation of the MPT were associated with PC.

In the setting of cardiac I/R, inhibition of GSK-3β through a PI3-kinase–Akt dependent pathway has been shown to be cardioprotective (Tong et al., 2002; Gross et al., 2004; Xi et al, 2009). Activation of the PI3K/Akt mediates PC-induced phosphorylation of GSK-3β on serine 9 and inactives it; an increase in the inactivated form of GSK-3β in mitochondria mediates convergence of protection signaling to inhibit the MPT (Juhaszova et al., 2004), by interaction of translocated GSK-3β with putative MPT components. Evaluation of GSK-3β and Akt status after I/R or PC showed that both pharmacological and IPC induced an

increase in active Akt and inactive mitochondrial GSK-3β, demonstrating that modulation of MPT is critical in PC (ischemic and pharmacological) protective signaling during hepatic I/R, and that it can be achieved by a GSK-3β-dependent manner.

With the goal of identifying successful therapeutic strategies to prevent hepatic I/R injury, we evaluated the effect of treatment of lean livers, before I/R, with indirubin-3'-oxime, an inhibitor of GSK-3β (chapter 3.2). This was based on the fact that pharmacological inhibitors of GSK-3β also increase phospho-GSK-3β (Zhang et al., 2003). Indirubin-3'-oxime was shown to protect the liver by maintaining mitochondrial calcium homeostasis, thus preserving mitochondrial function and hepatic energetic balance. I/R induced translocation of GSK-3β from the cytosol to the mitochondria, increasing mitochondrial GSK-3β activity. On the other hand, indirubin-3'-oxime preserved the level of inhibition of GSK-3β activity on mitochondria, by preserving the mitochondrial pool of phospho-Ser9-GSK-3β. Therefore, GSK-3β inactivation by indirubin-3'-oxime acts as pharmacological preconditioning, modulating the susceptibility to the MPT and preserving mitochondrial function after I/R. Although fatty livers exhibit mitochondrial dysfunction that enhances I/R injury, treatment with indirubin-3'-oxime was also a protective strategy in fatty livers, thus improving the efficiency of mitochondrial function.

We then sought to identify which type of interaction between translocated GSK-3β and the MPT would mediate the protective action of PC. Although it has been proposed that GSK-3β could interact with VDAC, VDAC does not appear to be necessary for MPT induction (Krauskopf et al., 2006; Baines et al., 2007), ruling out this type of interaction as MPT modulator. However, it has been shown that GSK-3β regulates VDAC and modulates transport through the outer membrane of the mitochondria, suggest that VDAC may be an important regulator in I/R injury (Das et al., 2008). Physical interaction of phospho-Ser9-GSK-3β with the ANT has been proposed to increase the MPT threshold by reducing ANT affinity to CypD (Nishiara et al.,2007; Terashima et al., 2010). Early on, it was demonstrated that ANT binds CypD in a CyA-sensitive manner and that role of CypD in MPTP formation is to induce a conformational change in the ANT (Crompton et al.,1998; Woodfiels et al., 1998). Furthermore, the proposed association between the mitochondrial phosphate carrier (PiC) and the ANT2 (liver mitochondria), than ANT1 (heart mitochondria) was proposed as the explanation for the increased sensitivity of liver mitochondria to the MPT, highlighting the importance of MPT targeting in the setting of I/R (Basso et al., 2008).

Based on the fact that CypD activity is modulated by phosphorylation via ERK/GSK-3β pathway, with Ser/Thr phosphorylation of CypD induced by GSK-3β favoring the MPT pore opening (Rasola et al., 2010), CypD status was evaluated after I/R in fatty livers. Active GSK-3β (in I/R situation) promoted an enhancement of CypD phosphorylation, resulting in increased CypD immunoprecipitated with the ANT and consequently MPT induction. Pharmacological preconditioning with indirubin-3'-oxime, maintained the levels of inhibited mitochondrial GSK-3β, inhibited the interaction CypD–ANT and preserved energetic balance in I/R fatty livers.

Dissociation of CypD from the ANT is also induced when SIRT3 in a $NAD^+$-dependent-manner deacetylates CypD (Shulga et al., 2010a; Shulga et al., 2010b; Hafner et al., 2010). Obesity decreases the cellular NAD+/NADH ratio, thereby contributing to a decrease in SIRT3 activity that increases the acetylation of mitochondrial proteins, and decreases mitochondrial efficiency. By exploring the role of the SIRT3-CypD-MPT axis in the protection against I/R injury in fatty livers, we observed that the increase in CypD acetylation in I/R group was accompanied by an inhibition of SIRT3 activity. (chapter 3.3) Conversely, adding $NAD^+$ to fatty livers prior to I/R significantly increases SIRT3 activity and decreases CypD acetylation, activity and binding of CypD to ANT, increasing the threshold to MPT induction. $NAD^+$ modulates mitochondrial function and preserves oxidative phosphorylation being protective against injury in a model of hepatic warm I/R.

In conclusion, modulation of CypD activity is a clear preventive strategy against I/R damage in fatty livers. Decreasing CypD phosphorylation (by inhibition of GSK-3β with indirubin-3'-oxime) or acetylation (by increasing SIRT3 deacetylase activity), increases the resistance to MPT induction. Since I/R injury is associated with increases in MPT pore activators (calcium, ROS, Pi) and reductions in MPT pore inhibitors, aggravated by fatty liver induced-mitochondrial dysfunction, CypD modulation has great therapeutic potential in the clinical setting of hepatic ischemia/reperfusion, acting as pharmacological preconditioning in lean and fatty livers.

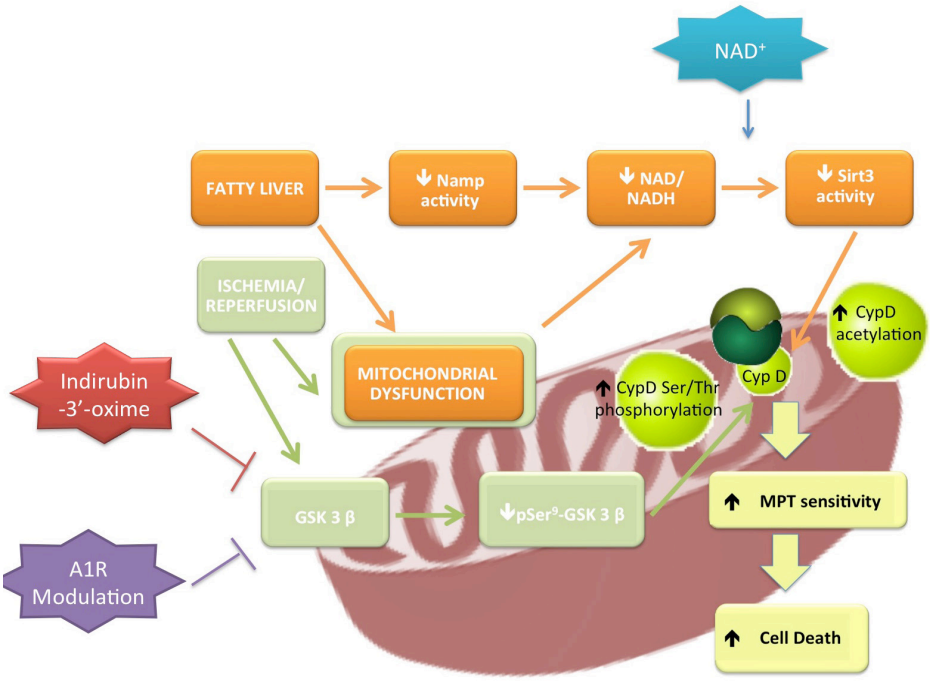
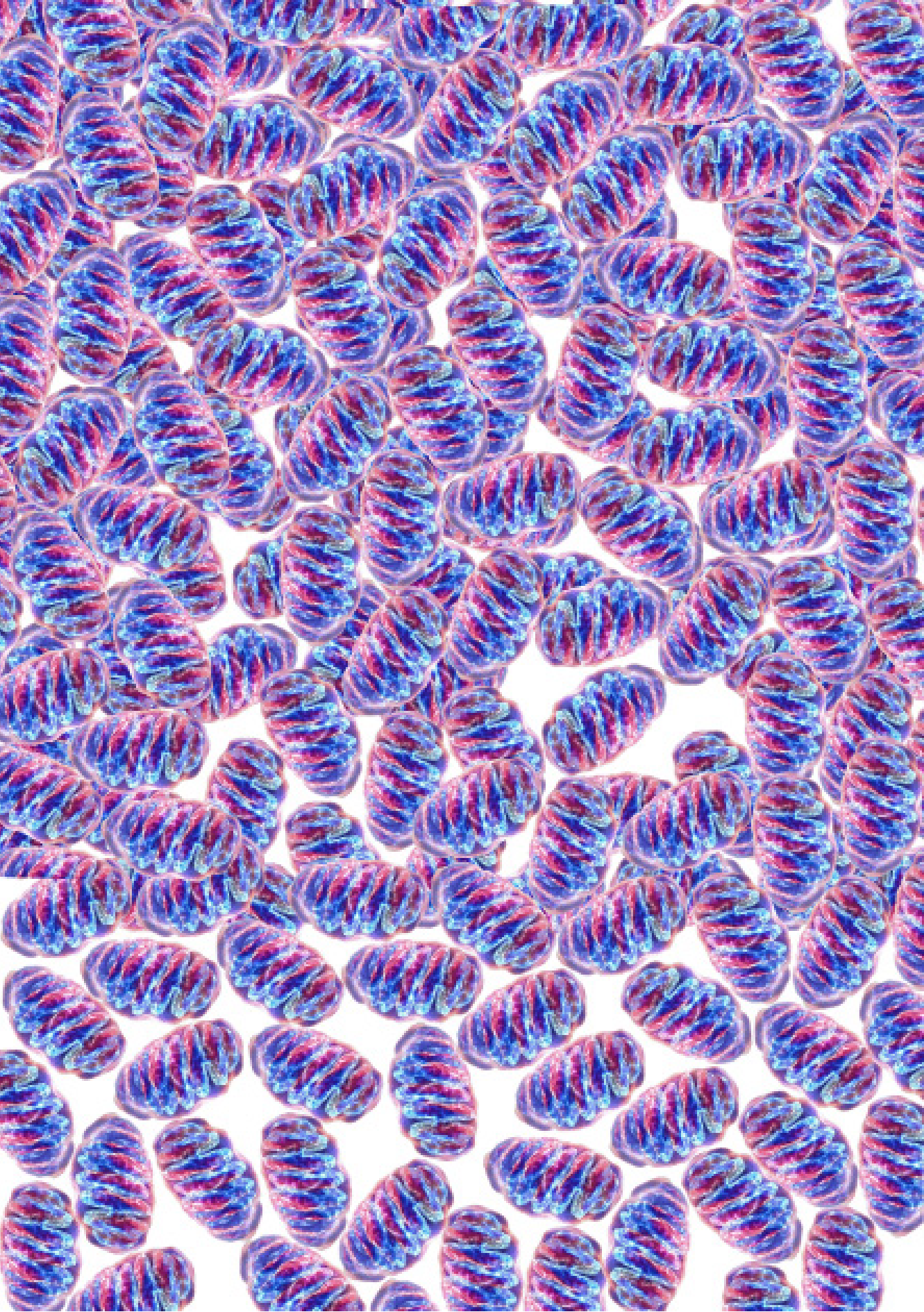
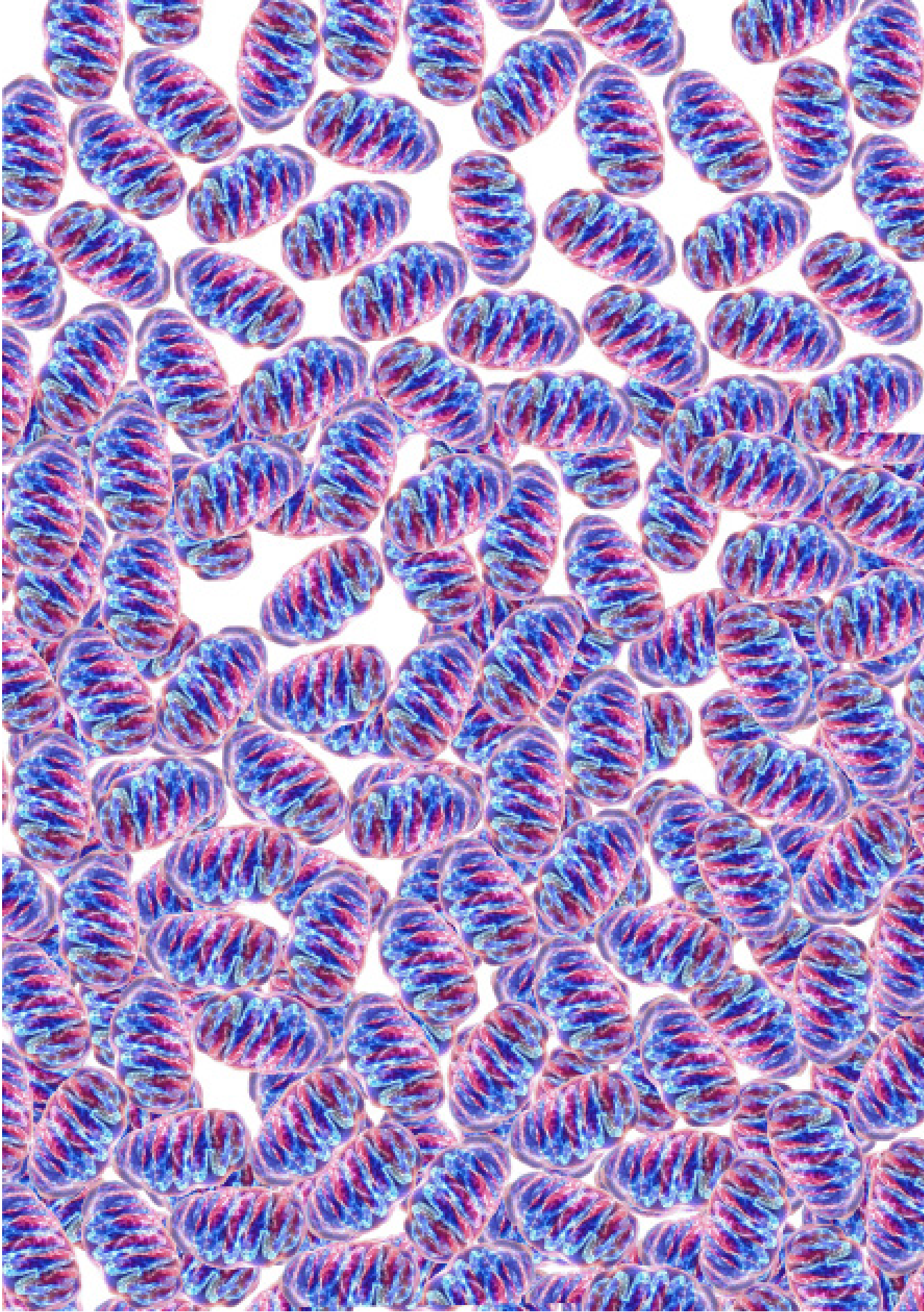



**Figure 4.1 – Preconditioning (ischemic and pharmacological) protects from I/R injury via activation of signaling cascades that converge to the mitochondria. This increase the resistence to MPT induction, thereby preventing cell death.**





Acín-Pérez R.; Fernández-Silva P.; Peleato M.L.; Pérez-Martos A.; Enriquez J.A. Respiratory active mitochon-drial supercomplexes. Molecular Cell, 2008, 32(4), 529– 539.

Aggarwal B.B. Tumour necrosis factors receptor associated signaling molecules and their role in activation of apoptosis, JNK and NF-kappaB. Annals of the Rheumatic Diseases, 2000, 59 (Suppl 1), i6–i16.

Akao M.; O'Rourke B.; Kusuoka H.; Teshima Y.; Jones S.P.; Marbán E. Differential actions of cardioprotective agents on the mitochondrial death pathway. Circ Res, 2003, 92, 195–202.

Anderson C.D.; Belous A.; Pierce J.; Nicoud I.B.; Knox C.; Wakata A.; Pinson C.W.; Chari R.S. Mitochondrial calcium uptake regulates cold preservation-induced Bax translocation and early reperfusion apoptosis. American Journal of Transplantation, 2004, 4, 52-362.

Anderson C.D.; Pierce J.; Nicoud I.; Belous A.; Knox C.D.; Chari R.S. Modulation of mitochondrial calcium management attenuates hepatic warm ischemia-reperfusion injury. Liver Transpl., 2005, 11(6), 663-8.

Anderson L. Identification of mitochondrial proteins and some of their precursors in two-dimensional electrophoretic maps of human cells. Proc. Natl. Acad. Sci. USA, 1981, 78, 2407-2411.

Andraus W.; de Souza G.F.; de Oliveira M.G.; Haddad L.B.; Coelho A.M.; Galvão F.H.; Leitão R.M.; D'Albuquerque L.A.; Machado M.C. S-nitroso-N-acetylcysteine ameliorates ischemia-reperfusion injury in the steatotic liver. Clinics, 2010, 65(7), 715-721.

Arai M.; Thurman R.G.; Lemasters J.J. Involvement of Kupffer cells and sinusoidal endothelial cells in ischemic preconditioning to rat livers stored for transplantation. Transplant Proc. 1999, 31, 425–427.

Arkadopoulos N.; Kostopanagiotou G.; Theodoraki K.; Farantos C.; Theodosopoulos T.; Stafyla V.; et al. Ischemic preconditioning confers antiapoptotic protection during major hepatectomies performed under combined inflow and outflow exclusion of the liver. World J. Surg., 2009, 33, 1909-1915.

Badiola N.; Malagelada C.; Llecha N.; Hidalgo J.; Comella J.X.; Sabriá J.; Rodríguez-Alvarez J. Activation of caspase-8 by tumour necrosis factor receptor 1 is necessary for caspase-3 activation and apoptosis in oxygen-glucose deprived cultured cortical cells. Neurobiol Dis., 2009, 35(3), 438-47.

Baines C.P.; Kaiser R.A.; Purcell N.H.; Blair N.S.; Osinska H.; Hambleton M.A.; Brunskill E.W.; Sayen M.R.; Gottlieb R.A.; Dorn G.W.; Robbins J.; Molkentin J.D. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature, 2005, 434, 658–662.

Baines C.P.; Kaiser R.A.; Sheiko T.; Craigen W.J; Molkentin J.D. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat. Cell Biol., 2007, 9, 550–555.

Balaban R.S. The role of Ca(2+) signaling in the coordination of mitochondrial atp production with cardiac work. Biochim Biophys Acta, 2009, 1787, 1334–1341.

Banga N.R.; Homer-Vanniasinkam S.; Graham A.; Al-Mukhtar A.; White S.A.; Prasad K.R. Ischaemic preconditioning in transplantation and major resection of the liver. British Journal of Surgery, 2005, 92 (5), 528–538.

Banga N.R.; Homer-Vanniasinkam S.; Graham A.; Al-Mukhtar A.; White S.A.; Prasad K.R. Ischaemic preconditioning in transplantation and major resection of the liver. British Journal of Surgery, 2005, 92 (5), 528–538.

Bao H.; Ge Y.; Zhuang S.; Dworkin L.D.; Liu Z. et al. Inhibition of glycogen synthase kinase-3β prevents NSAID-induced acute kidney injury. Kidney International, 2012, 81, 662-673.

Bao J.; Scott Lu Z.; Pang L.; Dimond C.C.; Gius D.; Sack M.N. SIRT3 is regulated by nutrient excess and modulates hepatic susceptibility to lipotoxicity. Free Radic. Biol. Med., 2010, 49(7), 1230-1237.

Barillas R.; Friehs I.; Cao-Danh H.; Martinez J.F.; Del Nido P.J. Inhibition of glycogen synthase kinase-3beta improves tolerance to ischemia in hypertrophied hearts. The Annals of Thoracic Surgery, 2007, 84, 126-133.

Basso E.; Fante L.; Fowlkes J.; Petronilli V.; Forte M.A.; Bernardi P. Properties of the permeability transition pore in mitochondria devoid of cyclophilin D. J. Biol. Chem., 2005, 280,18558-18561.

Basso E.; Petronilli V.; Forte M.A.; Bernardi P. Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. J. Biol. Chem., 2008, 283(39), 26307–11.

Becker L.B. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. Cardiovasc. Res., 2004, 61, 461-470.

Behrns K.E.; Tsiotos G.G.; DeSouza N.F.; Krishna M.K.; Ludwig J.; Nagorney D.M. Hepatic steatosis as a potential risk factor for major hepatic resection. J. Gastrointest Surg., 1998, 2, 292-298.

Bernardi P. Mitochondrial transport of cations: Channels, exchangers, and permeability transition. Physiol Rev, 1999, 79, 1127–1155.

Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. J. Biol. Chem., 1992, 267(13), 8834–9.

Bernardi P.; Krauskopf A.; Basso E.; Petronilli V.; BlalchyDyson E.; DiLisa F.; Forte M.A. The mitochondrial permeability transition from in vitro artifact to disease target. FEBS J., 2006, 273, 2077-2099.

Beutner G.; Sharma V.K.; Giovannucci D.R.; et al. Identification of a ryanodine receptor in rat heart mitochondria. J. Biol. Chem., 2001, 276, 21482–21488.

Bijur G.N.; Jope R.S. Glycogen synthase kinase-3 beta is highly activated in nuclei and mitochondria. NeuroReport, 2003, 278, 2415-2419.

Bijur G.N.; Jope R.S. Rapid accumulation of Akt in mitochondria following phosphatidyl-inositol 3-kinase activation. J. Neurochem, 2003, 87,1427-1435.

Blachly-Dyson E.; Forte M. VDAC channels. IUBMB Life, 2001, 52, 113–118.

Bolli R. The late phase of preconditioning. Circ. Res., 2000, 87, 972-983.

Borutaite V. ; Brown G.C. Mitochondria in apoptosis of ischemic heart. FEBS Letters, 2003, 541, 1-5.

Boveris A.; Cadenas E.; Stoppani A.O. Role of ubiquinone in the mitochondrial

generation of hydrogen peroxide. Biochem. J., 1976, 156, 435-444.

Boyer P.D. ATP-synthase – past and future. Biochim. Biophys. Acta, 1998, 1365, 3-9.

Boyer P.D. The ATP synthase – a splendid molecular machine. Ann. Rev. Biochem., 1997, 66, 717-749.

Bradham C.A.; Plumpe J.; Manns M.P.; Brenner D.A.; Trautwein C. Mechanisms of hepatic toxicity. I. TNF-induced liver injury. American Journal of Physiology, 1998, 275 (3 Pt 1), G387-G392.

Bradham C.A.; Schemmer P.; Stachlewitz R.F.; Thurman R.G.; Brenner D.A. Activation of nuclear factor-kappaB during orthotopic liver transplantation in rats is protective and does not require Kupffer cells. Liver Transplantation and Surgery, 1999, 5 (4), 282-293.

Browning J.D. & Horton J.D. Molecular mediators of hepatic steatosis and liver injury. J. Clin. Invest., 2004, 114, 147-152.

Brunt E.M.; Tiniakos D.G. Histopathology of nonalcoholic fatty liver disease. World J. Gastroenterol., 2010, 16(42), 5286-5296.

Caldwell S.H.; Chang C.Y.; Nakamoto R.K.; Krugner-Higby L. Mitochondria in nonalcoholic fatty liver disease. Clin. Liver Dis., 2004, 8, 595-617.

Caldwell-Kenkel J.C.; Currin R.T.; Tanaka Y.; Thurman R.G.; Lemasters J.J. Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. Hepatology, 1991, 13 (1), 83-95.

Canelo R.; Braun F.; Sattler B.; Klinge B.; Lorf T.; Ramadori G.; et al. Is a fatty liver dangerous for transplantation? Transplant Proc., 1999, 31, 414–415.

Caraceni P.; Bianchi C.; Domenicali M.; Maria Pertosa A.; Maiolini E.; Parenti Castelli G.; et al. Impairement of mitochondrial oxidative phosphorylation in rat fatty liver exposed to preservation-reperfusion injury. J. Hepatol., 2004, 41, 82-88.

Caraceni P.; Domenicali M.; Vendemiale G.; Grattagliano I.; Pertosa A.; Nardo B.; et al. The reduced tolerance of rat fatty liver to ischemia reperfusion is associated with mitochondrial oxidative injury. J Surg Res., 2005, 124, 160-168.

Caraceni P.; Nardo B.; Domenicali M.; Turi P.; Vici M.; Simoncini M.; De Maria N.; Trevisani F.; Van Thiel D.H.; Derenzini M.; Cavallari A.; Bernardi. Ischemia-reperfusion injury in rat fatty liver: role of nutritional status. Hepatology, 1999, 29(4), 1139.

Carini R.; Grazia De Cesaris M.; Splendore R.; Domenicotti C.; Nitti M.P.; Pronzato M.A.; Albano E. Signal pathway responsible for hepatocyte preconditioning by nitric oxide. Free Radic. Biol. Med., 2003, 34, 1047-1055.

Cesura A.M.; Pinard E.; Schubenel R.; Goetschy V.; Friedlein A.; Langen H.; et al. The voltage-dependent anion channel is the target for a new class of inhibitors of the mitochondrial permeability transition pore. J. Biol. Chem., 2003, 278(50), 49812–8.

Chance B.; Williams G.R. Respiratory enzymes in oxidative phosphorylation. VI. The effects of adenosine diphosphate on azide-treated mitochondria. J. Biol. Chem., 1956, 221, 477-489.

Chappell J.B.; Crofts A.R. Calcium ion accumulation and volume changes of isolated liver mitochondria: calcium ion-induced swelling. Biochem J., 1965, 95, 378–86.

Chavin K.D.; Yang S.; Lin H.Z.; Chatham J.; Chacko V.P.; Hoek J.B.; Walajtys-Rode E.; Rashid A.; Chen C.H.; Huang C.C.; Wu T.C.; Lane M.D.; Diehl A.M. Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. J. Biol. Chem., 1999, 274, 5692-5700.

Chen H.; Xing B.; Liu X.; Zhan B.; Zhou J.; Zhu H.; Chen Z. Ischemic postconditioning inhibits apoptosis after renal ischemia/reperfusion injury in rat. Transpl. Int., 2008, 21, 364-371.

Chen D.H.; Xie J.X. Chinese Trad. Herb. Drugs, 1984, 15, 6.

Chen Q.; Moghaddas S.; Hoppel C.L.; Lesnefsky E.J. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. American Journal of Physiology Cell Physiology, 2008, 294, C460-C466.

Choi C.H; Jung Y.K.; Oh S.H. Autophagy induction by capsaicin in malignant human breast cells is modulated by p38 and extracellular signal-regulated mitogen-

activated protein kinases and retards cell death by suppressing endoplasmic reticulum stress-mediated apoptosis. Mol. Pharmacol., 2010, 78, 114-125.

Choudhury M.; Jonscher K.R.; Friedman J.E. Reduced mitochondrial function in obesity-associated fatty liver: SIRT3 takes on the fat. Aging, 2011, 3, 175-178.

Choudhury S.; Bae S.; Ke Q.; Lee J.Y.; Kim J.; Kang P.M. Mitochondria to nucleus translocation of AIF in mice lacking Hsp70 during ischemia/reperfusion. Basic Res. Cardiol., 2011, 106(3), 397-407.

Chung H.T.; Pae H.O.; Choi B.M.; Billiar T.R., Kim Y.M. Nitric oxide as a bioregulator of apoptosis. Biochemical and Biophysical Research Communications, 2001, 282 (5), 1075-1079.

Cimen H.; Han M.J.; Yang Y.; Tong Q.; Koc H.; Koc E.C. Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria. Biochemistry, 2010, 49, 304-311.

Clarke S.J.; McStay G.P.; Halestrap A.P. Sanglifehrin A acts as a potent inhibitor of the mitochondrial permeability transition and reperfusion injury of the heart by binding to cyclophilin-D at a different site from cyclosporin A. J. Biol. Chem., 2002, 277(38), 34793–9.

Clavien P.A.; Yadav S.; Sindram D.; Bentley R.C. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. Ann. Surg., 2000, 232, 155-162.

Clemens M.G.; Zhang J.X. Regulation of sinusoidal perfusion: in vivo methodology and control by endothelins. Semin. Liver Dis., 1999, 19, 383-396.

Cohen M.V.; Downey J.M. Adenosine: trigger and mediator of cardioprotection. Basic Res. Cardiol. 2008, 103, 203-215.

Cohen M.V; Baines C.P; Downey J.M. Ischemic precondi-tioning: from adenosine receptor to KATP channel. Annu. Ver. Physiol, 2000, 62, 79-109.

Coito A.J.; Buelow R.; Shen X.D.; Amersi F.; Moore C.; Volk H.D.; Busuttil R.W.; Kupiec-Weglinski J.W. Heme oxygenase-1 gene transfer inhibits inducible nitric oxide synthase expression and protects genetically fat Zucker rat livers from ischemia-reperfusion injury. Transplantation, 2002, 74 (1), 96-102.

Connern C.P.; Halestrap A.P. Purification and N-terminal sequencing of peptidyl-prolyl cis-trans-isomerase from rat liver mitochondrial matrix reveals the existence of a distinct mitochondrial cyclophilin. Biochem. J., 1992, 284, 381-385.

Cortez-Pinto H.; Chatham J.; Chacko V.P.; Arnold C.; Rashid A.; Diehl A.M. Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. JAMA., 1999, 282, 1659-1664.

Crisostomo P.R.; Wairiuko G.M.; Wang M.; Tsai B.M.; Morrell E.D.; Meldrum D.R. Preconditioning versus postconditioning: mechanismsand therapeutic potentials. J. Am. Coll. Surg., 2006, 202, 797-812.

Crompton M.; McGuinness O.; Nazareth W. The Involvement of cyclosporin A binging proteins in regulating and uncoupling mitochondrial energy transduction. Biochim. Biophys. Acta, 1992, 1101, 214-217.

Crompton M.; Virji S.; Doyle V.; Johnson N.; Ward J.M. The mitochondrial permeability transition pore. Biochem. Soc. Symp., 1999, 66, 167-179.

Crompton M.; Virji S.; Ward J.M. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur. J. Biochem., 1998, 1, 258(2), 729-735.

Cutrin J.C.; Perrelli M.G.; Cavalieri B.; Peralta C.; Rosello-Catafau J.; Poli G. Microvascular dysfunction induced by reperfusion injury and protective effect of ischemic preconditioning. Free Radical Biology and Medicine., 2002, 33 (9), 1200-1208.

D'Alessandro A.M.; Kalayoglu M.; Sollinger H.W.; Hoffmann R.M.; Reed A.; Knechtle S.J.; et al.The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation, 1991, 51, 157-163.

Dahl T.B.; Haukeland J.W.; Yndestad A.; Ranheim T.; Gladhaug I.P.; Damås J.K.; Haaland T. et al. Intracellular nicotinamide phosphoribosyltransferase protects against hepatocyte apoptosis and is down-regulted in non-alcoholic fatty liver disease. J. Clin. Endocrinol Metab., 2010, 95, 3039-3047.

Dajani R.; Fraser E.; Roe S.M.; Young N.; Good V.; Dale T.C.; Pearl L.H. Crystal structure of glycogen synthase kinase 3beta: structural basis for phosphate-

primed substrate specificity and autoinhibition. Cell, 2001, 105, 721–732.

Das S.; Wong R.; Rajapakse N.; Murphy E.; Steenbergen C. Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation. Circ. Res., 2009, 103, 983-991.

D'Autréaux B.; Toledano M. B. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol., 2007, 8, 813–824.

Degle Esposti D.; Sebagh M.; Pham P.; Reffas M.; Pous C.; Azzoulay D.; Lemoine A. Ischemic preconditioning induces autophagy and limits necrosis in human recipients of fatty liver grafts,decreasing the incidence of rejection episodes. Cell Death Dis., 2011.

Demetris A.J.; Adeyi O.; Bellamy C.O.; Clouston A.; Charlotte F.; et al. Liver biopsy interpretation for causes of late liver allograft dysfunction. Hepatology, 2006, 44, 489–501.

Denton R.M. Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta, 2009, 1787, 1309–1316.

Dhalla N.S.; Elmoselhi A.B.; Hata T.; Makino N. Status of myocardial antioxidants in ischemia–reperfusioninjury. Cardiovascular Research, 2000, 47, 446-456.

Di Lisa F.; Canton M.; Menabo R.; Kaludercic N.; Bernardi P. Mitochondria and cardioprotection. Heart Fail Rev, 2007, 12(3–4), 249–60.

Diehl, A.M. Cytokine regulation of liver injury and repair. Immunological Reviews, 2000, 174, 160-171.

Doble B.W.; Woodgett J.R. GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci., 2003, 116, 1175-1186.

Downey J.M. The cellular mechanisms of ischaemic and pharmacological precon- ditioning. Cardiovasc. J. S. Afr., 2004, 15, S3.

Dudkina N. V.; Sunderhaus S.; Boekema E.J.; Braun H.P. The higher level of organization of the oxidative phosphorylation system: mitochondrial

supercomplexes. Journal of Bioenergetics and Biomembranes, 2008, 40(5), 419–424.

Echtay K.S.; Esteves T.C.; Pakay J.L.; Jekabsons M.B.; Lambert A.J.; Portero Otin M.; Pamplona R.; Vidal Puig A.J.; Wang S.; Roebuck S.J.; Brand M.D. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. EMBO J., 2003, 22, 4103-4110.

Estabrook R.W. Mitochondrial respiratory control and the polarographic measurements of ADP/O ratios. Methods Enzymol, 1967, 10, 41-47.

Fan C.; Zwacka R.M.; Engelhardt J.F. Therapeutic approaches for ischemia/reperfusion injury in the liver. Journal of Molecular Medicine, 1999, 77(8), 577-592.

Farber J.L. Membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. Lab. Invest., 1982, 47, 114-123.

Feldstein A.E. Novel insights into pathophysiology of nonalcoholic fatty liver disease. Semin. Liver Dis., 2010, 30, 391-401.

Fernandez L.; Heredia N.; Grande L.; Gomez G.; Rimola A.; Marco A.; Gelpi E.; Rosello-Catafau J.; Peralta C. Preconditioning protects liver and lung damage in rat liver transplantation: role of xanthine/xanthine oxidase. Hepatology, 2002, 36 (3), 562–572.

Fiore C.; Trézéguet V.; Le Saux A.; Roux P.; Schwimmer C.; Dianoux A.C.; Noel F.; Lauquin G.J.; Brandolin G.; Vignais P.V. The mitochondrial ADP/ATP carrier: structural, physiological and pathological aspects. Biochimie, 1998, 80, 137–150.

Fontaine E.; Ichas F.; Bernardi P. A ubiquinone-binding site regulates the mitochondrial permeability transition pore. J. Biol. Chem., 1998, 273(40), 25734–40.

Forbes R.A.; Steenbergen C.; Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. Circ. Res., 2001, 88, 802-809.

Frey T.G.; Mannella C. A. The internal structure of mitochondria. Trends Biochem. Sci., 2000, 25, 319-324.

Fukumori T.; Ohkohchi N.; Tsukamoto S.; Satomi S. Why is a liver with steatosis susceptible to cold ischemic injury? Transplant Proc., 1999, 31(1-2), 548-549.

Fukumori T.; Ohkohchi N.; Tsukamoto S.; Satomi S. Why is fatty liver unsuitable for transplantation? Disorientation of mitochondrial ATP synthesis and sinusoidal structure during cold preservation of a liver with steatosis. Transpl Proc, 1997, 29, 412-415.

Furukawa H.; Todo S.; Imventarza O.; Casavilla A.; Wu Y.M.; Scotti-Foglieni C.; Broznick B.; Bryant J.; Day R. and Starzl T.E. Effect of cold ischemia time on the early outcome of human hepatic allografts preserved with UW solution. Transplantation, 1991, 51, 1000-1004.

Gao W.; Bentley R.C.; Madden J.F.; et al. Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. Hepatology, 1998, 27(6), 1652-1660.

Garcia-Dorado D.; Rodriguez-Sinovas A.; Ruiz-Meana M.; Inserte J.; Agullo L.; Cabestrero A. The end-effectors of preconditioning protectionagainst myocardial cell death secondary to ischemia–reperfusion. Cardiovasc. Res., 2006, 70, 274-285.

Garrido C.; Schmitt E.; Cande C.; Vahsen N.; Parcellier A.; Kroemer G. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. Cell Cycle, 2003, 2, 579-584.

Gazotti P, Malmstron K, Crompton M. A Laboratory Manual on Transport and Bioenergetics. New York, NY:Springer Verlag, 1979. Falta Páginas

Gomez L.; Paillard M.; Thibault H.; Derumeauz G.; Ovize M. Inhibition of GSK3-beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. Circulation., 2008, 117, 2761-2768.

Gornall A.G.; Bardawill C.J.; David M.M. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., 1949, 177, 751-766.

Gosh S.; May M.J.; Koop E.B. NF-Kb and Rel proteins: evolucionary conserved mediators of immune responses. Annu Rev Imunol., 1998, 16, 225-260.

Grattagliano I.; Vendemiale G.; Lauterburg B.H. Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. Journal of Surgical Research, 1999, 86 (1), 2-8.

Green D.R.; Reed J.C. Mitochondria and apoptosis. Science., 1998, 28, 281(5381), 1309-1312.

Griffiths E.J.; Halestrap A.P. Further evidence that cyclosporin-A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase - Implications for the immunosuppressive and toxic effects of cyclosporine. Biochem. J., 1991, 274, 611-614.

Griffiths E.J.; Halestrap A.P. Mitochondrial non-specific pores remain closedduring cardiac ischaemia, but open upon reperfusion. Biochemical Journal, 1995, 307,93–98.

Griffiths E.J.; Halestrap A.P. Protection by cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. J. Mol. Cell Cardiol., 1993, 25(12), 1461–9.

Gross E.R.; Hsu A.K.; Fross G.J. Opioid-induced cardioprotection occurs via glycogen synthase kinase beta during reperfusion in intact rat hearts. Circ. Res., 2004, 94, 960–966.

Hafez T.S.; Glantzounis G.K.; Fusai G.; Taanman J.W.; Wignarajah P.; Parkes H.; Fuller B.; Davidson B.R.; Seifalian A.M. Intracellular oxygenation and cytochrome oxidase C activity in ischemic preconditioning of steatotic rabbit liver. Am. J. Surg., 2010, 4, 507-518.

Hafner A.V.; Dai J.; Gomes A.P.; Xiao C.Y.; Palmeira C.M.; Rosenzweig A.; Sinclair D.A. Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. Aging (Albany NY), 2010, 2(12), 914-923.

Hakamada K.; Sasaki M.; Takahashi K.; Umehara Y.; Konn M. Sinusoidal flow block after warm ischemia in rats with diet-induced fatty liver. Journal of Surgical Research, 1997, 70 (1), 12–20.

Halestrap A. A pore way to die. Nature, 2005, 434, 578-579.

Halestrap A.P. What is the mitochondrial permeability transition pore? J. Mol. Cell

Cardiol., 2009, 46, 821-831.
Halestrap A.P.; Clarke S.J.; Javadov S.A. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovascular Research, 2004, 61, 372-385.

Halestrap A.P.; Davidson A.M. Inhibition of Ca2(+)-induced large amplitude swelling of liver and heart mitochondria by Cyclosporin A is probably caused by the inhibitor binding to mitochondrial matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem. J., 1990, 268, 153-160.

Halestrap A.P.; McStay G.P.; Clarke S.J. The permeability transition pore complex: another view. Biochimie., 2002, 84,153-166.

Haque M.; Sanyal A. The metabolic abnormalities associated with non alcoholic fatty liver disease. Best Pract. Res. Clin. Gastroenlogy., 2002, 16, 709-731.

Harada H.; Wakabayashi G.; Takayanagi A.; Shimazu M.; Matsumoto K.; Obara H.; Shimizu N.; Kitajima M. Transfer of the interleukin-1 receptor antagonist gene into rat liver abrogates hepatic ischemia-reperfusion injury. Transplantation, 2002, 74 (10), 1434–1441.

Hatano E. Tumor necrosis factor signaling in hepatocyte apoptosis. J. Gastroenterol Hepatol., 2007, 22 Suppl 1, S43-44.

Hatefi Y. The mitochondrial electron transport and oxidative phosphorilation system. Ann. Rev. Biochem., 1985, 54, 1015-1069.

Hausenloy D.J.; Yellon D.M.; Mani-Babu S.; Duchen M.R. Preconditioning protects by inhibiting the mitochondrial permeability transition. American Journal of Physiology, 2004, 287, H841-H849.

Haworth R.A.; Hunter D.R. Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria. J. Bioenerg. Biomembr., 2000, 32, 91–96.

Haworth R.A.; Hunter D.R. The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. Arch Biochem Biophys, 1979, 195, 460–7.

He L.; Lemasters J.J. Heat shock suppresses the permeability transition in rat

liver mitochondria. J. Biol. Chem., 2003, 278, 16755.
He L.; Lemasters J.J. Regulated and unregulated mitochondrial permeability transition pores: A new paradigm of pore structure and function? FEBS Lett., 2002, 13, 512.

He S.; Atkinson C.; Evans Z.; Ellett J.D.; Southwood M.; Elvington A.; Chavin K.D.; Tomlinson S..A role for complement in the enhanced susceptibility of steatotic livers to ischemia and reperfusion injury. J. Immunol., 2009, 183(7), 4764-4772.

Hensley K.; Robinson K.A.; Gabbita S.P.; Salsman S.; Floyd R.A. Reactive oxygen species, cell signaling, and cell injury. Free Radic. Biol. Med., 2000, 28, 1456-1462.

Hirschey M.D.; Shimazu T.; Goetzman E.; Jing E.; Schwer B.; Lombard D.B.; Grueter C.A.; et al. SIRT3 regulates mitochondrial fatty–acid oxidation by reversiblr enzyme deacetylation. Nature, 2010, 464, 121-125.

Hoch F.L. Cardiolipins and biomembrane function. Biochim. Biophys. Acta, 1992, 1113, 71-133.

Honda H.M.; Korge P.; Weiss J.N. Mitochondria and ischemia/reperfusion njury. Annals of the New York Academy of Sciences, 2005, 1047, 248-258.

Hool L.C. Reactive oxygen species in cardiac signalling: from mitochondria to plasma membrane ion channels. Clin. Exp. Pharmacol. Physiol., 2006, 33, 146-151.

Hu K.; Zhan E.; McIntosh V.J.; Lasley R.D. Adenosine A2A and A2B receptors are both required for adenosine A1 receptor-mediated cardioprotection. Am. J. Physiol., 2011, 301, H1183-1189.

Huang J.; Klionsky D.J. Autophagy and human disease. Cell Cycle, 2007, 6, 1837-1849.

Iimuro Y.; Nishiura T.; Hellerbrand C.; Behrns K.E.; Schoonhoven R.; Grisham J.W.; Brenner D.A. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. Journal of Clinical Investigation, 1998, 101 (4), 802-811.

Imahashi K.; Schneider M.D.; Steenbergen C.; Murphy E. Transgenic expression

of Bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. Circ. Res., 2004, 95, 734-741.

Inagaki K.; Churchill E.; Mochly Rosen D. Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. Cardiovasc. Res., 2006, 70, 222-230.

Inserte J.; Gacrcia-Dorado D.; Hernando V.; Barba I.; Soler-Soler J. Ischemic preconditioning prevents calpain-mediated impairment of Na+/K+-ATPase activity during early reperfusion. Cardiovasc. Res., 2006, 70, 364-373.

Inserte J.; Garcia-Dorado D.; Ruiz-Meana M.; Padilla F.; Barrabes J.A.; Pina P.; et al. Effect of inhibition of Na+/Ca2+ exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc. Res., 2002, 55, 739-748.

Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. American Journal of Physiology: Gastrointestinal and Liver Physiology, 2003, 284 (1), G15-G26.

Jaeschke H. Reactive oxygen and ischemia/reperfusion injury of the liver. Chemico–Biological Interactions, 1991, 79 (2), 115-136.

Jaeschke H.; Farhood A.; Smith C.W. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J., 1990, 4(15), 3355-3359.

Jaeschke H.; Mitchell J.R. Mitochondria and xanthine oxidase both generate reactive oxygen species in isolated perfused rat liver after hypoxic injury. Biochemical and Biophysical Research Communications, 1989, 160 (1), 140-147.

Jaeschke H.; Smith C.V.; Mitchell J.R. Hypoxic damage generates reactive oxygen species in isolated perfused rat liver. Biochem. Biophys. Res. Commun., 1988, 150(2), 568-574.

Jassem W.; Fuggle S.V.; Rela M.; Koo D.D.; Heaton N.D. The role of mitochondria in ischemia/reperfusion injury. Transplantation, 2002, 27, 73(4), 493-499.

Javadov S.A.; Clarke S.; Das M.; Griffiths E.J.; Lim K.H.; Halestrap A.P. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J. Physiol, 2003, 549(Pt 2), 513–24.

Jennings R.B.; Reimer K.A. The cell biology of acute myocardial ischemia. Annu.

Rev. Med., 1991, 42, 225-246.
Jeon B.R.; Yeom D.H.; Lee S.M. Protective effect of allopurinol on hepatic energy metabolism in ischemic and reperfused rat liver. Shock, 2001, 15 (2), 112-117.

Johnson E.F.; Palmer C.N.; Griffin K.J.; Hsu M.H. Role of the peroxisome proliferator activated receptor in cytochrome P450 4A gene regulation. FASEB J., 1996, 10, 1241-1248.

Jope R.S.; Yuskaitis C.J.; Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. Neurochem. Res., 2007, 32, 577-595

Jope R.S; Jonhson, G.V.M. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem. Sci., 2004, 29, 95-102.

Juhaszova M.; Wang S.; Zorov D.B.; Nuss H.B.; Gleichmann M.; Mattson M.P.; Sollott S.J. The identity and regulation of the mitochondrial permeability transition pore: where the known meets the unknown. Ann. N. Y. Acad. Sci., 2008, 1123, 197-212.

Juhaszova M.; Wang S.; Zorov D.B.; Nuss H.B.; Gleichmann M.; Mattson M.P.; Sollott S.J. The identity and regulation of the mitochondrial permeability transition pore: where the known meets the unknown. Ann. N. Y. Acad. Sci., 2008, 1123, 197-212.

Juhaszova M.; Zorov D.B.; Kim S.H.; Pepe S.; Fu Q.; Fishbein K.W.; Ziman B.D.; Wang S.; Ytrehus K.; Antos C.L.; Olson E.N.; Sollott S.J. Glycogen synthase kinase-3 beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J. Clin. Invest., 2004, 113, 1535-1549.

Kamo N.; Muratsugu M.; Hongoh R.; Kobatake V. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J. Membr. Biol., 1979, 49, 105-121.

Ke B.; Lipshutz G.S.; Kupiec-Weglinski J.W. Gene therapy in liver ischemia and reperfusion injury. Curr. Pharm. Des., 2006, 12, 2969-2975.

Kendrick A.A.; Choudhury M.; Rahman S.M.; McCurdy C.E.; Friederich M.; Van Hove J.L.; Watson P.A.; Birdsey N.; Bao J.; Gius D.; Sack M.N.; Jing E.; Kahn

C.R.; Friedman J.E.; Jonscher K.R. Fatty liver is associated with reduced SIRT3 activity and mitochondrial protein hyperacetylation. Biochem. J., 2011, 433 (3), 505-514.

Kim H.P.; Morse D.; Choi A.M. Heat-shock proteins: new keys to the development of cytoprotective therapies. Expert Opin Ther Targets, 2006, 10(5), 759-769.

Kim S.C.; Sprung R.; Chen Y.; Xu Y.; Ball H.; Pei J.; Cheng T.; Kho Y.; Xiao H.; Xiao L.; Grishin N.V.; White M.; Yang X.J.; Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol. Cell., 2006, 23, 607-618.

Kleiner D.E.; Brunt E.M.; Van Natta M.; Behling C.; Contos M.J.; Cummings O.W.; Ferrell L.D.; Liu Y.C.; Torbenson M.S.; Unalp-Arida A.; Yeh M.; McCullough A.J.; Sanyal A.J. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology, 2005, 41, 1313-1321.

Kockeritz L.; Doble B.; Patel S.; Woodgett J.R. Glycogen Synthase Kinase-3 – An overview of an over-achieving protein kinase. Curr. Drug Targets, 2006, 7, 1377-1388.

Kodavanti P.; Mehendale H. Biochemical methods of studying hepatotoxicity. Hepatology, 1991, 241-326.

Kokoszka J.E.; Waymire K.G.; Levy S.E.; Sligh J.E.; Cai J.; Jones D.P.; MacGregor G.R.; Wallace D.C. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature, 2004, 427, 461-465.

Kong X.; Wang R.; Xue Y.; Liu X.; Zhang H.; Chen Y.; Fang F.; Chang Y. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS ONE, 2010, 5, e11707.

Koolman J.; Röhm K.H. Color Atlas of Biochemistry. Thieme Medical Publishers Inc., 1996, 130-131, 278-279.

Kostyak J.C.; Hunter J.C.; Korzick D.H. Acute PKCdelta inhibition limits ischaemia-reperfusion injury in the aged rat heart: role of GSK-3beta. Cardiovascular Research, 2006, 70, 325-334.

Koti R.S.; Seifalian A.M.; Davidson B.R. Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. Dig. Surg.,

2003, 20, 383–396.

Kuno A.; Critz S.D.; Cui L.; Solodushko V.; Yang X.M.; Krahn T.; Albrecht B.; Philipp S.; Cohen M.V.; Downey J.M. Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A2b-dependent signaling during early reperfusion. J. Mol. Cell. Cardiol., 2007, 43, 262-271.

Lambert A. J.; Brand M. D. Inhibitors of the quinone binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). J. Biol. Chem., 2004, 279, 39414–39420.

Lanir A.; Jenkins R.L.; Caldwell C.; Lee R.G.; Khettry U.; Clouse M.E. Hepatic transplantation survival: correlation with adenine nucleotide level in donor liver. Hepatology, 1988, 8, 471-475.

Leclerc S.; Garnier M.; Hoessel R.; Marko D.; Bibb J.A.; Snyder G.L.; Greengard P.; Biernat J.; Wu Y.Z.; Mandelkow E.M.; Eisenbrand G.; Meijer L. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? J. Biol. Chem., 2001, 276, 251-260.

Lee J.S.; Lee J.J.; Seo J.S. HSP70 deficiency results in activation of c-Jun N-terminal Kinase, extracellular signal-regulated kinase, and caspase-3 in hyperosmolarity-induced apoptosis. J. Biol. Chem., 2005, 280, 6634-6641.

Lemasters J.J. Modulation of mitochondrial membrane permeability in pathogenesis, autophagy and control of metabolism. J. Gastroenterol Hepatol, 2007, 22, S31-S37.

Lemasters J.J.; Thurman R.G. The many facets of reperfusion injury. Gastroenterology, 1995, 108, 1317-1320.

Lemasters, J.J. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. American Journal of Physiology, 1999, 276 (1 Pt 1), G1-G6.

Lentsch A.B.; Kato A.; Yoshidome H.; McMasters K.M.; Edwards M.J. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology, 2000, 32 (2), 169-173.

Lentsch A.B.; Yoshidome H.; Cheadle W.G.; et al. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. Hepatology, 1998, 27(4), 1172-1177.

Lentsch A.B.; Yoshidome H.; Kato A.; et al. Requirement for interleukin-12 in the pathogenesis of warm hepatic ischemia/reperfusion injury in mice. Hepatology, 1999, 30(6), 1448-1453.

Leung A.W.C.; Varanyuwatana P.; Halestrap A.P. The mitochondrial phosphate carrier interacts with cyclophilin D and my play a key role in the permeability transition. J. Biol. Chem., 2008, 283, 26312-26323.

Levene A.P.; Goldin R.D. The epidemiology, pathogenesis and histopathology of fatty liver disease. Histopathology, 2012, 156(9),A4568

Li X.; Zhang J.F.; Lu M.Q.; Yang Y.; Xu C.; Li H.; Wang G.S.; Cai C.J.; Chen G.H. Alleviation of ischemia-reperfusion injury in rat liver transplantation by induction of small interference RNA targeting Fas. Langenbecks Arch Surg., 2007, 392(3), 345-351

Lieber A.; He C.Y.; Meuse L.; Schowalter D.; Kirillova I.; Winther B.; Kay M.A. The role of Kupffer cell activation and viral gene expression inearly liver toxicity after infusion of recombinant adenovirus vectors. Journal of Virology, 1997, 71 (11), 8798-8807

Lim S.Y.; Davidson S.M.; Hausenloy D.J.; Yellon D.M. Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. Cardiovascular Research, 2007, 75, 530-535.

Lin K.M.; Lin B.; Lian I.Y.; et al. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia – reoxygenation. Circulation, 2001, 103, 1787-1792.

Ma Y.; Chen H.; He X.; Nie H.; Hong Y.; Sheng C.; Wang Q.; Xia W.; Ying W. NAD+ Metabolism and NAD+-Dependent Enzymes: Promising Therapeutic Targets for Neurological Diseases. Curr. Drug Targets, 2012, 13, 222-229.

Mari M.; Bai J.; Cederbaum A.I. Adenovirus-mediated overexpression of catalase in the cytosolic or mitochondrial compartment protects against toxicity

caused by glutathione depletion in HepG2 cells expressing CYP2E1. Journal of Pharmacology and Experimental Therapeutics, 2002, 301(1), 111-118.
Marni A.; Ferrero M.E.; Gaja G. Metabolic function of grafted liver in rats. Transplantation, 1988, 46, 830-835.

Massip-Salcedo M.; Casillas-Ramirez A.; Franco-Gou R.; Bartrons R.; Ben Mosbah I.; Serafin A.; Rosello-Catafau J.; Peralta C. Heat shock proteins and mitogen-activated protein kinases in steatotic livers undergoing ischemia-reperfusion: some answers. Am. J. Pathol., 2006, 168, 1474–1485.

Matsui T.; Rosenzweig A. Convergent signal transduction pathways controlling cardiomyocyte survival and function: the role of PI 3-kinase and Akt. J. Mol. Cell Cardiol., 2005, 38, 63-71.

McCormack L.; Dutkowski P.; El-Badry A.M.; Clavien P.A. Liver transplantation using fatty livers: always feasible? J. Hepatol., 2011, 54, 1055–1062.

McCuskey R.S.; Urbaschek R.; Urbaschek B. The microcirculation during endotoxemia. Cardiovascular Research, 1996, 32 (4), 752-763.

Metzger J.; Dore S.P.; Lauterburg B.H. Oxidant stress during reperfusion of ischemic liver: no evidence for a role of xanthine oxidase. Hepatology, 1988, 8 (3), 580-584.

Michels G.; Khan I.F.; Endres-Becker J.; et al. Regulation of the human cardiac mitochondrial Ca2+ uptake by 2 different voltage-gated Ca2+ channels. Circulation, 2009, 119, 2435–2443.

Mitchell P. Coupling of phosphorilation to electron and hydrogen transfer by a chemi-osmotic type mechanism. Nature, 1961, 191, 144-145.

Mittler R.; Vanderauwera S.; Suzuki N.; Miller G.; Tognetti V. B.; Vandepoele K.; Gollery M.; Skulachev V.; Van Breusegem F. ROS signaling: the new wave? Trends Plant Sci., 2011, 16, 300–309.

Miura T.; Nishihara M.; Miki T. Drug development targeting the glycogen synthase kinase-3beta (GSK-3beta)-mediated signal transduction pathway: role of GSK-3beta in myocardial protection against ischemia/reperfusion injury. J. Pharmacol Sci., 2009, 109(2), 162-167.

Miyamoto S.; Murphy A.N.; Brown J.H. Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II, Cell Death Differ., 2008, 15, 521-529.

Mochida S.; Arai M.; Ohno A.; Masaka N.; Ogata I.; Fujiwara K. Oxidative stress in hepatocytes and stimulatory state of Kupffer cells after reperfusion differ between warm and cold ischemia in rats. Liver., 1994, 14 (5), 234-240.

Moreau B.; Nelson C.; Parekh A.B. Biphasic regulation of mitochondrial ca2+ uptake by cytosolic Ca2+ concentration. Curr. Biol., 2006, 16, 1672–1677.

Morihira M.; Hasebe N.; Baljinnyam E.; Sumitomo K.; Matsusaka T.; Izawa K.; Fujino T.; Fukuzawa J.; Kikuchi K. Ischemic preconditioning enhances scavenging activity of reactive oxygen species and diminishes transmural difference of infarct size. Am. J. Physiol., 2006, 290, H577-H583.

Mosbah I.B.; Alfany-Fernandez I.; Martel C.; Zaouali M.A.; Bintanel-Morcillo M.; Rimola A.; et al. Endoplasmic reticulum stress inhibition protects steatotic and non-steatotic livers in partial hepatectomy under ischemia-reperfusion. Cell Death and Dis., 2010, 1 (e52), 1-12.

Mosher B.; Dean R.; Harkema J.; Remick D.; Palma J.; Crockett E. Inhibition of Kupffer cells reduced CXC chemokine production and liver injury. J. Surg. Res., 2001, 99(2), 201-10.

Murata M.; Akao M.; O'Rourke B.; Marban E. Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca2+ overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. Circ. Res., 2001, 89, 891-898.

Murphy E.; Steenbergen C. Ion transport and energetics during cell death and protection. Physiology (Bethesda), 2008, 2, 115-123.

Murphy E.; Steenbergen C. Preconditioning: the mitochondrial connection. Annu. Ver. Physiol., 2007, 69, 51-67.

Murphy M. P. How mitochondria produce reactive oxygen species. Biochem. J., 2009, 417, 1–13.

Nakagawa T.; Shimizu S.; Watanabe T.; Yamaguchi O.; Otsu K.; Yamagata

H.; Inohara H.; Kubo T.; Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature, 2005, 434, 652-658.

Nakayama H.; Yamamoto Y.; Kume M.; Yamagami K.; Yamamoto H.; Kimoto S.; et al. Pharmacologic stimulation of adenosine A2 receptor supplants ischemic preconditioning in providing ischemic tolerance in rat livers. Surgery, 1999, 126, 945-954.

Nicchitta C.V.; Williamson J.R. Spermine. A regulator of mitochondrial calcium cycling. J. Biol. Chem., 1984, 259, 12978–12983.

Ning X.H.; Xu C.S.; Song Y.; Xiao Y.; Hu Y.J.; Lupinetti F.; Portman M. Hypothermia preserves function and signaling for mitochondrial biogenesis during subsequent ischemia. Am. J. Physiol., 1998, 274 (Heart Circ. Physiol. 43), H786-H793.

Nishihara M.; Miura T.; Miki T.; Tanno M.; Yano T.; Naitoh K.; et al. Modulation of the mitochondrial permeability transition pore complex in GSK-3 β -mediated myocardial protection. J Mol. Cell Cardiol., 2007, 43, 564 – 570.

Noack E.; Greeff K. Inhibition of calcium transport in mitochondria by -receptor blocking substances and its reactivation by phospholipids. Experientia, 1971, 27, 810–811.

Nussler A.K.; Di S.M.; Billiar T.R.; et al. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. J. Exp. Med., 1992, 176 (1), 261-4.

Nylandsted J.; Rohde M.; Brand K.; Bastholm L.; Elling F.; Jaattela M. Selective depletion of HSP70 activates a tumor-specific death program that is independent of caspases and bypasses Bcl-2. Proc. Natl. Acad. Sci., 2000, 97, 7871-7876.

Orrenius S.; McConkey D.J.; Bellomo G.; Nicotera P. Role of Ca in toxic cell injury. TIPS, 1989,10, 281-285.

Pachori A.S.; Melo L.G.; Hart M.L.; Noiseux N.; Zhang L.; Morello F.; Solomon S.D.; Stahl G.L.; Pratt R.E.; Dzau V.J. Hypoxia-regulated therapeutic gene as a preemptive treatment strategy against ischemia/reperfusion tissue injury. Proceedings of the National Academy of Sciencesof the United States of America., 2004, 101 (33), 12282-12287.

Pagel P.S.; Krolikowski J.G.; Neff D.A.; Weihrauch D.; Bienengraeber M.; Kersten J.R.; Wartlier D.C. Inhibition of glycogen synthase kinases enhances isoflurane-induced protetion against myocardial infarction during early reperfusion in vivo. Anesth. Analg., 2006, 102,1348-1354.

Palmeira C.M.; Moreno A.J.; Madeira V.M.C. Interactions of herbicides 2,4-D and dinoseb with liver mitochondrial bioenergetics. Toxicol. Appl. Pharmacol., 1994, 127, 50-57.

Palmeira C.M.; Rolo A.P. Mitochondrial membrane potential (ΔΨ) fluctuations associated with the metabolic states of mitochondria. Methods Mol. Biol., 2012, 810, 89-101.

Palmeira C.M; Wallace K.B. Benzoquinone inhibits the voltage-dependent induction of the mitochondrial permeability transition caused by redox-cycling naphtoquinones. Toxicol. Appl. Pharmacol., 1997, 143, 338-347.

Paradies G.; Petrosillo G.; Pistolese M.; Di Venosa N.; Federici A.; Ruggiero F.M. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart-Involvement of reactive oxygen species and cardiolipin. Circ. Res., 2004, 94, 53-59.

Paradies G.; Ruggier F.M.; Petrosillo G.; Quagliariello E. Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. FEBS Lett., 1998, 424, 155-158.

Parekh A.B. Mitochondrial regulation of intracellular Ca2+ signaling: More than just simple Ca2+ buffers. News Physiol Sci, 2003, 18, 252–256.

Park S.S; Zhao H.; Mueller R.A.; Xu Z. Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3beta. J. Mol. Cell Cardiol., 2006, 40, 708-716.

Park S.W.; Chen S.W.C.; Kim M.; Brown K.M.; D.D'Agati V.; Lee H. T. Protection against Acute Kidney Injury via A1 Adenosine Receptor-Mediated Akt Activation Reduces Liver Injury after Liver Ischemia and Reperfusion in Mice. J. Pharmacol. Exp. Ther., 2010, JPET 333, 736-747.

Paschen S.A.; Neupert W. Protein import into mitochondria. IUBMB Life, 2001, 52, 101-112.

Pastorino J.G.; Hoek J.B.; Shulga N. Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. Cancer Res., 2005, 65, 10545-10554.

Penzo D.; Petronilli V.; Angelin A.; Cusan C.; Colonna R.; Scorrano L.; et al. Arachidonic acid released by phospholipase A(2) activation triggers Ca(2+)-dependent apoptosis through the mitochondrial pathway. J. Biol. Chem., 2004, 279(24), 25219–25.

Peralta C.; Fernandez L.; Panes J.; Prats N.; Sans M.; Pique J.M.; Gelpi E.; Rosello-Catafau J. Preconditioning protects against systemic disorders associated with hepatic ischemia-reperfusion through blockade of tumor necrosis factor-induced P-selectin up-regulation in the rat. Hepatology, 2001, 33, (1), 100-113.

Peralta C.; Hotter G.; Closa D.; Prats N.; Xaus C.; Gelpí E.; Roselló-Catafau J. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. Hepatology, 1999, 29, 126-132.

Peralta C.; Prats N.; Xaus C.; Gelpi E.; Rosello-Catafau J. Protective effect of liver ischemic preconditioning on liver and lung injury induced by hepatic ischemia-reperfusion in the rat. Hepatology, 1999, 30 (6), 1481-1489.

Peralta C.; Rull R.; Rimola A.; Deulofeu R.; Rosello-Catafau J.; Gelpi E.; Rodes J. Endogenous nitric oxide and exogenous nitric oxide supplementation in hepatic ischemia-reperfusion injury in the rat. Transplantation, 2001, 71 (4), 529-536.

Pérez-Carreras M.; Del Hoyo P.; Martín M.A.; Rubio J.C.; Martín A.; Castellano G.; Colina F.; Arenas J.; Solis-Herruzo J.A. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. Hepatology., 2003, 38, 999-1007.

Petronilli V.; Costantini P.; Scorrano L.; Colonna R.; Passamonti S.; Bernardi P. The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. J. Biol. Chem., 1994, 269(24), 16638–42.

Petrosillo G.; Portincasa P.; Grattagliano I.; Casanova G.; Matera M.; Ruggiero F.M.; Ferri D.; Paradies G. Mitochondrial dysfunction in rat with nonalcoholic fatty liver. Involvemnt of complex I, reactive oxygen species and cardiolipin. Biochimica et Biophysica., 2007, Acta 1767, 1260-1267.

Petrosillo G.; Ruggiero F.M.; Di Venosa N.; Paradies G. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. FASEB J., 2003, 17, U395-U413.

Philipp S.; Yang X.M.; Cui L.; Davis A.M.; Downey J.M.; Cohen M.V. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade, Cardiovasc. Res., 2006, 70 308-314.

Pillai V.B.; Sundaresan N.R.; Kim G.; Gupta M.; Rajamohan S.B.; Pillai J.B; Samant S.; et al. Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. J. Biol. Chem., 2010, 285, 3133-3144.

Ploeg R.J.; D'Alessandro A.M.; Hoffmann R.M.; Eckhoff D.; Isaacs R.; Knechtle S.J.; Pirsch J.D.; Stegall M.D.; Kalayoglu M.; Belzer F.O. Impact of donor factors and preservation on function and survival after liver transplantation. Transplant Proc., 1993, 25, 3031-3033.

Polster B.M.; Basanez G.; Etxebarria A.; Hardwick J.M.; Nicholls D.G. Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J. Biol. Chem., 2005, 280(8), 6447–54.

Porte R.J.; Ploeg R.J.; Hansen B.; van Bockel J.H.; Thorogood J.; Persijn G.G.; Hermans J.; Terpstra O.T. Long-term graft survival after liver transplantation in the UW era: late effects of cold ischemia and primary dysfunction. Transpl. Int., 1998, 11, Suppl. 1, S164-S167.

Preiss D.; Sattar N. Non-alcoholic fatty liver disease: an overview of prevalence, diagnosis, pathogenesis and treatment considerations. Clinical Science, 2008, 115, 141-150.

Putney J.W. Jr; Thomas A.P. Calcium signaling: Double duty for calcium at the mitochondrial uniporter. Curr. Biol., 2006, 16, R812–815.

Qiu X.; Brown K.; Hirschey M.D.; Verdin E.; Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab., 2010, 12, 662-667.

Rachek L.I.; Yuzefovych L.V.; Ledoux S.P.; Julie N.L.; Wilson G.L. Troglitazone, but not rosiglitazone, damages mitochondrial DNA and induces mitochondrial dysfunction and cell death in human hepatocytes. Toxicol. Appl. Pharmacol., 2009, 240, 348-354.

Rajdev S.; Reynolds I.J. Calcium green-5N, a novel fluorescent probe for monitoring high intracellular free Ca2+ concentrations associated with glutamate excitotoxicity in cultured rat brain neurons. Neuroscience Letters, 1993, 162, 149-152.

Rasola A.; Sciacovellia M.; Chiara F.; Pantica B.; Brusilown W.S.; et al. Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. PNAS, 2010, 11, 727-731.

Reddy J.K.; Mannaerts G.P. Peroxisomal lipid metabolism. Annu. Rev. Nutr., 1994, 114, 343-370.

Rehman H.; Connor H.D.; Ramshesh V.K.; Theruvath T.P.; Mason R.P.; Wright G. L.; Lemasters J.J.; Zhong Z. Ischemic Preconditioning Prevents Free Radical Production and Mitochondrial Depolarization in Small-for-Size Rat Liver Grafts. Transplantation, 2008, 85, 1322-1331.

Ren F.; Duan Z.; Cheng Q.; Shen X.; Gao F.; et al. Inhibition of glycogen synthase kinase 3 beta ameliorates liver ischemia reperfusion injury by way of an interleukin-10-mediated immune regulatory mechanisms. Hepatology, 2011, 54 (2), 687-696.

Ricci C.; Pastukh V.; Leonard J.; Turrens J.; Wilson G.; Schaffer D.; Schaffer S.W. Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis. Am. J. Physiol. Cell Physiol., 2008, 294, C413-C422.

Rizzuto R.; Brini M.; Murgia M.; et al. Microdomains with high Ca2+ close to ip3-sensitive channels that are sensed by neighboring mitochondria. Science, 1993, 262, 744–747.

Rizzuto R.; Simpson A.W.; Brini M.; et al. Rapid changes of mitochondrial Ca2+ revealed by specifically targeted recombinant aequorin. Nature, 1992, 358, 325–

327.

Rolo A.P.; Teodoro J.S.; Peralta C.; Rosello-Catafau J.; Palmeira C.M. Prevention of I/R injury in fatty livers by ischemic preconditioning is associated with increased mitochondrial tolerance: the key role of ATPsynthase and mitochondrial permeability transition. Transpl Int., 2009, 22(11), 1081-1090.

Rossi C.S.; Vasington F.D.; Carafoli E. The effect of ruthenium red on the uptake and release of Ca2+ by mitochondria. Biochem Biophys Res Commun, 1973, 50, 846–852.

Rostovtseva T.K.; Tan W.; Colombini M. On the role of VDAC in apoptosis: fact and fiction. J. Bioenerg Biomembr, 2005, 37, 129–142.

Ryu S.Y.; Beutner G.; Dirksen R.T.; et al. Mitochondrial ryanodine receptors and other mitochondrial Ca2+ permeable channels. FEBS Lett., 2010, 584, 1948–1955.

Ryu S.Y.; Beutner G.; Kinnally K.; et al. Single channel characterization of the mitochondrial ryanodine receptor in heart mitoplasts. J Biol Chem, 2011.

Saito T.; Ishii S.; Abe T.; Tsuchiya T.; Kanno H.; Miyazawa M.; Suzuki M.; Gotoh M. Effect of preconditioning in the liver against alterations of gene transcription. Transplant. Proc., 2001, 33, 849.

Sastrasinh M.; Weinberg J.M.; Humes H.D. The effect of gentamicin on calcium uptake by renal mitochondria. Life Sci., 1982, 30, 2309–2315.

Schanne F.A.X.; Kane A.B.; Young E.A.; Farber J.L.; Calcium dependence of toxic cell death, a final common pathway. Science, 1979, 206, 700-702.

Schellenberg G.D.; Anderson L.; Cragoe E.J. Jr; et al. Inhibition of brain mitochondrial Ca2+ transport by amiloride analogues. Cell Calcium, 1985, 6, 431–447.

Scherz-Shouval R.; Shvets E.; Fass E.; Shorer H.; Gil L.; Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J., 2007, 26, 1749-1760.

Schinzel A.; Takeuchi O.; Huang Z.; Fisher J.K.; Zhou Z.; Rubens J.; et al.

Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc. Natl. Acad. Sci., 2005, 102, 12005-12010.

Schon E.A.; Dencher N.A. Heavy breathing: energy conversion by mitochondrial respiratory supercomplexes. Cell Metabolism, 2009, 9(1), 1–3.

Schulz R.; Cohen M.V.; Behrends M.; Downey J.M.; Heusch G. Signal transduction of ischemic preconditioning. Cardiovasc. Res., 2001, 52, 181–198.

Schwarz E.R.; Whyte W.S.; Kloner R.A. Ischemic preconditioning. Curr.Opin. Cardiol., 1997, 12, 475-481.

Schwer B.; Verdin E. Conserved metabolic regulatory functions of sirtuins. Cell Metab., 2008, 7, 104-112.

Selzner M.; Rüdiger H.; Sindram D.; Maddan J.; Clavien P. A. Mechanisms of ischemic injury are different in the steatotic and normal rat liver. Hepatology, 2000, 32, 1280-1288.

Selzner M.; Clavien P.A. Fatty liver in liver transplantation and surgery. Semin. Liver. Sis., 2001, 21, 105-113.

Selzner N.; Rudiger H.; Graf R.; Clavien P.A. Protective strategies against ischemic injury of the liver. Gastroenterology, 2003, 125 (3), 917-936.

Selzner N.; Selzner M.; Jochum W.; Amann-Vesti B.; Graf R.; et al. Mouse livers with macrosteatosis are more susceptible to normothermic ischemic injury than those with microsteatosis. J. Hepatol., 2006, 44, 694.

Serafín A.; Fernández-Zabalegui L.; Prats N.; Wu Z.Y.; Roselló-Catafau J.; Peralta C. Ischemic preconditioning: tolerance to hepatic ischemia-reperfusion injury. Histol Histopathol, 2004, 19(1), 281-289.

Shimizu S.; Eguchi Y.; Kamiike W.; Funahashi Y.; Mignon A.; Lacronique V.; Matsuda H.; Tsujimoto Y. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proceedings of the National Academy of Sciences USA, 1998, 95, 1455-1459.

Shulga N.; Pastorino J.G. Acyl coenzyme A-binding protein augments bid-

induced mitochondrial damage and cell death by activating mu-calpain. J. Biol. Chem., 2006, 281(41), 30824–33.

Shulga N.; Pastorino J.G. Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3. J. Cell Sci., 2010, 123, 4117-4127.

Shulga N.; Wilson-Smith R.; Pastorino J.G. Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria. J. Cell. Sci., 2010, 123(Pt 6), 894-902.

Sinay L.; Kurthy M.; Horvath S.; Arato E.; Shafei M.; Lantos J.; Ferencz S.; Bator A.; Balatonyi B.; Verzar Z.; Suto B.; Kollar L.; Weber G.; Roth E.; Jancso G. Ischaemic postconditioning reduces peroxide formation, cytokine expression and leukocyte activation in reperfusion injury after abdominal aortic surgery in rat model. Clin. Hemorheol. Microcirc., 2008, 40,133-142.

Skulachev V. P. Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. Q. Rev. Biophys., 1996, 29, 169–202.

Skulachev V. P. Uncoupling: new approaches to an old problem of bioenergetics. Biochim. Biophys. Acta, 1998, 1363, 100–124.

Skulachev V.P. Uncoupling: new approaches to an old problem of bioenergetics. Biochim. Biophys. Acta, 1998, 1363, 100-124.

Smeitink J.; Van Den Heuvel L.; Di Mauro S. The genetics and pathology of oxidative phosphorylation. Nature Rev. Genet., 2001, 2(5), 342-352.

Someya S.; Yu W.; Hallows W.C.; Xu J.; Vann J.; Leeuwenburgh C.; Tanokura M.; Denu J.M.; Prolla T.A. SIRT3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell, 2010, 143, 802-812.

Sparagna G.C.; Gunter K.K.; Sheu S.S.; et al. Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. J. Biol. Chem., 1995, 270, 27510–27515.

Stankiewicz A.R.; Lachapelle G.; Foo C.P.; Radicioni S.M.; Mosser D.D. HSP70

inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J. Biol. Chem., 2005, 280, 38729-38739.

Stocchi V.; Cucchiarini L.; Magnani M.; Chiarantini L.; Palma P.; Crescentini G. Simultaneous extraction and reverse-phase highperformance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. Analytical Biochemistry, 1985, 146, 118-124.

Stryer, L. Biochemistry (3rd Edition). W.H. Freeman Company, 1988, 529-556.
Suleiman M.S.; Halestrap A.P.; Griffiths E.J. Mitochondria: a target for myocardial protection. Pharmacol. Ther., 2001, 89, 29-46.

Sun C.K.; Zhang X.Y.; Zimmermann A.; Davis G.; Wheatley A.M. Effect of ischemia-reperfusion injury on the microcirculation of the steatotic liver of the Zucker rat. Transplantation, 2001, 27, 72(10), 1625-1631.

Tafani M.; Schneider T.G.; Pastorino J.G.; Farber J.L. Cytochrome c-dependent activation of caspase-3 by tumor necrosis factor requires induction of the mitochondrial permeability transition. Am. J. Pathol., 2000, 156, 2111-2121.

Takano H.; Manchikalapudi S.; Tang X.L.; Qiu Y.; Rizvi A.; Jadoon A.K.; Zhang Q.; Bolli R. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. Circulation, 1998, 98, 441–449.

Takeshige K.; Minakami S. NADH and NADPH dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. Biochem. J., 1979, 180, 129-135.

Teodoro J.; Rolo A.P.; Oliveira P.J.; Palmeira C.M. Decreased ANT content in Zucker fatty rats: relevance for altered hepatic mitochondrial bioenergetics in steatosis. FEBS Lett., 2006, 580, 2153-2157.

Teodoro J.S.; Rolo A.P.; Duarte F.V.; Simões A.M.; Palmeira C.M. Differential alterations in mitochondrial function induced by a choline-deficient diet: understanding fatty liver disease progression. Mitochondrion., 2008, 8, 367-376.

Teoh N.; Leclercq I.; Pena A.D.; Farell G. Low-dose TNF-alpha protects against hepatic ischemia-reperfusion injury in mice: implications for preconditioning. Hepatology, 2003, 37 (1), 118-128.

Teoh N.C.; Farrel G.C. Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection. Journal of Gastroenterology and Hepatology, 2003, 18 (8), 891-902.

Teramoto K.; Bowers J.; Kruskal J.; Clouse M. Hepatic microcirculatory changes after reperfusion in fatty and normal liver transplantation in the rat. Transplantation, 1993, 56, 1076-1082.

Terashima Y.; Sato T.; Yano T.; Maas O.; Itoh T.; Miki T.; Tanno M.; Kuno A.; Shimamoto K.; Miura T. Roles of phospho-GSK-3β in myocardial protection afforded by activation of the mitochondrial K ATP channel. J. Mol. Cell Cardiol., 2010, 49(5), 762-770.

Tong H.; Imahashi K.; Steenbergen C.; Murphy E. Phosphorylation of glycogen synthase kinase-3b beta during preconditionoing through a phosphatidylinositol-3-kinase dependent pathway is cardioprotective. Circ. Res., 2002, 90, 377-379.

Toth A.; Jeffers J.R.; Nickson P.; Min J.Y.; Morgan J.P.; Zambetti G.P.; Erhardt P. Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. Am. J. Physiol., 2006, 291, H52–H60.

Trenker M.; Malli R.; Fertschai I.; et al. Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca2+ uniport. Nat. Cell Biol., 2007, 9, 445–452.

Tsuchiya D.; Hong S.; Matsumori Y. Overexpression of rat heat shock protein 70 is associated with reduction of early mitochondrial cytochrome c release and subsequent DNA fragmentation after permanent focal ischemia. J. Cereb. Blood Flow Metab., 2003, 23, 718-727.

Tsung A.; Sahai R.; Tanaka H.; Nakao A.; Fink M.P.; Lotze M.T.; Yang H.; Li J.; Tracey K.J.; Geller D.A.; Billiar T.R. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia–reperfusion. J. Exp. Med., 2005, 201, 1135-1143.

Turrens J.F. Mitochondrial formation of reactive oxygen species. J. Physiol., 2003, 552, 335-344.

Turrens J.F.; Alexandre A.; Lehninger A.L. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch. Biochem. Biophys., 1985, 237, 408-414.

Varela A.T.; Gomes A.P.; Simões A.M.; Teodoro J.S.; Duarte F.V.; Rolo A.P.; Palmeira C.M. Indirubin-30-oxime impairs mitochondrial oxidative phosphorylation and prevents mitochondrial permeability transition induction. Toxicology and Applied Pharmacology, 2008, 233, 179-185.

Varela A.T.; Rolo A.P.; Palmeira C.M. Fatty liver and ischemia/reperfusion: are there drugs able to mitigate injury? Curr. Med. Chem., 2011, 18, 4987-5002.

Varela A.T.; Simões A.M.; Teodoro J.S.; Duarte F.V.; Gomes A.P.; Palmeira C.M.; Rolo A.P. Indirubin-3'-oxime prevents hepatic I/R damage by inhibiting GSK-3beta and mitochondrial permeability transition. Mitochondrion., 2010, 10(5), 456-463.

Vendemiale G.; Grattagliano I.; Caraceni P.; Caraccio G.; Domenicali M.; Dall'Agata M.; et al. Mitochondrial oxidative injury and energy metabolism alteration in rat fatty liver: Effect of the nutritional status. Hepatology, 2001, 33, 808-815.

Waldmeier P.C.; Zimmermann K.; Qian T.; TintelnotBlomley M.; Lemasters J.J. Cyclophilin D as a drug target. Curr. Med. Chem., 2003, 10, 1485-1506.

Wallace D.C. Mitochondrial DNA mutations in diseases of energy metabolism. J. Bioenerg. Biomemb., 1994, 26, 241-250.

Wang J.Y.; Shen J.; Gao Q.; Ye Z.G.; Yang S.Y.; Liang H.W.; Bruce I.C.; Luo B.Y.; Xia Q. Ischemic postconditioning protects against global cerebral ischemia/reperfusion-induced injury in rats. Stroke, 2008, 39, 983-990.

Wang L.; Du F.; Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell, 2008, 16, 133(4), 693-703.

Wanner G.A.; Ertel W.; Muller P.; et al. Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. Shock., 1996, 5(1), 34-40.

Woodfield K.; Ruck A.; Brdiczka D.; Halestrap A.P. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. Biochem. J., 1998, 336, 287–290.

Woodfield K.Y.; Price N.T.; Halestrap A.P. cDNA cloning of rat mitochondrial cyclophilin. Biochim. Biophys. Acta, 1997, 1351, 27-30.

Xi J.; Wang H.; Mueller R.A.; Norfleet E.A.; Xu Z. Mechanism for resveratrol-induced cardioprotection against reperfusion injury involves glycogen synthase kinase 3beta and mitochondrial permeability transition pore. Eur. J. Pharmacol., 2009, 604(1-3), 111-116.

Xing B.; Chen H.; Zhang M.; Zhao D.; Jiang R.; Liu X.; Zhang S. Ischemic postconditioning inhibits apoptosis after focal cerebral ischemia/reperfusion injury in the rat. Stroke, 2008, 39, 2362-2369.

Yang Z.; Sun W.; Hu K. Molecular mechanism underlying adenosine receptor-mediated mitochondrial targeting of protein kinase C. Biochim. Biophys. Acta, 2012, 1823(4), 950-958.

Ye S.Y.; Wu J.; Zhang J.; Zheng S.S. Locally synthesized HSP27 in hepatocytes: Is it possibly a novel strategy against human liver ischemia/reperfusion injury? Med. Hypotheses, 2011, 76(2), 296-298.

Yin D.P.; Sankary H.N.; Chong A.S.; Ma L.L.; Shen J.; Foster P.; Williams J.W. Protective effect of ischemic preconditioning on liver preservation-reperfusion injury in rats. Transplantation, 1998, 66, 152-157.

Ying W. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. Antioxid. Red. Signal, 2008, 337, 179-206.

Ying W.; Wei G.; Wang D.; Wang Q.; Tang X.; Shi J.; Zhang P.; Lu H. Intranasal administration with NAD+ profoundly decreases brain injury in a rat model of transient focal ischemia. Front. Biosci., 2007, 12, 2728-2234.

Yoshidome, H.; Kato, A.; Edwards, M.J.; Lentsch, A.B. Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: implications of a central role for nuclear factor kappa B. Hepatology, 1999, 30 (1), 203-208.

Yoshizumi T.; Yanaga K.; Soejima Y.; Maeda T.; Uchiyama H.; Sugimachi K. Amelioration of liver injury by ischaemic preconditioning. Br. J. Surg., 1998, 85, 1636-1640.

Zeng Z.; Huang H.F.; Chen M.Q.; Song F.; Zhang Y.J. Postconditioning Prevents Ischemia/Reperfusion Injury in Rat Liver Transplantation. Hepatogastroenterology, 2010, 57 (101), 875-881