New strategies to reduce liver ischemia – reperfusion injury in fatty and non-fatty livers: a focus on sirtuin 1 implication

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A Chara, José, Aris, Miguel y a Edoardo

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Summary

Liver transplantation is the last-resort treatment for end-stage of both acute and chronic liver diseases. However, this therapy is always hampered by the extreme sensitivity of liver to ischemia-reperfusion injury (IRI). Moreover, the lack of suitable donors and the increasing number of patients in the waiting list for transplantation has obliged the physicians to transplant liver that previously have been considered unacceptable due to their higher incidence of dysfunction after transplantation. One example of these suboptimal grafts are the steatotic livers, which are characterized by an excessive lipid accumulation and have been associated with poor transplantation outcome. In fact, the coincidence of multiple marginal characteristics further exacerbates the extent of IRI in the liver graft and reduces the chances of a successful outcome. In this context, the improvement of preservation solutions is a key step in the attempt to meet these clinical demands. In addition, the development of surgical strategies such as the use of reduced-size liver grafts, in order to expand the pool of donors, is considered as one of the most important advances in LT.

IRI associated with liver transplantation is a complex phenomenon that occurs when blood flow is interrupted for a prolonged period of time (ischemia) and then it is restored (reperfusion). During ischemia, the initiation of anaerobic metabolism and the adenosine triphosphate (ATP) depletion results in cell swelling and death. However, the injury is more severe during the reperfusion phase, where the increase in oxygen delivery provokes generation of reactive oxygen species (ROS) that damage cells and proteins, enhance microcirculatory disturbances and the initiation of inflammatory reponses. The final result is cell death and organ injury.

Due to the fact that IRI remains a serious complication in liver surgery and suboptimal grafts are more vulnerable to IRI, it is an urgent need to identify new pharmaceutical targets and develop new strategies in order to diminish its detrimental effects. Various investigations have demonstrated that ischemic preconditioning, a surgical approach that consists on the application of brief episodes of ischemia followed by short periods of reperfusion defore a sustained period of ischemia-reperfusion, decreases significantly IRI. The main PC beneficial effects are mediated

through endogenous adenosine and nitric oxide increases, activation of adenosine monophosphate-activated protein kinase (AMPK), attenuation of oxidative stress, apoptosis and inflammation. However, the potential application of PC in clinical practice remains controversial.

Recent experimental investigations have associated sirtuin 1 (SIRT1) with protective effects against IRI. SIRT1 belongs to the family of class III histone deacetylases of sirtuins, whose activity is dependent of nicotinamide adenine dinucleotide (NAD^+). Sirtuins regulate a wide variety of cellular functions, such as cell cycle, metabolism and cellular stress response. SIRT1 has been shown to exert its beneficial effect against oxidative stress, hypoxic injury, apoptosis and inflammation associated with IRI in heart and brain, but no data has been reported concerning the implication of SIRT1 in hepatic IRI and liver transplantation. Consequently, the present tesis aims to investigate the potential role of SIRT1 in a model of warm ischemia-reperfusion in steatotic livers when PC has been applied (first study), in a model of orthotopic liver transplantation, OLT, (second study) and in reduced-size orthotopic liver transplantation, ROLT, (third study).

In the first study, we applied PC (5 minutes ischemia and 10 minutes of reperfusion) prior to 1 hour of partial ischemia followed by 24-hour reperfusion in Zucker obese rats. In additional groups, we administered either sirtinol or EX527 (SIRT1 inhibitors). We observed that SIRT1 protein levels and activity are augmented during PC. SIRT1 inhibition during PC increases liver injury, oxidative stress and apoptosis and abolishes the activation of cytoprotective mediators of PC, such as AMPK. Consequently, SIRT1 contributes to the beneficial mechanisms of PC against IRI in fatty livers.

In the second study, livers from Sprague-Dawley male rats were preserved for 8 hours (4 ^{0}C) in Institute Georges Lopez-1 (IGL-1) preservation solution enriched or not with trimetazidine (an anti-ischemic drug) and then subjected to OLT. We observed that the addition of TMZ in IGL-1 solution reduced liver injury and mitochondrial damage and increased SIRT1 protein expression levels and SIRT1 activity-related parameters. Also, SIRT1 overexpression was accompanied by a significant increase in

autophagy. This study evidences for the first time the involvement of SIRT1 in hepatic IRI associated to OLT.

In the third study, we further aimed to examine a possible association of SIRT1 with angiotensin II, the main effector of the renin-angiotensin system which has been correlated with increased hepatic injury. Losartan, an angiotensin II type I receptor antagonist, has been shown to exert protective effects against IRI, but the underlying mechanisms are not fully understood. Livers of Sprague-Dawley rats were preserved in University of Wisconsin preservation solution for 1 hour (4 ^{0}C) and then subjected to ROLT. In an additional group, losartan was orally administered 24 hours and 1 hour before the surgical procedure to both the donor and the recipient rats. We observed that losartan pretreatment diminished hepatic injury in ROLT and promoted both SIRT1 protein expression and activity. This fact was consistent with decreases in the endoplasmic reticulum stress parameters and in liver apoptosis. This study evidences the existence of an angiotensin II/SIRT1 axis in liver transplantation, and that the benefits of angiotensin II inhibition against liver IRI are mediated, at least in part, through SIRT1 activation.

According these results, the present thesis concludes that SIRT1 is implicated in the hepatic IRI and that strategies that enhance its activity can be a promising approach to reduce liver IRI.

Abbreviations

ACE: angiotensin converting enzyme

ALT: alanine aminotransferase

AMPK: adenosine monophosphate-activated protein kinase

APAF1: apoptotic protease activation factor 1

AST: aspartate aminotransferase

AT1R: angiotensin II type I receptors

AT2R: angiotensin type II receptors

ATF-6: activating transcription factor 6

ATP: adenosine triphosphate

Bcl-2: B cell lymphoma-2

BcL-xL: Bcl-like X

Cat: catalase

CHOP: C/EBP homologous protein

CytC: cytochrome c

eIF2α: eukaryotic translation initiation factor 2α subunit

eNOS: endothelial nitric oxide synthase

ERK ½: Extracellular signal regulated kinases

ERS: endoplasmic reticulum stress

ET: endothelins

FoxO: Forkhead box-containing protein O

GADPH: 3-phosphate dehydrogenase

GRP78: glucose regulated protein 78

HES: hydroxyethyl starch

HIFs: hypoxia-inducible factors

HSPs: Heat shock proteins

ICAM-1: intracellular adhesion molecule

IGL-1: Institut Georges Lopez-1

IL-1: interleukin-1

IL-10: interleukin-10

IL-6: interleukin-6

INF-γ: interferon-γ

iNOS: inducible nitric oxide synthase

IRE1α: inositol requiring enzyme 1

IRI: ischemia-reperfusion injury

LDH: lactate dehydrogenase

LDLT: living donor liver transplantation

LT: liver transplantation

MAPKS: mitogen activated protein kinases

MDH: malate dehydrogenase

MnSOD: Mn-superoxide dismutase

mPTP: mitochondria permeability transition pore

mTOR: (mammalian Target of rapamycin)

NADPH: nicotinamide adenine dionucleotide phosphate

NF-kB: nuclear factor kappa B

NO: nitric oxide

p70S6k: protein S6 kinase

PC: ischemic preconditioning

PERK: RNA-activated protein kinase (PKR)-like ER kinase

PGC1α: peroxisome proliferator-activated receptor-γ coactivator

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

ppar-α:peroxisome proliferator-activated receptor-α

RAS: renin-angiotensin system

ROLT: reduced orthotopic liver transplantation

ROS: reactive oxygen species

SECs: Sinusoidal endothelial cells

SIRT1: sirtuin 1

SIRT3: sirtuin 3

SLT: split liver transplantation

STAT3: signal transducer and activator of transcription-3

TBA: thiobarbituric acid

TCA: trichloroacetic acid

TNF-α: tumour necrosis factor

Trx1: thioredoxin 1

UCP2: uncoupling protein 2

UPR: unfolded protein response

UW: University of Wisconsin

XBP-1: X box-binding protein 1

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1. INTRODUCTION

1. INTRODUCTION

1.1. Anatomy of the liver and hepatic vasculature

The liver is the largest solid organ in the body, situated in the upper-right abdomen and forms part of the digestive system. In humans it is separated incompletely into lobes, covered on their external surfaces by a thin connective tissue capsule. The main hepatic function is the uptake of substrates from the intestine in order to be stored, metabolized and distributed to the peripheral circulation for being used by other tissues. Furthermore, it is the main detoxifying organ of the body, which removes wastes and xenobiotics by metabolic conversion and biliary excretion [1].

Anatomically human liver is divided into right and left lobes by the falciform ligament, which connects the liver to the anterior abdominal wall and the diaphragm. The right lobe is further subdivided into two smaller lobes, the caudate and the quadrate lobes. The left part of the liver can also be divided into medial and lateral sections by the tissue named as ligamentum teres. Furthermore, the right lobe is firmly attached to the gall bladder, a pear-shaped pocket that stores and evacuates bile. The liver can also be divided into eight segments, where each one has its own vascular and biliary supply (Figure 1A).

The hepatic circulation has some unique characteristics. The liver has two blood supplies; one is the hepatic artery whose function is mainly nutritional and the other is the portal vein that provides blood from intestine, pancreas and spleen. Approximately 80% of the blood entering the liver is supplied by the portal vein, is poorly oxygenated, but facilitates exposure of nutrients and toxins to hepatocytes. The remaining 20% of the blood supply is delivered by the hepatic artery and is oxygenated.

The hepatic artery and the portal vein accompanied by the hepatic bile duct enter the liver at portal triad. Then, branches of each one travels together in portal tracts through the liver parenchyma. After repeated branching, terminal branches of the blood vessels supply blood to sinusoids. After flowing through the sinusoids, blood is collected in small branches of hepatic veins, which finally leave the liver on the dorsal surface and join the inferior vena cava. Apart from blood vessels, it can also be found the bile canaliculi, spaces of 1-2 μm wide which are formed between adjacent

hepatocytes. They are interconnected and form a network of intercellular channels that receive the bile secreted from hepatocytes (Figure 1B).

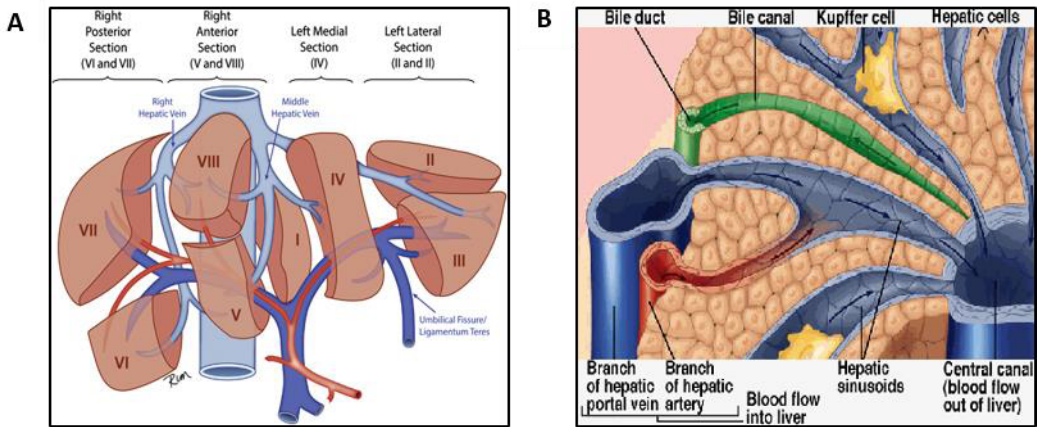

Figure 1: (A) Scheme division of the liver in eight segments, (B) Hepatic circulation in lobules.

Liver is divided histologically into lobules. The classic hepatic lobule is a polygonal structure where the hepatic venule forms its central axis and in its periphery boundaries are regularly distributed the portal triads, containing a bile duct and a terminal branch of the hepatic artery and portal vein, as shown in Figure 2A. The lobule is further composed of multiple smaller units, called acini. The boundaries of the hepatic acinus can not be visualized; its axis is the portal tract and its peripheral boundary is an imaginary line connecting the neighboring central hepatic venule. The acinus is divided in three zones, with zone 1 closest to the portal vein and zone 3 closest to the hepatic venule in the center of the lobule (Figure 2B). The blood circulates through the sinusoids from zone 1 to zone 3, whereas bile flows in the opposite direction via a separate route to the portal bile ducts. Thus, each zone has different levels of oxygenation and metabolic function; hepatocytes closest to the hepatic artery (zone 1) are the best oxygenated, while those in zone 3 have the poorest supply of oxygen [2, 3].

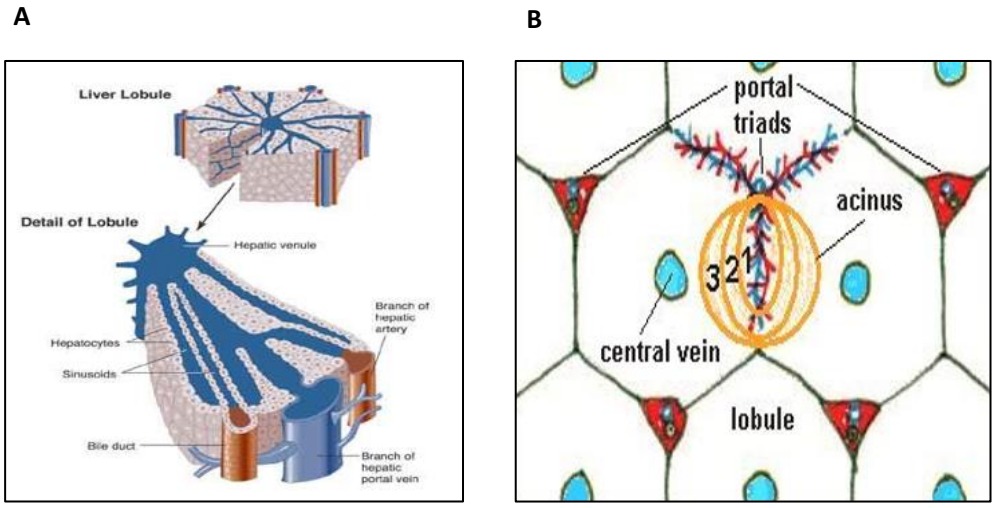

Figure 2: (A) Representation of a hepatic lobule. (B) Scheme of hepatic acinus.

1.2. Hepatic Cells

The liver is composed of several cell types that contribute to different functions. The hepatic parenchymal cell, or hepatocyte which makes up almost 60% of cells and 80% of the volume of the organ, is considered as the main cell type of the liver that carries out most of the hepatic functions. The other 20% comprises represent the non-parenchymal cells, which include sinusoids, Kuppfer cells, lymphocytes and perisinusoidal stellate cells (fat-storing cells of Ito) [2].

Sinusoids are the large capillaries of the liver, are located between the cords of parenchymal cells, nourishing thus each hepatocyte on various sides. Sinusoids are lined by a thin layer of endothelial cells and contain Kupffer cells which are the resident macrophage of the liver. The space between the hepatocyte and the sinusoid is named Disse space, where stellate cells are found. Furthermore, in space of Disse it can be observed different components of the extracellular matrix, like collagen, proteoglycans and fibronectin that contribute to the cellular adherence, intercellular communication and cellular differentiation. Lymphocytes are also part of the innate immune system that resides within the liver to help resist infection (Figure 3).

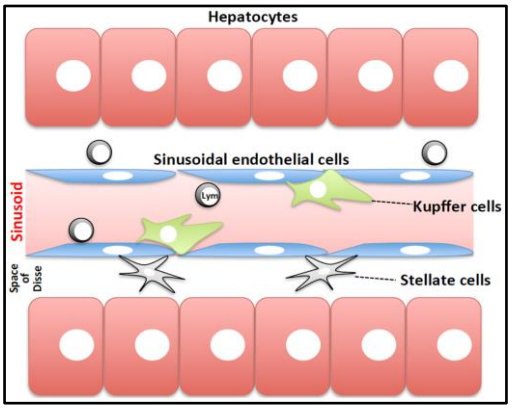

Figure 3: Schematic structure of the hepatic cells.

1.2.1. Hepatocytes

Hepatocytes carry out most of the hepatic functions; they extract and process the nutrients from the blood and produce both exocrine and endocrine secretions, as follows [1, 2]:

i) Protein synthesis: Hepatocytes synthesize various liver specific enzymes that process many synthetic and detoxifying functions of the liver. Furthermore, hepatocytes secrete the majority of plasma proteins, except immunoglobulins, including albumin, prothrombin, fibrinogen, lipoproteins and complement proteins.

ii) Bile synthesis and secretion: Hepatocytes synthesize bile acids from cholesterol. Bile acids emulsify fats in the lumen of the small intestine. Insoluble bilirubin is produced from red blood cell breakdown in the spleen. Then, it circulates in the blood by forming complex with albumin and is taken up from the blood hepatocytes in order to be secreted into the bile canaliculi.

iii) Glucose homeostasis: Liver contributes to the maintenance of the blood glucose levels. In response to pancreatic islet hormones, hepatocytes synthesize glucogen from glucose or break down glycogen in order to produce glucose (glycogenolysis). Glucose can also be generated from other sugars, such as fructose, and from amino acids (gluconeogenesis).

iv) Metabolism of drugs and toxins: Hepatocyte enzymes metabolize drugs and toxins delivered from the gut via the portal circulation. In the endoplasmic reticulum of liver

cells are located enzymes that convert lipid-soluble exogenous and endogenous compounds to water-soluble metabolites that can be easily excreted by the kidney. For example, in phase I biotransformation reactions the cytochrome P450 superfamily of monooxygenases oxidates lipid-soluble compounds to polar compounds. Phase II reactions conjugate these polar metabolites to glucuronic acid, sulfate, glutathione, glycine, or taurine. In Phase III reactions, these conjugated metabolites are transported into bile by specific transporters in phase III reactions. Due to the fact that the enzymes that participate in these reactions have a wide array of substrate specificity, the liver is able to metabolize various drugs. Depending on the rate of their metabolism in the endoplasmic reticulum, drugs are converted to less active or inactive compounds. Metabolism of the drugs by this enzyme system can also lead to more toxic compounds which can produce liver injury, for example in the case of carbon tetrachloride [4, 5].

1.2.2. Kupffer cells

Kupffer cells are macrophages that are situated in the luminal surface of the hepatic sinusoids and thus are exposed to the bloodstream. As Kupffer cells´ main function is to remove toxic substances, they are strategically located in the areas of entrance of foreign substances; the majority is found in the periportal region where they are larger and present greater phagocytic activity than those found in the central area of the lobule.

Kupffer cells remove through endocytosis toxicants and bacteria from the circulation, as well as toxic and infective substances of intestine origin. Furthermore, they secrete inflammatory mediators that influence the function of adjacent cells. Kupffer cells also produce both beneficial and toxic substances that contribute to host defense and liver injury respectively [3].

1.2.3. Sinusoidal endothelial cells

Sinusoidal endothelial cells (SECs) are a layer of cells between the hepatocytes and the blood flowing in sinusoids. SECs contain numerous fenestrae (pores) which are clustered together in groups known as “sieve plates” and allow the exchange between

the blood and the surrounding tissue. The endothelial fenestrae are dynamic structures whose diameters are affected by luminal blood pressure, vasoactive substances, drugs and toxins.

SECs represent an important blood clearance system, as all transport between the lumen and the hepatocytes has to pass through this filter. Furthermore, permit rapid access to substances in the blood. SECs play an important role in immunity and inflammation, as secrete pro-inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6), interferons and eicosanoids. They facilitate also adhesion of leucocytes and lymphocytes by secreting chemokines and expressing molecules, such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Thus, along with the Kupffer cells, the endothelium participates in host defense mechanisms. Furthermore, they contribute to the formation of new blood vessels and regulate sinusoidal blood flow by releasing vasoconstrictor and vasodilator factors [1].

1.2.4. Stellate cells

Stellate cells are located in the space of Disse, in the space between hepatocytes and sinusoidal endothelial cells. In this way, they are able to interact with the surrounding cell types. Stellate cells contain fat droplets and constitute the most important storage site of retinoids, including vitamin A. In healthy liver, they are quiescent. However, when activated, they synthesize collagen and thus contribute to the development of cirrhosis [1, 6].

1.3. Liver transplantation

Liver transplantation (LT) is the last resort option for patients with short edge diseases, such as chronic hepatitis B/C, autoimmune hepatitis and alcoholic cirrhosis. Between them, cirrhosis represents the main indication of LT, where predominate patients with alcoholic and virus C related cirrhosis [7]. The first successful LT was carried out with a whole liver graft (orthotopic LT, OLT) in 1967 and the procedure has been improved dramatically over the last years, due to the refinement and standardization of the surgical procedure [8]. The number of LT has been significantly

grown since 1980s and OLT consists the most common procedure for transplantation. According to European Liver Transplant Registry, more than 5000 liver transplants per year are performed in Europe and in USA and in most cases the 5-year patient survival is over 70% [7]. However, the overwhelming success of LT is limited by the increased number of patients added to the waiting list every year.

Consequently, several surgical techniques have been developed to enlarge the pool of organs that are based on the use of partial liver grafts that can be obtained from either a deceased donor (in this case the liver graft can be split and used for two recipients, usually an adult and a child) or a living donor [9]. The need for the development of new techniques is more important in case of pediatric transplantation, due to the fact that in this population the number of the cadaveric grafts with the adequate size is much more limited [10]. Although more technically demanding, these techniques give results similar to those for cadaveric LT and allow a larger number of patients to undergo LT [11].

1.3.1. Reduced-size orthotopic liver transplantation

Reduced orthotopic liver transplantation (ROLT) where only one lobe of the liver is used as a graft, whereas the rest is discarded, was firstly reported by Bismuth and Houssin in 1984, and aimed to overcome size disparity and provide grafts from older donors to younger recipients [12]. ROLT takes advantage of the segmental anatomy of the liver, where each segment is an independent functional unit with its own vascular and biliary supply. The most commonly employed parts of a graft used in children are segments 2 and 3 (left lateral segment) and segments 2, 3, and 4 (left lobe). However, the type of the graft chosen for transplantation depends mainly on the size disparity between donor and recipient. For example, a full right graft will usually fit in a recipient at least half the size of the donor, whereas a full left graft permits a donor-recipient size disparity of up to 4:1. Besides this, the relative size of the individual segments can vary significantly and for this reason the donor liver and the recipient's hepatic fossa should be examined carefully [13].

Results with ROLT in children have been comparable to those reported with whole-organ cadaveric grafts [14, 15]. Furthermore, ROLT provides the advantage of

lower incidence of hepatic arterial complications due to the larger caliber of the adult hepatic artery [16, 17]. ROLT has increased the number of pediatric donor organs, but not the total number of organs available for transplantation [10].

The surgical techniques applying partial grafts are based on the unique capacity of the liver to regenerate within a short period. During donor surgery, a part of the lobe is resected and the remaining hepatic cells are proliferated, so that the liver can be expanded in mass and compensate for the lost tissue, without harming the viability of the whole body. Through resection, it is important to achieve the minimal bleeding and leave adequate functional liver [18]. In experimental models, as rodents possess five lobules, three of them can be easily removed through a technique known as 2/3 partial hepatectomy. Thus, the remnant hepatocytes initiate the phenomenon of regeneration and within 5–7 days after surgery the liver size has been completely restored [19].

Liver regeneration is a very complex and well-orchestrated process, which involves the activation of various signaling cascades involving growth factors, cytokines and extracellular matrix remodeling. It is divided into three phases: priming, proliferation and growth termination. The priming phase involves extracellular matrix degradation and the activation of tumour necrosis factor (TNF)-α and IL-6, which present pro-mitogenic effects and facilitate the entering of hepatocytes to the proliferation stage. During proliferation stage, various growth factors are released, including hepatocyte growth factor, epidermal growth factor and transforming growth factor α that enhance DNA synthesis, the matrix remodelling and the restoration of liver function. Furthermore, in this stage many pro-angiogenic growth factors such as vascular endothelial growth factor, are up-regulated so that the microcirculatory system can be restored. Finally, in the ‘termination stage’, factors that inhibit proliferation, including transforming growth factor β are released and the hepatocytes are brought back into a state of quiescence [19].

1.3.2. Living donor liver transplantation

Living donor liver transplantation (LDLT) is a natural extension of ROLT, where a living person donates a portion of his liver to others. Potential donors can be

considered persons that are completely healthy and have a compatible blood group. The LDLT procedure for adults usually involves transplantation of the right hepatic lobe from an adult donor to the recipient, whereas for children recipient the left lateral lobe. For this reason, the hepatic size, liver anatomy and vasculature of the donor are factors to be considered for LDLT [20].

The development of LDLT was a result of the increased demand for organs in the late 80 ´s, when LT was increasingly successful. The first LDLT was realized in United States in 1989, where a child received a segment of his mother's liver [21]. LDLT presents the advantage of increased histocompatibility between donor and recipient, favoring thus the lower incidence of rejection [10] Furthermore, the mean waiting time for LDLT is much shorter than in case of deceased donor LT. Thus, LDLT constitutes an alternative therapy for patients with end-stage liver diseases in conditions of increased lack of cadaver livers [22].

Furthermore, the eventual postoperative complications of donor, as well as the possible mortality of the recipient after the surgery have decreased the health-related quality of life of donors [23]. In addition, although most donors present a satisfying level of liver regeneration [24], they can face post-operative complications, including biliary leakage and incision infection; in most cases patients are recovered after adequate treatment, but the hospitalization is prolonged [22].

1.3.3. Split liver transplantation

Split liver transplantation (SLT) is a technique that combines the procedures of ROLT and LDLT. In this case, a whole adult cadaveric liver is divided into two functioning allografts, allowing the transplantation in two recipients. It was firstly reported by Pichlmayr in 1988, where the right graft was placed into a 63-year-old woman with primary biliary cirrhosis and the left graft into a small child. Consequently, not only the drawbacks of ROLT and LDLT are overcoming, but also the total number of donor organs is augmented, especially in pediatric transplantation. While SLT is commonly accepted when one pediatric and one adult patient are transplanted, there is a strong debate about the possible success of adult-to-adult SLT. Indeed, two experienced centers in Italy have provided inferior outcomes in case of SLT when

compared to those achieved with whole liver grafts transplanted into adults [25, 26]. Furthermore, graft size must be adequate to fit into the recipient and to provide sufficient functional hepatic mass [27].

Besides this, the application of SLT for two adults can be hampered by many difficulties [25]. First of all, the procurement of the full left graft and the full right graft is a procedure that is highly technically demanding and requires surgeons with a high experience in liver resection and transplantation both during the graft procurement, as well as during the transplant. In addition, it is necessary an efficiently coordinated system between the donor and recipient teams, which are often working in different institutions. Furthermore, it still lacks an agreement between the physicians respecting on indications, surgical technique, and results, and therefore it is difficult to evaluate the final outcome and the possible advantages/inconvenients. At last, the theoretical feasibility of SLT for two adults has been estimated to be very low (less than 15%), may be due to the decreased availability of healthy big donors.

Partial liver grafts have a higher incidence of biliary complications as a result of the risks of biliary leakage from the transected liver surface and as a result of the risks of surgical dissection in the hepatic hilum. However, various studies have evidenced that the survival rates of grafts from SLT are comparable to those achieved with LT with complete cadaveric grafts [28-30].

1.4. Suboptimal grafts in LT: Steatotic livers

In an attempt to overcome the discrepancy between liver organ availability and demand, many liver transplant programmes are increasingly using donor livers of “marginal” quality; liver grafts that previously have been considered unsuitable for transplantation. The marginal grafts, such as the steatotic livers, are associated with higher liver graft dysfunction and postoperative complications [31-33]. This could justify the need for optimizing the techniques related with the transplantation of the suboptimal grafts. As steatotic or fatty livers are defined the livers that present an excessive (above 5% of wet liver weight) accumulation of lipids, mainly triglycerides, as lipid synthesis overcomes liver export and consumption. Liver steatosis can be provoked by various factors such as insulin resistance, a high fat diet, obesity and

alcohol abuse [34]. Depending on the percentage of hepatocytes that contain fat vacuoles within the cytoplasm, steatosis is considered as mild (< 30%), moderate (30 - 60%) or severe (>60%). In addition to this, fatty infiltration can be separated into, macro- and micro-vesicular steatosis. In macrovesicular steatosis, hepatocytes contain one large vacuole of fat, which displaces the nuclei to the cell periphery and it is mostly correlated with obesity, diabetes, or alcohol abuse. In case of microvesicular steatosis, the cytoplasm contains many small fatty inclusions and the nuclei remain in the center of the cell. Microvesicular steatosis is provoked when mitochondrial β-oxidation is impaired, like in presence of toxins or metabolic disorders [35]. In contrast to microvesicular steatosis, macrovesicular steatosis has been associated with poor outcome following LT [36].

The pathophysiology of hepatic steatosis is complex. Due to the fact that fat accumulates excessively in vacuoles within hepatocytes, cell volume is increased and subsequently the sinusoidal lumen is narrowed. Thus, microcirculation is impaired, nutrient and oxygen transfer is limited. Other characteristics include the dysfunction of adenosine triphosphate (ATP) synthesis, the development of a low energy balance with subsequent activation of cytosolic glycolysis and lactate accumulation, limited mitochondrial oxidative phosphorylation, decreased oxygen consumption and the production of the reactive oxygen species (ROS) due to elevated mitochondrial dysfunction [37].

1.5. Pathophysiology of ischemia-reperfusion injury

Apart from the immunologic reject, the success of transplantation is significantly restricted by the syndrome of ischemia-reperfusion injury (IRI). IRI develops when blood flow is interrupted or significantly attenuated for a period of time (ischemia) and then it is restored (reperfusion).The pathophysiology of liver IRI includes both direct cellular damage as a result of the ischemic insult, as well as delayed dysfunction and damage resulting from the ROS production and activation of inflammatory pathways during the reperfusion phase [38]. IRI remains a serious complication in clinical settings such as LT and hepatic resection and is the main factor responsible for primary graft non-function or malfunction following LT [39]. During

surgical resection, the portal triad is usually clamped in order to control excessive bleeding from the cut hepatic surface, provoking thus an ischemic insult. The posterior removal of clamp permits the blood return, but also causes reperfusion injury. In the case of transplantation, following graft ex-plantation from the donor, the liver graft is preserved to a cold preservation solution (and thus subjected to cold ischemia) in order to diminish metabolic activity. Then, it is implanted into the recipient and subjected to warm reperfusion [40].

The degree of IRI depends on various parameters, being the length and method of ischemia applied and the liver condition the most important ones. For example, patients who undergo short intermittent periods of ischemia, suffer less liver dysfunction compared to those with prolonged ischemia [41]. Furthermore, animal models with chronic liver disease or older/fatty livers exhibit exaggerated liver IRI when compared to younger or normal ones [40].

The mechanisms underlying IRI are multifactorial and more profound investigations are necessary in order to elucidate the implicated mediators and define their interactions. Reducing or preventing IRI is a central strategy for improving the graft performance after transplantation.

1.6. Ischemic injury

To begin with, the absent blood flow during ischemia results in insufficient oxygen supply to hepatocytes. Consequently, the mitochondrial oxidative phosphorylation is inhibited and the ATP synthesis is interrupted. Thus, there is a shift towards anaerobic metabolism. As cellular ATP stores are decreased, the ATP-dependent Na^+/K^+ ATPase is inhibited, causing alterations in intracellular Na^+ and Ca^{2+} homeostasis. The energy deficiency during ischemia also provokes SEC vacuolization and swelling, as well as sinusoidal lumenal narrowing [42]. Further, ATP degradation products, like adenosine, hypoxanthine and xanthine are accumulated, contributing to ROS production after reperfusion. The final result is cell swelling and death via necrosis or apoptosis. In addition, ischemia stimulates the formation of pro-inflammatory mediators and expression of adhesion molecules that mainly contribute to the injury in the reperfusion phase [43].

Liver ischemic injury can be categorized into warm (or normothermic), cold (or hypotermic) injury, and rewarming. Warm ischemia occurs in various clinical situations, such as hepatectomia, trauma and shock [44, 45]. Cold ischemic injury happens during LT, when the graft is conserved. Rewarming ischemia occurs when the graft is implanted during the anastomosis in LT. Ischemic injury influences differently hepatic cell types; nonparenchymal cells (sinusoidal endothelial, Kupffer, stellate and biliary epithelial cells) are more affected during cold ischemia, whereas in warm and rewarming ischemia are the hepatocytes [42].

1.7. Reperfusion injury

The hypoxic organ damage is further accentuated after the return of blood flow to the previously ischemic tissue. First of all, the increase in oxygen delivery exceeds the rate at which cellular metabolism returns to aerobic pathways, which results in ROS production. Apart from the direct cellular injury caused by ROS, the reperfusion phase is characterized by a cascade of mediators leading to microvascular changes and activation of inflammation, which are described as follows:

1.7.1. Reactive oxygen species

Crucial mediators of IRI are the ROS. Superoxide, hydrogen peroxide and reactive nitrogen species, such as peroxynitrite, are produced by cytosolic xanthine oxidase, mitochondria or are released by Kupffer cells and adherent leukocytes [46-48]. First of all, xanthine oxidase, with the molecular oxygen that is introduced on tissue during reperfusion, catalyzes the oxidation of hypoxanthine to xanthine, whereas superoxide is released. The production of ROS depends on the concentration of xanthine and hypoxanthine, which are accumulated during ischemia, but they are fast metabolized. Besides this, mitochondria are considered to play the most important role in ROS production. Under stress conditions, the electron leakage from the respiratory chain enzyme complexes leads to superoxide formation. However, mitochondria dispose anti-oxidant enzymes, including Mn-superoxide dismutase (MnSOD), glutathione and glutathione peroxidase, thioredoxin-2, and glutaredoxin in order to confront ROS [49]. In addition, Kupffer cells are the main source of ROS in the

early stages of liver IRI, whereas neutrophils are the main source in the very later stages [50].

Oxygen radical formation leads to damage various biomolecules, including nucleic acids, membrane lipids, enzymes, and receptors. Peroxidation of membrane lipids disrupts membrane fluidity and cell compartmentalization, which can result in cell lysis. Also, lipid peroxidation and protein oxidation contributes to the impaired cellular function and cell death. ROS can also ruin the microvasculature integrity by damaging endothelial cells [51] [52-54].

Moreover, oxidative stress in combination with calcium over-load induces opening of huge channels, named mitochondria permeability transition pores (mPTPs) that are localized to contact sites between the inner and outer mitochondrial membranes. Once opened, mPTPs permit ion exchange between the cytoplasm and the mitochondrial matrix. Consequently, the mitochondrial membrane potential is collapsed and ATP synthesis is inhbited. mPTPs induction also results in matrix swelling and rupture of the outer membrane, which subsequently leads to release of pro-apoptotic factors like cytochrome c (CytC)[43, 55].

1.7.2. Nitric oxide and endothelins

One of the earliest processes of liver reperfusion is the reduction in sinusoidal diameter and consequently the decreased blood flow. These microcirculatory changes are provoked due to an imbalance between vasoconstrictor factors, such as endothelins (ET) and vasodilator substances like nitric oxide (NO)[50]. First of all, SECs damage (that has been initiated during ischemia) results in deficient NO generation during reperfusion. In addition to this, SECs, as well as Kupffer and stellate cells, release augmented levels of ET. The final outcome is a significant reduction of the sinusoidal diameter[42]. Microcirculatory disturbances may also be triggered by the activation of the coagulation cascade[56].

NO is a free-radical diatomic gas that endogenously is synthesized from the amino acid precursor L-arginine. In endothelial cells, NO is produced in small quantities for short periods of time by endothelial nitric oxide synthase (eNOS) under physiologic conditions, as well as in response to extracellular stimuli, such as shear or metabolic

stress. Besides this, NO can also be generated in large amounts for sustained periods by inducible nitric oxide synthase (iNOS), in response to inflammatory mediators. It has been proposed that eNOS-derived NO has a protective effect against liver IRI by regulating sinusoidal diameter and abrogating thus the microcirculatory changes during reperfusion [57]. In this sense, it has been demonstrated that NOS inhibitors exaggerate IRI [58]. Furthermore, NO production by eNOS can prevent neutrophil adhesion and platelet aggregation. On the other hand, the excessive levels of iNOS-derived NO have been considered to be detrimental, as they have been associated with production of nitrogen species like superoxide and peroxynitrite [59].

1.7.3. Inflammatory mediators

The activation of inflammatory cells is a key event in the development of liver injury during ischemia and reperfusion. Kupffer cells are activated during reperfusion and release reactive oxygen and nitrogen species and pro-inflammatory cytokines, such as TNFα, interferon-γ (INF-γ), interleukin-12 and IL-1. These chemokines promote the expression of adhesion molecules, such as ICAM-1, potentiating thus the activation, recruitment, and adhesion of neutrophils to the endothelial cells. Adhered neutrophils trigger cell death by releasing various proteases (elastases, proteinases, and collagenases), which degrade components of the extracellular matrix, attack cells, and inactivate various proteins such as immunoglobulins and proteins of complement. Furthermore, neutrophils generate ROS, like hydrogen peroxide, through activation of nicotinamide adenine dionucleotide phosphate (NADPH) oxidase. In addition, the produced cytokines, ROS and the increased translocation of P-selectin (endothelial adhesion molecule) to the surface of endothelial cells and platelets promote the adherence of leukocytes to the microvascular endothelium. The inflammatory cascade induces significant organ infiltration and injury [38, 45, 60].

The discussed mechanisms of IRI are summarized in Figure 4:

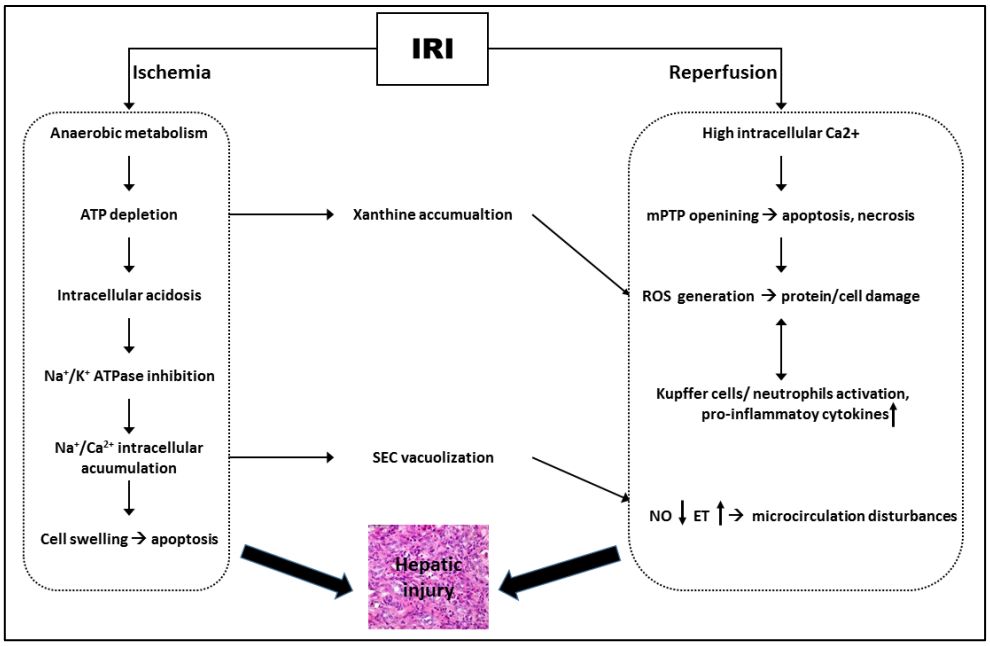

Figure 4: Mediators involved in ischemia-reprefusion injury (IRI).

1.8. Cellular processes involved in ischemia-reperfusion injury

IRI is a multifactorial process, as involves alterations in various signaling pathways, including apoptosis, necrosis, autophagy and endoplasmic reticulum stress

1.8.1. Apoptosis

Apoptosis is a relevant cell death mechanism during hepatic IRI. Two main pathways have been described for apoptosis; the intrinsic (mitochondrial) pathway that is activated by various stressors such as DNA damage and p53 activation and the extrinsic pathway that is triggered through death receptors. In intrinsic pathway, pro-apoptotic proteins, such as the Bcl-2 family (Bax, Bak, Bad, Bid) are activated and then translocate to mitochondria, contributing to increased permeability of the outer mitochondrial membrane. Consequently, mitochondrial cytochrome C (CytC) is released and interacts with apoptosis-activating factor-1 (APAF-1) to promote caspase 9 activation which then activates caspase 3 and the final stages of apoptosis.

The extrinsic pathway is initiated by the binding of various transmembrane receptors, (death receptors) to their cognate ligands. Death receptors include Fas, TNFα-receptor 1 and the death receptor 4 and 5. When the ligand is binding to its receptor, procaspases 8 and 10 and several adaptor proteins are recruited in order to

form a large complex, which results in activation of caspase 8 and 10 and finally in a proteolytic cascade that leads to cell death [5].

1.8.2. Necrosis

Apart from apoptosis, cell death also occurs through necrosis. Necrosis and apoptosis share characteristics and mechanisms, which complicates the discrimination between these forms of cell death. The basic characteristics of necrosis are cell swelling, vacuolation, karyolysis (dissolution of the nucleus) and release of cell content that can affect neighbouring cells and favour the initiation of inflammatory response. This contrast with the apoptotic features of apoptosis, where both the nucleus and cytoplasm are fragmented into apoptotic bodies which are then phagocyted by phagocytes or neighboring cells. Furthermore, the apoptotic cells usually present normal appearance; they do not release intracellular contents and consequently do not promote the inflammatory response. The initiation of necrosis and apoptosis depends from mPTP opening and ATP levels. In case of an excessive mPTP opening that involves most mitochondria, a complete ATP depletion is remarked. This fact halts caspase activation and promotes the opening of a glycine-sensitive organic anion channel which promotes plasma membrane rupture and the onset of necrotic cell death. However, if ATP is preserved, at least in part, CytC is released in order to activate the caspase-dependent apoptosis. The intracellular acidosis that occurs during ischemia delays the onset of necrotic cell death, whereas the pH normalization during reperfusion promotes necrosis [61]. Moreover, it has been reported that in warm ischemia reperfusion necrosis occurs predominantly in hepatocytes whereas in cold ischemia, necrotic death occurs nearly exclusively in SEC [62].

Besides this, CytC liberation to the cytosol can also lead to mitochondrial membrane depolarization and ATP depletion and finally promote necrotic cell killing. Thus, it is considered that apoptosis and necrosis coexist in liver pathology and share common signals, a phenomenon called necrapoptosis. For example, mPTP opening during IRI causes both apoptosis and necrosis, although in a particular circumstance one or the other may predominate [61].

1.8.3. Autophagy

Autophagy is a tightly regulated pathway implicated in many physiological and pathological processes. The term “autophagy” comes from Greek, meaning self-eating, as the cell degrades its own intracellular components. Autophagy is essential for normal development and embryogenesis, as contributes to the clearance of apoptotic cells. Autophagic degradation of cellular constituents can efficiently recycle essential nutrients so that basic biological processes can be sustained. [63].

One of the major regulatory components of autophagy is the protein kinase mammalian Target of rapamycin (mTOR), a serine/threonine protein kinase that modulates various cellular processes including cell cycle, growth and survival [64]. mTOR is activated through phosphorylation in the presence of growth factors and nutrient-rich conditions and results in inhibition of autophagy. Furthermore, activation of Akt/protein kinase B results in mTOR activation and subsequent activation of ribosomal protein S6 kinase (p70S6k). [65]. On the other hand, food restriction or starvation are well-known inducers of autophagy. Under these conditions, autophagy is activated to provide cells with all the necessary nutrients by degrading intracellular components. During starvation, AMPK is activated and suppresses mTOR, and thus activates autophagy [65].

The process of autophagy can be divided into four basic steps: induction, formation of autophagosome, autophagosome fusion with the lysosome, and degradation, where more than 30 autophagy-related proteins participate. The first step, the induction of autophagy, requires the beclin-1–class III PI3K (phosphoinositide 3-kinase) complex. After induction, the isolation membrane is elongated in order to sequester the cytosolic components and form the double membrane autophagosome. This step is primarily mediated by LC3II. LC3, the full length precursor protein, is converted to LC3-I which then is conjugated with phosphatidylethanolamine and thus is converted into LC3-II. LC3-II is inserted into the autophagosomal membrane, a process that play an essential role in the expansion of the autophagosomes [65]. Next, the outer membrane of autophagosomes fuse with lysosomes to generate the autophagolysosome and finally the contents of the autophagolysosome are degradated onto the lysosome [66].

The role of autophagy has been better described in cardiac IRI. It has been reported that autophagy is induced during cardiac ischemia, as the low nutrient provision and the subsequent activation of AMPK are necessary for autophagy induction. During the degradation process of autophagy, free fatty acids and amino acids are released, so they can be recycled to generate ATP and thus compensate for the energy deficient. It has been shown that cardiac autophagy serves as an energy-recovering process during ischemic phase and is essential for cardiomyocyte survival [67].

Furthermore, autophagy has been described to contribute to remove the dysfunctional mitochondria during reperfusion, as in this phase mitochondria become an important source of ROS, which results in initiation of inflammatory response and the damage of proteins and protein membranes. Under these conditions, a process known as mitochondrial autophagy or mitophagy is induced in order to take away the damaged mitochondria and prevent the release of the novice ROS [68].

Besides this, it has been reported that autophagy can be a double-edged sword in the pathological process of IRI [67]. Autophagy can be detrimental during reperfusion, but the underlying mechanisms remain to be elucidated. In case that autophagy is hyper-activated, can result in degradation of necessary proteins and thus lead to cell dysfunction and final cell death. During reperfusion, as AMPK is not activated, beclin-1 might be the most important mediator of autophagy [69]. The enhanced autophagic response by beclin-1 has been related with downregulation of the anti-apoptotic Bcl-2 protein, which could implicate a cross talk between autophagy and apoptosis [70, 71]. Furthermore, in an “ex-vivo” liver perfusion model decreases in autophagy parameters have been associated with increased hepatic IRI [72].

1.8.4. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an organelle where the secretory and membrane proteins are succumbed to posttranslational modifications. Proteins must be folded properly in order to be able to reach their destiny and fulfil their function. Thus, in ER nascent proteins are folded with the assistance of molecular chaperones

and folding enzymes. Furthermore, ER is the site where Ca^{2+} is stored and is released under various stimuli and in order to participate in cellular signal transduction [73].

Besides this, in case that the folding apparatus cannot deal with an excessive increase of protein translation or with perturbations in the ER environment, such as alterations in redox state and Ca^{2+} levels or improper post-translational modifications, then unfolded proteins are accumulated. The unfolded proteins expose hydrophobic amino-acid residues and form toxic protein aggregates, which results in stress conditions in the ER. To cope with ER stress (ERS), cells activate a series of signaling pathways referred to as unfolded protein response (UPR) that aim to decrease the load of nascent and unfolded proteins or clear out the damaged ER. The UPR involves the activation of three main resident transmembrane sensors in the ER: inositol requiring enzyme 1 (IRE1α), activating transcription factor 6 (ATF-6), and RNA-activated protein kinase (PKR)-like ER kinase (PERK), which normally bind in ER chaperones, like glucose regulated protein 78 (GRP78 or BiP) and thus are held in an inactive state. Upon UPR, GRP78 is displaced in order to manage the exposed hydrophobic regions of the unfolded proteins. The displacement of GRP78 results in IRE1α, PERK, and ATF-6 release and activation [74].

The activated IRE1α functions as a nuclease in order to splice X box-binding protein 1 (XBP-1) mRNA which contributes to degradation of mRNA of secretory and membrane proteins. The accumulation of unfolded proteins leads to proteolytical cleavage of ATF6 and its translocation in nucleus, where upregulates chaperones, such as GRP78, in order to restore the folding of proteins in the ER lumen [75]. PERK activation leads to phosphorylation of eukaryotic translation initiation factor 2α subunit (eIF2α) in order to halt protein translation and upregulate chaperones through increases in ATF4 mRNA (Figure 5A). In addition, it has been shown that the UPR induces the autophagic pathway in order to remove damaged organelles [73].

Although the early activation of UPR diminishes ER stress and contributes to cell survival, UPR prolongation due to excessive injury can result in cell suicide, usually in the form of apoptosis [74]. In this case, activated IRE1α binds the tumor necrosis factor associated factor 2 (TRAF2), and promotes apoptosis through the JunNH2-terminal kinases ½ (JNK), MAPK p38 and caspase-12 activation [76]. The proapoptotic

Bax and Bak translocate to the ER membrane causing Ca2+ release, provoking activation of caspases. Sustained activation of PERK and subsequent upregulation ATF4 results in increased expression of the C/EBP homologous protein (CHOP or GADD153). CHOP is a transcription factor that induces apoptosis, through inhibition of Bcl-2 expression [77]. CHOP over-expression has also been associated with enhancement of oxidative stress and inflammatory response (Figure 5B) [78].

It has been evidenced that IRI is associated with ER stress, including increased GRP78, CHOP, sXBP-1 and PERK [78, 79]. In rat OLT, decreases in ER stress parameters including GRP78, CHOP, ATF4, p-eIF2, caspase-12, has been related with attenuation of apoptosis and improved LT outcome [76]. Furthermore, in human LT UPR can lead to both adaptive or pro-apoptotic responses depending on the phase of transplantation. For example, during ischemia IRE1α enhances survival pathways in order to increase the folding capacity of the ER, whereas during reperfusion IRE1α can also activate the pro-apoptotic kinase JNK [80]. Consequently, it can be assumed that the regulation of the balance between the pro-apoptotic and pro-survival signalling pathways may be critical for organ recovery and function during transplantation.

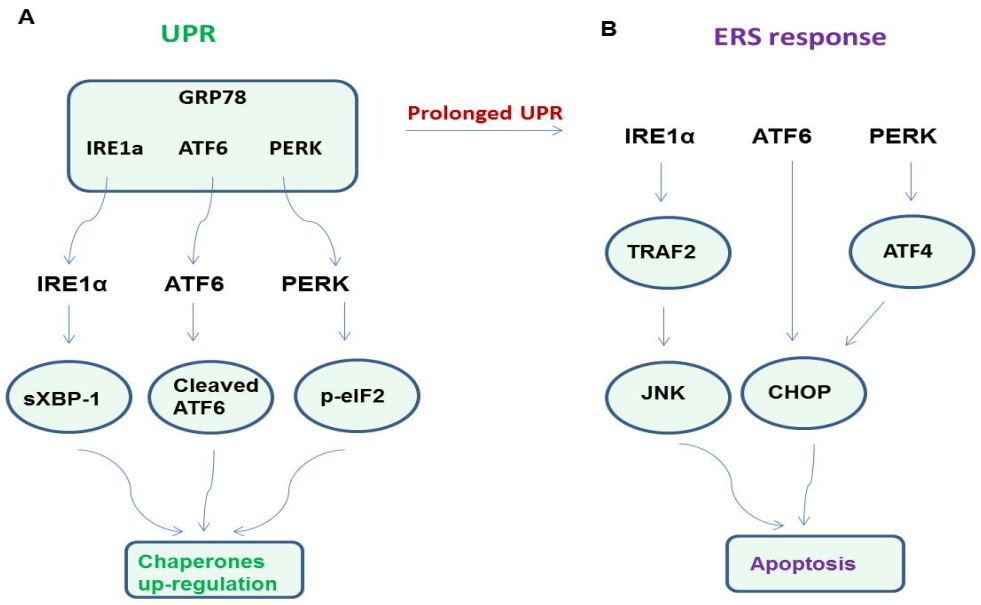

Figure 5: Main pathways of (A) unfolded protein response (UPR) and (B) endoplasmic reticulum stress (ERS) response (ERS response)

1.9. The renin-angiotensin system and IRI

The renin-angiotensin system (RAS) plays a central role in the regulation of blood pressure by affecting vascular smooth muscle tone and extracellular fluid homoeostasis. RAS includes the combination of various signal transductions, which initiates when angiotensinogen (released from the liver) is cleaved in the circulation by the enzyme renin (secreted from the kidney) in order to form the decapeptide angiotensin I. Angiotensin I is then activated to the octapeptide angiotensin II by angiotensin converting enzyme (ACE) (highly expressed in the lung). Besides this, it has been found that various tissues like heart, liver, kidney and brain can produce AngII (local RAS system) through pathways dependent or not of ACE. These local RAS systems act in a paracrine fashion and regulate inflammation, fibrosis, angiogenesis and cell proliferation, apoptosis and survival in various stimuli [81, 82].

Angiotensin II is the most powerful biologically active product of the RAS, although other bioactive angiotensin peptides have been described, such as angiotensin III, angiotensin IV, and angiotensin 1-7. Angiotensin II increases arterial blood pressure and maintains glomerular filtration, enhances vasoconstriction and myocardial contractility and regulates sodium transport by epithelial cells in intestine and kidney. Angiotensin II exerts its effects through its binding to two receptors, angiotensinII type I receptors (AT1R) and angiotensinII type II receptors (AT2R). Angiotensin II binds to the two receptors with similar affinity, but the majority of its biological actions are mediated through AT1R. Activation of the AT1R by angiotensin II leads to a variety of intracellular signalling events, which finally result in proliferation, inflammation, angiogenesis, and regulation of apoptosis. On the other hand, AT2R are mainly expressed during fetal development and have been associated with cellular differentiation and regeneration [83]. In addition, AT2R antagonizes the actions stimulated by AT1R, contributing thus to counterbalance some of the effects of angiotensinII mediated by the AT1R [84].

Various studies have demonstrated that the RAS is significantly involved in hepatic injury and inflammation. Previous study demonstrated that angiotensinII exerts proinflammatory actions by stimulating the secretion of cytokines and the expression of proinflammatory proteins, such as iNOS, as well as it activates hepatic

stellate cells in order to increase oxidative stress [85]. AngII has also been associated with liver fibrosis [86]. The biological effects of AngII in the liver are mainly mediated by AT1R, which are expressed on hepatocytes, hepatic stellate cells and Kupffer cells [86, 87]. Consequently, antagonists of AT1R, like losartan, have been associated with anti-fibrotic effects, as well as with attenuation of inflammation and oxidative stress in experimental models of IRI [88] [87, 89]. In addition, Losartan also has been found to protect steatotic livers against IRI [87, 90]. In clinical practice, it has been shown that losartan, lessens portal pressure in cirrhosis and liver fibrosis, including cases of non-alcoholic steatohepatitis [91, 92].

1.10. Steatotic livers in IRI

Steatotic livers are more vulnerable to IRI than non steatotic ones. Livers with severe steatosis are considered inappropriate for transplantation, as they are associated with a high risk of primary non-function, postoperative complications and patient death following LT. However, transplantation with livers containing mild steatosis (<30%) provide similar results than non steatotic ones, but the final outcome depends on the existence of additional risk factors [35]. Fatty livers tolerate to a less extent ischemic injury, as the decreased ATP levels lead to acidosis and cellular edema, which significantly impairs hepatic microcirculation. In addition to this, during reperfusion, the oxidative stress and the inflammatory response (including Kupffer cells, adhesion of neutrophils, cytokines) are much more excessive than in non-steatotic ones. For these reasons, it is an urgent need the development of strategies in order to minimize the detrimental effects of IRI in case of steatotic livers or to eliminate the fat content.

1.11. Surgical strategies to prevent IRI: Ischemic Preconditioning

Ischemic preconditioning (PC) is a surgical strategy developed to diminish IRI in various organs including liver, heart and brain. PC is based on the application of short periods of ischemia (5-10 minutes), separated by short reperfusions (10-15 minutes) prior to a sustained episode of IR. In this way, hepatocytes are prepared to respond favorably against the sequential prolonged IRI. Various duration times of PC have been applied in the rat and it has been shown that PC consisting of 5 minutes of ischemia

followed by 10 minutes of reperfusion conferred the strongest protection against hepatic ischemia -reperfusion after 60 minutes of partial ischemia followed by 24 hours of reperfusion in both normal and steatotic livers [2]. Moreover, the duration of the brief ischemic period is critical for the induction of preconditioning, as hypoxic periods shorter than 5 minutes or exceeding 15 minutes failed to induce protection. PC process involves multiple extracellular signals and intracellular second messengers [93-95].

The effects of PC can be differentiated in 2 phases characterized by different time frames and mechanisms: an early phase (early preconditioning) and the late phase (late preconditioning). Early preconditioning immediately follows the brief ischemic time and lasts 2–3 hours. The late preconditioning begins 12–24 hours from the transient ischemia and lasts for about 3–4 days [93, 96].

The effectiveness of PC led to the development of various strategies capable of mimicking its beneficial effects. Between them we can report the heat shock or hyperbaric preconditiong where the organ is temporarily exposed to hyperthermia or at 100% oxygen at 2.5 atmosphere absolute prior to ischemia [97, 98],as well as the pharmacological preconditioning which includes the administration of a chemical compound like doxorubicin [99] and simvastatin [100]. Although their protective effects against IRI have been evidenced in experimental models, their possible clinical application seems to be limited due to difficulties in implementing them in clinical practice, and other toxicity and side-effects problems [57].

1.11.1. Mediators of ischemic preconditioning

1.11.1.i. Adenosine

Various studies have evidenced the potential protective mechanisms of PC against hepatic IRI. The protective effect of PC is based on the fact that as ATP decreases during the brief ischemic period, induces endogenous adenosine and NO increases. In fact, the optimal ischemic time window to induce PC in the liver depends on adenosine and xanthine concentration levels. It is required an adenosine concentration high enough to induce NO generation through the activation of

adenosine A2 receptors, but also a low xanthine concentration in order to avoid the deleterious effects of NO.

Adenosine receptors are divided into 4 major subclasses: A1, A2A, A2B and A3, and in liver A2A receptor has been involved in the hepatoprotective effects of PC. Stimulation of liver adenosine results in activation of various kinases, including protein kinase C (PKC). PKCs are serine/threonine kinase isoenzymes that are divided into 3 main subclasses, from which novel PKCs (PKC -δ and -ε), that require diacylglycerol and phosphatidylserine for their activation, are involved in liver PC [101]. Activation of PKC during PC leads to elevated tolerance to IRI through activation of several intracellular signaling pathways like nuclear factor kappa B (NF-kB) and mitogen activated protein kinases (MAPKs), such as p38 [102].

1.11.1.ii. AMPK and eNOS

Furthermore, during the brief period of PC, adenosine monophosphate-activated protein kinase (AMPK) is activated and phosphorylates various downstream substrates in order to maintain ATP levels and reduce the lactate levels that have been accumulated during sustained ischemia. Furthermore, it has been shown that administration of an AMPK activator before ischemia simulated the benefits of preconditioning on energy metabolism and hepatic injury [103]. AMPK, a serine/threonine protein kinase, is an important regulator of cellular energy homeostasis and coordinates various metabolic pathways in order to provide a balance between the energy supply and demand. AMPK is activated by a high AMP/ATP ratio and in turn switches off ATP-dependent processes [64]. Apart from regulating hepatic energy metabolism, PC also regulates Na^+ homeostasis and contributes to the neutralization of intracellular pH by inhibiting the Na^+/H^+ exchanger [104].

NO also has been involved in the protective effects of PC against rat IRI by inhibiting ET levels and ameliorating hepatic microcirculation [105-107]. In addition, augmented eNOS expression was detected in preconditioned rat liver [108]. Apart from its vasodilator effects, NO contributes to decreased inflammatory response by increasing the anti-inflammatory interleukin-10 (IL-10) and thus inhibiting interleukin-1β release [109]. NO can also induce preconditioning of hepatocytes by promoting the

sequential activation of guanylate cyclase, cyclic GMP-dependent kinase and p38 MAPK [101]. Moreover, in a rat model of LT it has been shown that AMPK is activated during PC and induces the synthesis of NO and thus protects against IRI [110]. Also, NO contributed to the hepatoprotective effects of PC in a rat ROLT model; PC enhanced liver regeneration and decreased oxidative stress through inhibition of interleukin-1α [111].

1.11.1.iii. Mitogen activated protein kinases

MAPKs play an important role in intracellular signal transduction in response to extracellular stimuli and dual phosphorylation of their threonine and tyrosine residues is necessary for their activation. Once activated, these kinases are translocated to the nucleus, where they phosphorylate and activate different transcription factors and thus the transcription of various genes. MAPKs are classified as: (1) Extracellular signal regulated kinases, ERK ½, (2) (JNK 1/2) and (3) p38 MAPK. ERK 1/2 is usually activated by mitogenic and proliferative stimuli, like growth hormone receptors, whereas JNK and p38 are stimulated by various cellular stresses like: ROS, heat shock, inflammatory cytokines, and ischemia and for this reason are also referred as stress-activated protein kinases [112] [113]. p38 kinase regulates cell proliferation and differentiation and can modulate either pro-proliferative or pro-apoptotic signals [114]. Besides this, p38 activation has been mainly associated with the production and activation of inflammatory mediators [115].

The hepato-protective effects of PC have been associated with activation of JNK-1 and p-p38 and subsequent entry of hepatocytes into the cell cycle, thus favoring hepatocyte survival against IRI [102]. However, more recent studies in steatotic livers revealed that PC reduced p38 and JNK expression [116]. Furthermore, the pro-apoptotic ERK has been shown to be activated during cardiac PC [117].

1.11.1.iv. Heat shock proteins

Heat shock proteins (HSPs) are closely related to PC. HSPs are induced during exposure to a wide variety of stresses, including thermal stress, ischemia-reperfusion, hypoxia, in order to protect cells from damage. HSPs have been associated with anti-

apoptotic effect by binding to CytC, APAF-1 and inhibiting caspase activation [118]. In addition to this, HSPs activation decreases pro-inflammatory mediators, such as NF-kB and enhances the anti-oxidant capacity of the cell [119] [120]. Various studies have evidenced that the induction of HSP72 and HO-1 expression during PC contributed to the acquisition of improved hepatic function and increased tolerance against IRI [121, 122]. In addition, HSP70 and HO-1 activation during PC contributed to augmented liver regeneration in a rat ROLT model [111].

1.11.1.v. Signal transducer and activator of transcription-3

Furthermore, the IL-6/STAT3 pathway has also been involved in the protective mechanisms of PC. IL-6 is a cytokine that plays a central role in host defense, inflammation and liver regeneration [123, 124]. IL-6 carries out these functions through activation of the signal transducer and activator of transcription-3 (STAT3) and subsequent translocation of cytoplasmic STAT3 to the nucleus [125]. STAT3 is a factor of transcription that causes gene transcription of various genes associated with cell growth and differentiation, as well as with anti-oxidant and anti-apoptotic effect [126, 127]. It has been demonstrated that the up-regulation of IL-6 and subsequent induction of phosphorylated STAT3 contribute to decreased hepatocellular injury during PC [128].

1.11.2. PC effect on liver apoptosis

Furthermore, hepatic PC prevents hepatocyte and sinusoidal endothelial cell apoptosis, by down-regulating caspase-3 [129]. In addition, PC promotes anti-apoptotic signals through the Akt pathway. Akt is activated during hepatic PC and inhibits through phosphorylation pro-apoptotic factors such as Bad and glycogen synthase kinase β, as well as downregulates the JNK and NF-kB activities related with inflammation and tissue necrosis [130].

1.11.3. PC correlation with oxidative stress and inflammation

In addition, PC inhibits the oxidative stress derived through the xanthine oxidase pathway by limiting the accumulation of xanthine and the conversion of xanthine dehydrogenase to xanthine oxidase [131, 132]. PC can also reduce oxidative

stress by inhibiting the release of free radicals by Kupffer cells. Moreover, it has been reported that PC decreases the expression of P-selectin, thereby diminishes the oxidative neutrophil-mediated damage and the leukocyte adhesion, migration and activation[133] [134]. The anti-inflammatory capacity of PC has also been attributed to attenuated production of pro-inflammatory chemokines, such as TNF-α [135].

1.11.4. PC in fatty livers

Experimental models in warm ischemia and LT have evidenced the favorable effects of PC against the increased vulnerability of fatty livers to hepatic IRI. In rat warm ischemia, PC counteracted the mechanisms associated with the low resistance of fatty livers to hepatic IRI, including oxidative stress, neutrophil accumulation and microcirculatory failure, as well as prevented the release of pro-inflammatory cytokines (IL-1β) and increased the IL-10 generation (anti-inflammatory cytokine). Further, NO has been demonstrated to be an important mediator of these PC benefits [136]. Additional PC protective mechanisms include the decreases in adiponectin levels and in MAPKs activation, the enhanced expression of peroxisome proliferator-activated receptor-α (ppar-α) and of HSPs [116] [122]. Furthermore, PC contributes to increased tolerance of fatty livers to IRI by lessening the induction of mitochondrial permeability transition pore and by preserving ATP synthase activity [137]. In rat steatotic LT, PC through NO generation attenuated lipid peroxidation and neutrophil accumulation, resulting in decreased hepatic and lung damage [138]. AMPK is another important mediator involved in the protection against lipid peroxidation and hepatic injury in steatotic livers during transplantation [110].

1.12. PC in clinical practice

In clinical practice, the effectiveness of PC against IRI remains controversial. Several clinical studies have evidenced the beneficial effects of PC in hepatic resection. PC was demonstrated to be an effective straregy for lessening hepatic injury in both healthy and cirrotic livers [139, 140] and decreasing postoperative complications such as hemorrhage and biliary leakage [141]. Another randomized study evidenced that PC protective effect was stronger for young patients (less than 60 years) and patients with steatotic livers (>25% esteatosis) and was associated with ameliorated preservation of

hepatic ATP contents after reperfusion [142]. Besides these encouraging findings, more recent studies were not able to demonstrate any beneficial effect of PC in liver resection [143-145]. Significant controversy regarding the use of PC has also been evident in transplantation. A randomized prospective study of deceased donor LT showed a significant improvement in hepatic injury, decreased apoptosis and reduced incidence of primary non-function when PC was performed at the end of the procurement procedure [146]. Moreover, PC has also been shown to attenuate graft rejection incidence in recipients of steatotic grafts through augmenting autophagy and thus preventing parenchymal necrosis [147]. In a nonrandomized study of OLT, the use of PC was associated with lower increases in hepatic injury parameters, but it did not ameliorate the perioperative outcome [148]. In other study of deceased donor LT PC was found to be insufficient to provide clinical benefits [149]. Consequently, more randomized clinical studies are necessary in order to confirm whether PC is appropriate for LT in clinical practice.

1.13. Strategies against cold ischemia-reperfusion injury: preservation solutions

Cold IRI is inherent to LT and an appropriate organ preservation solution is necessary for the maintenance of the functional and morphological integrity of the graft. Although cold is a fundamental requirement for tissue preservation, it has harmful consequences, such as induction of cell swelling. For this reason, various commercial organ preservation solutions have been produced in order to prevent many of the cellular alterations associated to hypothermia [150].

Various preservation solutions have been proposed, with the University of Wisconsin (UW) solution being considered as the gold standard for liver grafts. UW has been developed in the early 1980s by Belzer and Southard and has improved significantly organ preservation and has been widely used in USA and Europe [151, 152]. UW solution is a phosphate buffer, with high K+ concentration (intracellular like solution) which further contains lactobionic acid, raffinose and hydroxyethyl starch (HES) as osmotic supporters and glutathione and allopurinol in order to eliminate free radicals. Although the colloid HES prevents interstitial edema [153], it has also been shown that enhances the aggregation of erythrocytes, a fact that could result in stasis

of blood and incomplete washout of donor organs before transplantation [154]. Furthermore, the high K^+ levels can cause blood vessel constriction when the organ is cold flushed [155].

The inconvenients of UW solution led to the development of the Institut Georges Lopez-1 (IGL-1) solution. IGL-1 solution is characterized by inversion of K^+ and Na^+ concentrations compared to UW solution and contains polyethylene glycol of 35 kDa (PEG35) as osmotic support rather than HES. IGL-1 has been applied in clinical kidney [156] and LT models [157] with satisfying results. Furthermore, in several experimental orthotopic LT models IGL-1 has been evidenced as good alternative to UW solution [76, 158, 159]. The beneficial action of IGL-1 has been related with enhanced production of NO through eNOS activation, prevention of oxidative stress and decreases in apoptosis and endoplasmic reticulum stress [76, 160]. In addition, IGL-1 provided a more effective preservation of steatotic livers in an "ex-vivo" perfusion model [58]. Furthermore, in order to ameliorate liver graft preservation, various experimental studies have proposed the enrichment of UW and IGL-1 solutions with various additives, including anti-ischemic drugs (like trimetazidine, TMZ) or hormones (like melatonin)[150].

1.14. New therapeutical targets for ischemia-reperfusion injury: Sirtuins

Although the IRI has been extensively investigated, it still remains a serious complication in liver surgery and for this reason it is an urgent need to identify new pharmaceutical targets in order to diminish its detrimental effects. Recent experimental investigations in heart and brain have evidenced that sirtuins are implicated in IRI and can potentially be appealing targets for therapeutic interventions against IRI.

The sirtuins belong to the highly conserved class III histone deacetylases with homology to the yeast silent information regulator 2 (Sir2). To date, seven sirtuins have been described in mammals (SIRT1 through SIRT7). They possess nicotinamide adenine dinucleotide (NAD^+) deacetylase activity, with the exception of SIRT4 which has only ADP-ribosyltransferase activity, and SIRT1 and SIRT6 which have both

deacetylation and a relatively weak ADP-ribosyltransferase activity [161]. Their enzymatic activity depends on their protein expression levels, the availability of NAD^+ and the presence of proteins that modulate sirtuin enzymatic activity. For instance, expression of SIRT1 increases during starvation or when cells are exposed to conditions of oxidative stress and DNA damage [162, 163].

Sirtuins are found in different subcellular locations, including the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2), and mitochondria (SIRT3–5), although in some studies, SIRT1 has been found to possess cytosolic activities, and SIRT2 has been found to associate with nuclear proteins [164].

Several studies in the past few years have shown that sirtuins regulate a wide variety of cellular functions, such as gene transcription, metabolism and cellular stress response [165-167]. SIRT1, the most studied member of the family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis. SIRT3 has recently emerged as a sirtuin with considerable impact on mitochondrial energy metabolism and function.

1.14.1. Role of sirtuins in ischemia

The low energy state during ischemia results in activation of AMPK. Sirtuins activity is directly related to the metabolic state of the cell due to their dependence on NAD+. In this regard, Suchankova and collaborators have found that glucose-induced changes in AMPK are linked to alterations in NAD +/NADH ratio and SIRT1 abundance and activity [168]. From these results, we might consider a possible implication between AMPK and SIRT1 in ischemic conditions. Indeed, an activator of AMPK, AICAR, has been found to ameliorate IRI and decrease SIRT1 expression in the rat kidney [169]. Furthermore, enhancing the activity of SIRT1 through the application of resveratrol, a SIRT1 activator, has been demonstrated to protect against cerebral ischemia [170].

Another element that plays an essential role in triggering cellular protection and metabolic alterations from the consequences of oxygen deprivation are hypoxia-inducible factors (HIFs). Mammals possess three isoforms of HIFα, of which HIF1α and HIF2α are the most structurally similar and best characterized. During hypoxia, protein

levels of HIF-2α increase slightly, but it becomes significantly activated, suggesting that additional post-translational mechanisms regulate its activity. One of these post-translational modulations could be deacetylation, since in hypoxic Hep3B cells SIRT1 deacetylates lysine residues in the HIF2α protein, enhancing its transcriptional activity [171].

Additionally, SIRT1 interacts with HIF1α, but in this case SIRT1 represses HIF-1α transcriptional activity [172]. Under hypoxic stress, decreased cellular NAD regulates SIRT1, increases HIF1α acetylation, and thereby promotes the expression of HIF1α target genes [172]. Interestingly, other studies have shown that HIF2α compete with HIF1α for binding to SIRT1 [173]. Moreover, it has been evidenced that SIRT6 is also linked to HIF1a by repressing the transcription of HIF1a target genes [174].

Likewise, the effects of SIRT3 appear to be protective in the context of hypoxic stress in human cancer cells. SIRT3 overexpression resulted in decreased ROS and impediment of stabilization of HIF1a, with a subsequent suppression of tumorigenesis [175, 176]. On the contrary, the role of SIRT3 on HIF1α stabilization in IRI has not been reported.

One of the most important factors involved in the metabolic control regulated by SIRT1 is peroxisome proliferator-activated receptor-γ coactivator (PGC1α), a transcriptional co-activator of many nuclear receptors and transcriptional factors. SIRT1 functionally interacts with PGC1α and deacetylates it thus inducing the expression of mitochondrial proteins involved in ATP-generating pathways [177]. Increased PGC1α activity is also related with lessened oxidative damage during ischemia, as it has been shown by decreasing ROS scavenge in rodents lacking PGC1α and subjected to global ischemia [178]. Furthermore, the uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, regulates proton electrochemical gradient and in neuronal cells PGC1α is required for the induction of UCP2 and a subsequent protection against oxidative stress [179]. It has also been shown that enhanced activity of SIRT1 during PC or resveratrol preconditioning confers protection against cerebral ischemia through decrease of UCP2 levels, resulting in increased ATP levels [170]. However, a more recent study associated the protective effect of resveratrol against oxidative stress in cerebral ischemia with increased levels of both SIRT1/ PGC1a and

UCP2 levels [180]. Moreover, the exact role of UCP2 during ischemia has not been fully understood, as studies of its effects have produced conflicting results [181-184]. The overall mechanisms are summarized in Figure 6.

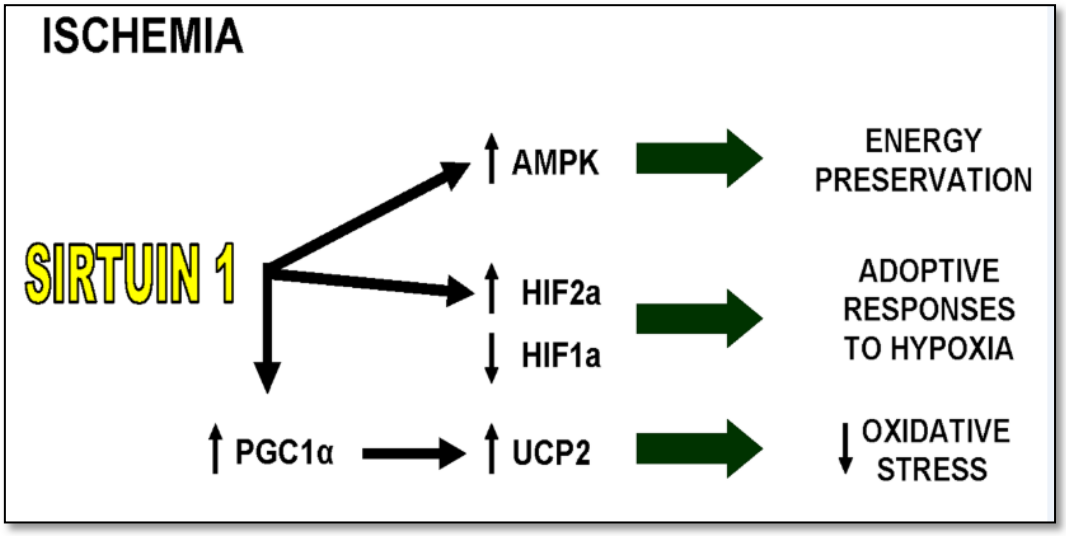

Figure 6: Protective role of sirtuin 1 during ischemia. Sirtuin 1 (SIRT1) activates adenosine monophosphate protein kinase (AMPK) as a cell response to counteract the energy deficiency. SIRT1 upregulates hypoxia-inducible factor 2α (HIF2α) and downregulates HIF1α to increase their transcriptional activity. SIRT1 upregulates peroxisome proliferator-activated receptorγ coactivator, leading to enhancement of anti-oxidant capacity of uncoupling protein 2 (UCP2). PGC1α: Peroxisome proliferator-activated receptorγ coactivator.

1.14.2. Role of sirtuins in reperfusion

Deprivation of oxygen to the grafts during ischemia induces severe lesions, but the most important damage is caused during reperfusion, when oxygen entry to the organ is restored and ROS are generated. ROS can be eliminated by enzymatic pathways including MnSOD, catalase (Cat) and peroxidases. Imbalance between ROS generation and elimination produces oxidative stress [50, 185].

Various reports in cardiomyocytes have demonstrated the protective role of SIRT1 against oxidative stress [186, 187]. Hearts overexpressing SIRT1 were more resistant to oxidative stress in response to IRI, as SIRT1 upregulated the expression of anti-oxidants like MnSOD and thioredoxin 1 (Trx1) [188]. SIRT1 also deacetylated Forkhead box-containing protein O (FoxO) 1 (FoxO1) transcription factor, inducing its

translocation and subsequent transcription of anti-oxidants molecules [188, 189]. Moreover, the question of whether SIRT1 can induce the transcription of other FoxO transcription factors, like FoxO3α, has not yet been investigated. However, the levels of SIRT1 activation are decisive for its protective role, as very high cardiac SIRT1 expression induces mitochondrial dysfunction and increases oxidative stress [186]. Furthermore, in a model of kidney IRI, the protective effect of SIRT1 against oxidative stress has also been demonstrated, since SIRT1 up-regulated Cat levels and maintained peroxisomes number and function [190].

Although mitochondrial sirtuins (SIRT3-SIRT5) have not been studied as extensively as SIRT1, an increasing body of evidence indicates the importance of SIRT3 in mitochondrial biology and function. Lombard and collaborators demonstrated that SIRT3 is the dominant mitochondrial deacetylase, as a significant number of mitochondrial proteins are hyperacetylated in SIRT3−/− mice [191]. SIRT3 deacetylates and thus enhances the activity of various proteins that appear to be an important part of the antioxidative defense mechanisms of mitochondria, such as MnSOD [192, 193], regulatory proteins of the glutathione [194-196] and Trx [197].

Transcriptional upregulation of the antioxidant enzymes MnSOD, Cat and peroxiredoxin can also be achieved by FoxO3α transcription factor, which is translocated to the nucleus after being deacetylated by SIRT3 [198, 199]. Furthermore, SIRT3 is necessary for the enhanced expression of Cytc, which presents peroxidase- and superoxidase-scavenging capacity [194, 196, 200]. However, a similar anti-oxidant effect of SIRT3 has not yet been established in models of IRI.

A wide array of functional alterations develops in mitochondria during reperfusion injury [201, 202]. In healthy cells, their primary function is the provision of ATP through oxidative phosphorylation in order to meet the high energy demands. Moreover, mPTP is involved in the decline in mitochondrial function, which is a common finding during reperfusion injury [203-205]. SIRT3 is known to deacetylate the regulatory component of the mPTP, cyclophilin D, and thereby reduces its activity and the subsequent mitochondrial swelling in heart [206]. It has also been shown that SIRT4 interacts with the adenine nucleotide translocator, another component of mPTP,

and that SIRT5 deacetylates CytC, but the physiological importance of these interactions has not yet been established [207, 208], especially in models of IRI.

Microcirculatory alterations play an important part in IRI. During the ischemic period, vascular hypoxia can cause increased vascular permeability. After reperfusion, complement system activation, leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation further aggravate microvascular dysfunction [209].

NO opposes the vasoconstrictive actions of ET can abrogate the microcirculatory stresses generated during reperfusion [210]. There is a large body of evidence in favour of the relationship between eNOS and SIRT1; SIRT1 interacts and modifies the acetylation state of eNOS, resulting in the activation of the enzyme [211-213]. In $SIRT1^{+/+}$ hearts subjected to IRI SIRT1 was associated with eNOS activation [214]. SIRT1 activation by resveratrol protected against subacute intestinal IRI by reducing the NO production through iNOS [215] Moreover, various experimental models showed that resveratrol inhibits endothelin-1 levels, providing a better regulation of vascular tone [216-218]. However, a recent study in human umbilical vein endothelial cells has shown that the inhibitory effects of resveratrol in endothelin-1 levels are SIRT1-independent [219]. The overall mechanisms are summarized in Figure 7.

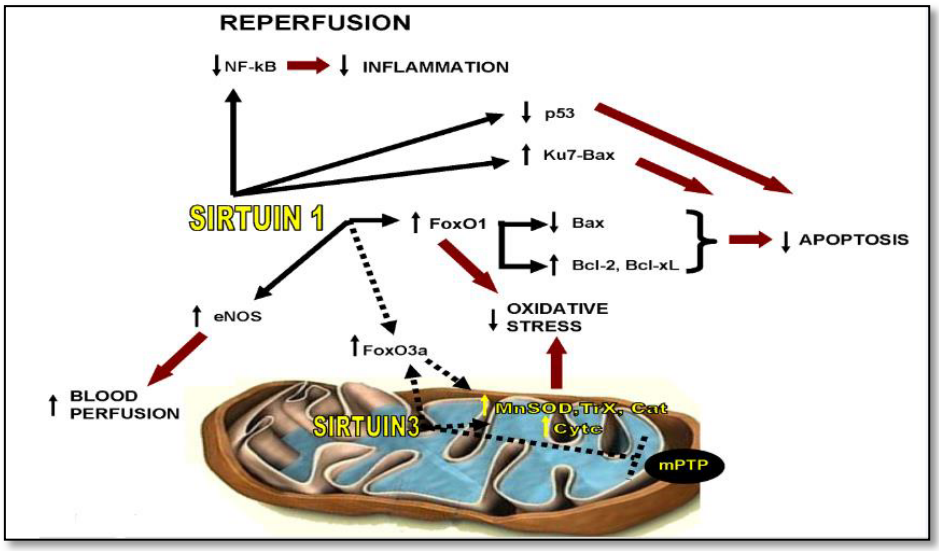

Figure 7: Protective role of sirtuin 1 and suggestive role of sirtuin 3 during reperfusion. Sirtuin 1 (SIRT1) inhibits inflammation through inhibition of nuclear factor kappa B and activates

endothelial nitric oxide synthase for a better microcirculation. SIRT1 downregulates apoptosis through multiple pathways, for example, inhibiting p53 transcriptional activity or favoring the binding between Ku70 and Bax. SIRT1 also enhances forkhead box-containing protein O 1 (FoxO1) transcriptional activity, resulting in Bax downregulation and in the upregulation of B cell lymphoma-2 and Bcl-like X. Deacetylation of FoxO1 by SIRT1 also results in lessening oxidative stress, whereas the same effect may be achieved by deacetylation of forkhead box-containing protein 3 alpha (FoxO3α). Sirtuin 3 (SIRT3) is suggested to contribute to decrease in oxidative stress either by a direct interaction with mitochondrial anti-oxidant enzymes [manganese superoxide dismutase (MnSOD), thioredoxin system (Trx), cytochrome (Cyt)] or by enhancing FoxO3α to transcribe MnSOD and Cat. Mitochondrial permeability transition pore (mPTP) may also be inhibited by SIRT3 and result in less production of oxidative stress. NFκB: Nuclear factor kappa B; eNOS: Endothelial nitric oxide synthase; Bcl-2: B cell lymphoma-2; Bcl-xL: Bcllike X; Bax: Bcl-2-associated X; Cat: Catalase

1.14.3. Role of sirtuins in IRI-associated inflammation

IRI results in a profound inflammatory tissue reaction with immune cells infiltrating the tissue. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors with NF-kB being one of the key modulators of inflammation. After activation, the transcription factor migrates to the nucleus and enhances the transcription of pro-inflammatory genes, potentiating the inflammatory response. This is followed by an infiltration of lymphocytes, mononuclear cells/macrophages, and granulocytes into the injured tissue [220-222].

SIRT1 plays an important role in neuroprotection against brain ischemia by deacetylation and subsequent inhibition of p53 and NF-κB pathways [223]. In SIRT1+/+ hearts subjected to IRI SIRT1 was correlated with decreased acetylation of NF-κB and a possible prevention of inflammation [214]. Moreover, SIRT1´s anti-inflammatory action, by deacetylating NF-κB and thus inhibiting the expression of endothelial adhesion molecules, has also been demonstrated in human aortic endothelial cells [222].

1.14.4. Sirtuins: cell survival or death?

Apoptotic cell death is a well-known mechanism involved in IRI which occurs via activation of caspases that cleave DNA and other cellular components [50, 224, 225]. There is evidence that SIRT1 is associated with life longevity in mammals and enhances mammalian cell survival under stress conditions by regulating the specific substrates [226-228]. In fact, several studies have mentioned SIRT1´s anti-apoptotic effect in IRI. SIRT1 deacetylates known mediators of apoptosis, such as the tumor-suppressor p53, resulting in inhibition of its transcriptional activity [229, 230] and the DNA repair factor Ku70 [162, 231, 232], causing it to sequester the pro-apoptotic factor Bcl-2-associated X / Bax away from the mitochondria. In ischemic kidney and brain SIRT1 has been identified as an important survival mediator, given that increased SIRT1 was associated with reduced p53 expression and apoptosis [223, 233]. SIRT1 also modulates apoptosis-related molecules through the deacetylation of FoxO family of transcription factors. During IRI in SIRT1+/+ heart transgenic mice, SIRT1 induces nuclear translocation of FoxO1, which upregulates the anti-apoptotic factors B cell lymphoma-2 (Bcl-2) and Bcl-like X (Bcl-xL) and downregulates Bax [188]. As regards other members of FoxO family, Brunet *et al.* revealed a dual role of SIRT1 in the cell cycle in stress conditions; SIRT1 inhibited FOXO3's ability to induce cell death, thus promoting cell survival, and surprisingly, it increased FOXO3's ability to induce cell cycle arrest and resistance to oxidative stress [234].

A possible pro-apoptotic role of SIRT1 in IRI has not been reported previously. However, studies in human embryonic kidney cells, have revealed that SIRT1can promote cell death by inhibiting NF-kB in response to TNFα [235]. Further investigation is required in order to define the conditions under which SIRT1 may promote apoptosis.

Since apoptotic pathways are initiated upon the opening of the mPTP and SIRT3 is located in the mitochondria, it may be involved in anti-apoptotic pathways. In this regard, SIRT3 protects various types of cells from apoptotic cell death triggered by genotoxic or oxidative stress [236-239] . The pro-apoptotic role of SIRT3 has also been associated with tumor suppression and restraint of ROS [240]. However, SIRT3 has also been reported to contribute to Bcl-2- and JNK-related apoptotic pathways in human

colorectal carcinoma cells [241]. In any case, the potential anti-apoptotic mechanisms of SIRT3 during IRI are yet to be elucidated.

2. OBJECTIVES

Taking into account the crucial importance of identifying new mediators implicated in IRI and that SIRT1 has been involved in models of IRI in heart and brain but no data is reported concerning its role in liver IRI, the objectives of the present thesis are the following:

- Objective 1: Examine whether SIRT1 is involved in the hepato-protective mechanisms of PC against IRI in obese rats.
- Objective 2: Investigate the potential implication of SIRT1 in a model of OLT when an IGL-1 preservation solution enriched with trimetazidine was used.
- Objective 3: Evaluate the possible involvement of SIRT1 in ROLT when an antagonist of angiotensin II was administrated.

3. MATERIALS AND METHODS

3. Materials and Methods

3.1. Animals

In order to realize the study related to PC, male homozygous obese (Ob) Zucker rats aged 12 weeks were used. Ob rats are characterized by the lack of the cerebral leptin receptor and show severe macro- and microvesicular fatty infiltration in hepatocytes (40–60% steatosis). As far as transplantation experiments are concerned, male Sprague–Dawley rats (200–250 g) were used as donors and recipients. All animals come from Charles River (France) and were housed in conventional animal facilities (Faculty of Medicine, University of Barcelona) for at least one week before the surgical procedure. The environmental conditions of animal facilities were constantly regulated; temperature at 22^0C, humidity 70 % and 12-hour light/dark cycle. All animals had free access to water and a standard laboratory diet (12% fat, 28% protein and 60% of carbohydrates). All procedures were performed under isofluorane inhalation anesthesia at 1.5-2%, and oxygen flow at 2-2.5 L/min. Animal experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 400/12 and 396/12), University of Barcelona, as well as complied with European Union regulations for animal experiments (EU guideline 86/609/EEC).

3.2. Ischemic Preconditioning

In order to induce PC, a microvascular clamp was applied to the hepatic artery and the portal vein of the rats for 5min. In this way, we induce partial ischemia (70 %) in the liver. Then the clamp was removed and 10 minutes later we induced partial hepatic ischemia of 60 min followed by 24-hours of reperfusion.

3.3. Orthotopic liver transplantation design

The orthotopic liver transplantation was performed according to the technique of double "cuff" without reconstruction of the hepatic artery, as has been previously described by Kamada *et al.* [242]. The technique can be divided into three phases: donor surgery, bank surgery and surgery of the receptor.

3.3.i. Donor Surgery

After the rat being anesthetized, the rat abdomen was shaved and transverse laparotomy was performed. The hepatic ligaments were sectioned and the inferior vena cava was freed. The right renal pedicle was dissected and then the right renal artery and vein and the right lumbar and suprarenal veins were ligated. Next, in hepatic hilum, portal vein was dissected away from hepatic artery and the bile duct. The pyloric and splenic veins were ligated. Then, it followed the cannulation of bile duct with a catheter of polyethylene (approximately 2 cm long) and fixed with a double 6/0 silk ligature. The right diaphragmatic vein was freed and ligated, whereas aorta was separated from inferior vena cava (Figure 8A).

Afterwards, we administered intravenously 300 units of heparin. Once the liver was ready for its extraction, the aorta was cannulated (with a catheter of 20 G) and perfusion of the graft was started with 50 ml of UW solution. For this reason, it was necessary the occlusion of thoracic aorta and the cut of the suprahepatic inferior vena cava (Figure 8B). Once the perfused liver was extracted, it was placed in a bath with UW solution at 4 ^{0}C.

3.3.ii. Bench surgery

Bench surgery is performed in order to prepare the graft for its implantation into the receptor. The diaphragm surrounding the suprahepatic inferior vena cava was cut. Then, we cut the length of the inferior vena cava, leaving a small rim for its posterior anastomosis. In order to facilitate the anastomosis, we place two surgical 7.0 polypropylene sutures at both ends of the suprahepatic inferior vena cava. Furthermore, the anastomosis of the portal vein and infrahepatic inferior vena cava were performed in the receptor applying the technique of double cuff. During the bench surgery, we localized in both veins some tubular structures of polyethelene called cuffs (Figure 8C). In this way, during the implantation process, each cuff is inserted into portal vein and infrahepatic inferior cava, avoiding thus a continuous suture that could increase the duration of the intervention and could also be detrimental for the survival of the receptor.

3.3.iii. Surgery of the receptor

Similarly as in case of donor, we perform laparotomy and liver dissection. Furthermore, in hepatic hilum, bile duct was sectioned. Two surgical 7.0 polypropylene sutures were located in the extreme sites of portal vein and infrahepatic inferior vena cava in order to make easier the introduction of cuffs and the proper orientation of both veins. By applying microvascular clamps we obtained the occlusion of portal vein (in the point of its confluence with splenic vein), of the infrahepatic inferior vena cava (in the upper site of right renal vein) and of the suprahepatic inferior vena cava (in this case we used a Satinsky clamp). At this point begins the anhepatic phase; the portal vein as well as both the infra- and supra- hepatic inferior vena cava were sectioned in sites as much nearer to liver as possible (Figure 8D).

Following, donor´s liver is perfused with Lactate Ringer Hartmann solution in order to eliminate the excessive concentration of K^+ that possesses the UW solution. Donor´s liver is now ready to be implanted to the receptor, through a continuous surgical 7.0 polypropylene sutures between the donor´s and the receptor´s suprahepatic inferior vena cava (Figure 8E). Then, with a vascular clamp holding the cuff extension, the cuff of the donor portal vein was inserted into the lumen of the recipient portal vein and secured with a 6.0 silk ligature. Then, the clamps that occluded the portal vein and the suprahepatic inferior vena cava were removed, allowing graft reperfusion. Next, the anastomosis of infrahepatic inferior vena cava is realized, as in case of portal vein, which was followed by the removal of the correspondent microvascular clamp and the subsequent blood flow restoration. Then, the graft is rehydrated by an intravenous administration of 0.5 ml of sodium bicarbonate (1 M) and 2.5 ml of isotonic Ringer Lactate solution; in this way we achieve to restore the volume that has been lost and counteract the acidosis produced during surgery. At last, bile duct anastomosis took place (Figure 8F) and the surgical intervention finalized with a continuous suture (2.0) of both muscle and skin.

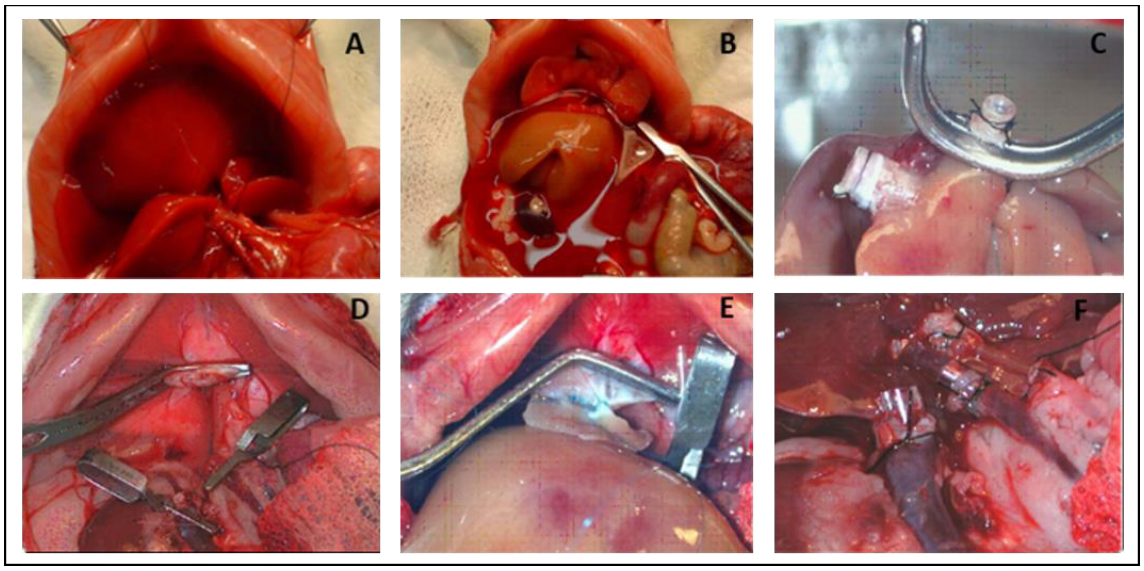

Figure 8: Orthotopic liver transplantation in Sprague-Dawley rats. (A): Hepatic hilum of the donor, (B) Liver graft perfusion with UW solution, (C) Cuffs of portal vein and of infrahepatic inferior vena cava, (D) Hepatectomy of the receptor, (E) Anastomosis of inferior suprahepatic vena cava, (F) Anastomosis of infrahepatic vena cava, portal vein and bile duct

3.4. Reduced-size orthotopic liver transplantation design

The procedure of reduced orthotopic liver transplantation was realized similarly as in case of OLT, according the technique reported by Kamada *et al.* and Xia *et al.,* [242, 243] but with the only difference regarding the previously described protocol:

During donor surgery, after the cannulation of the bile duct with the catheter, the reduction of the liver was carried out. The left lateral lobule and the two caudate lobules, which account for 70 % of liver mass, were elevated to expose their vascular pedicle, which was encircled with a ligature (5.0) and then they were removed. Then, we administered heparin and and we perfused the graft with 50 ml de UW solution, as mentioned before.

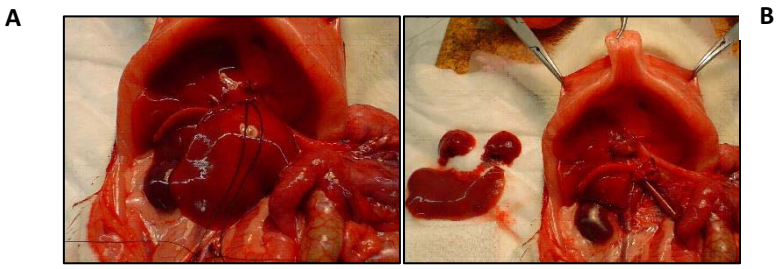

Figure 9: (A) Ligature of left lateral lobule and (B) Removed hepatic lobules (left lateral lobule and the two caudate)

3.5. Experimental Groups

3.5.1. First study

Silent information regulator 1 protects the liver against ischemia–reperfusion injury: implications in steatotic liver ischemic preconditioning

The first study aimed to evaluate the potential role of SIRT1 in fatty liver ischemic preconditioning. For this reason, the following experimental groups were realized with Zucker obeses (ob) rats:

1. Sham (n = 6): Ob rats were anesthetized and subjected to transversal laparotomy and hepatic hilum vessels were dissected. We finally realized a continuous suture of both muscle and skin and animals were returned to their cage and after 24 hours they were sacrificed.
2. IR (n = 6): After laparotomy and the dissection of hepatic hilum vessels, a microvascular clamp was localized for 1 hour to both the hepatic artery and the portal vein. In this way, the hepatic inflow to the median and left lobes was obstructed, provoking thus a partial (70 %) ischemia. After 1 hour, the clamp was removed, allowing thus the blood flow recovery and the initiation of reperfusion phase. Animals were sacrificed after 24 hours of reperfusion.
3. PC (n = 6): In order to induce PC, a clamp was applied to the hepatic artery and the portal vein for 5 min and was removed for 10 min. Then, the clamp was returned to be localized and provoke partial ischemia for 1 hour, as similarly occurred in IR group. Animals were sacrificed after 24 hours of reperfusion.
4. Sirtinol + PC (n = 6). As in group of PC, but 5 minutes before PC application, a SIRT1 inhibitor, sirtinol (dissolved in DMSO), was administered in rats intravenously at 0.9 mg/kg [244].
5. EX + PC [n = 6]. As in group sirtinol + PC, but in this case rats were treated with an intravenously administration of a more specific inhibitor of SIRT1, EX527 (dissolved in DMSO/saline), at 5 mg/Kg, 30 min before PC [245].

In all experimental groups, before rats being sacrificed, liver samples were collected; some of them were immediately frozen at dry ice and then stored at -80 ^{0}C for further determinations. Other hepatic samples were fixed in

paraformaldehyde at 4 % for their posterior histological analysis and evaluation of liver damage. Before liver extraction, blood samples were collected and centrifuged immediately at 4 °C for 10 min at 3000 rpm. Plasma extracts (supernatant) were then stored at -20 ^{0}C for further determinations. Hepatic injury was evaluated through determination of hepatic transaminase levels in plasma. We further determine lipid peroxidation, SIRT1 activity and, through western blot, various proteins involved in PC mechanisms, such as AMPK, eNOS, MAPKs, HSP70 and caspase levels.

3.5.2. Second study

Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach

The second study aimed to examine the possible implication of SIRT1 in orthtopic liver transplantation when IGL-1 preservation solution enriched or not with TMZ was used. Thus, male Sprague-Dawley rats (200-250 gr) were distributed in the following groups:

1. Sham (n = 6): Animals underwent transverse laparotomy and received silk ligatures in the right suprarenal vein, diaphragmatic vein and hepatic artery.
2. IGL-1 (n = 6): Livers were extracted from donors, flushed with IGL-1 solution and then stored in IGL-1 preservation solution for 8 hours at 4℃. Then, they underwent OLT according to Kamada's cuff technique without arterialization. Rats were sacrificed 24 hours after the blood flow in the graft was successfully restored after its implantation to the recipient.
3. IGL-1+TMZ (n = 6): Similarly as in previous group, but during bench surgery livers were preserved in IGL-1 solution supplemented with TMZ at 10^{-6} mol/L.

After the 24 hours of transplantation, liver samples were collected and stored at -80 ^{0}C, as well as plasma extracts for a posterior determination of transaminase levels and glutamate dehydrogenase activity (mitochondrial damage).Through western blot technique, we analyzed the protein expression pattern of SIRT1 and of proteins related to its activity (NAMPT, acetylated p53 and FoxO1), along with the expression of AMPK,

p-mTOR, p-p70S6K, MAPKs and of autophagy parameters (beclin-1, LC3B). Lipid peroxidation and NAD^+ levels were also evaluated.

3.5.3. Third study

Losartan activates SIRT1 in rat reduced-size orthotopic liver transplantation

In order to explore whether SIRT1 is involved in the protective mechanisms of Losartan against IRI associated with ROLT, the following groups with Sprague-Dawley rats were assessed:

1. Sham (n=6): Animals were subjected to transverse laparotomy and silk ligatures were located in the right suprarenal vein, diaphragmatic vein and hepatic artery.
2. ROLT (n =12, 6 transplants): Rats were subjected to ROLT, according to Kamada *et al.* and Xia *et al.,* [242, 243], which also involved the reduction of the liver mass at 40 %. Before harvesting the graft, the pedicle of the left lateral lobe was ligated with 5.0 silk ligature, and the lobe was removed. Two caudate lobes were then separately removed with the ligation. The donor livers were flushed and preserved with cold (4 ^{0}C) UW for 1 hour; the short time of ischemia coincided with the ischemic time that occurs in clinical practice of living donor liver transplantation. Furthermore, the time of the anhepatic phase was 20 min. Rats were sacrificed 24 hours after the initiation of graft reperfusion in the receptor.
3. Losartan + ROLT (n=12, 6 transplants): Similarly as occurred in ROLT, but in this case, an AT1R antagonist (Losartan) was orally administered (5 mg/kg) 24 hours and 1 hour before the surgical procedure to both the donor and the recipient.

After 24 hours of reperfusion, liver and blood samples were collected. We evaluated liver injury by determining hepatic transaminases in liver plasma. In hepatic tissue samples, we examined the protein expression pattern of SIRT1 and of its direct substrates (acetylated p53 and FoxO1), which was associated with the protein expression of ERS parameters (GRP78, IRE1a, p-eIF2), of MAPKs (p-p38 and p-ERK), of

HSPs (HSP70 and HO-1) and with liver apoptosis (caspase 12 and caspase 3). SIRT1 activity and NAD+ levels were also measured.

3.6. Biochemical determinations

3.6.1. Transaminases (AST, ALT)

The transaminases, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) are hepatic enzymes whose elevated plasma concentration has been associated with hepatic damage. In case of hepatocellular damage, plasma membrane is disrupted, which allows the leakage of intracellular enzymes such as ALT or AST into the bloodstream.

The reactions related with ALT are the following (LDH: lactate dehydrogenase):

$$\text{2-oxoglutarate + L-arginine} \xrightarrow{\text{ALT}} \text{Pyruvate + L-glutamate}$$

$$\text{Pyruvate + NADH + H+} \xrightarrow{\text{LDH}} \text{D-Lactate + } NAD^+$$

In case of AST the following reactions are realized (MDH: malate dehydrogenase):

$$\text{2-oxoglutarate + L-arginine} \xrightarrow{\text{AST}} \text{Oxaloacetic acid + L-glutamate}$$

$$\text{Oxaloacetic acid + NADH + H+} \xrightarrow{\text{MDH}} \text{D-malate + NAD+}$$

The activity of these enzymes was determined in rat plasma by a commercial kit (RAL, Barcelona, Spain), where the NADH decreases were determined through spectrophotometry at 365 nm for 5 minutes. The final result was calculated as Δ abs/min x 1745.

3.6.2. Glutamate Dehydrogenase

Glutamate dehydrogenase (GLDH) is located in the mitochondria and is involved in carbon and nitrogen metabolism, as catalyzes the reversible inter-conversion of glutamate to α-ketoglutarate and ammonia, as showing:

$$\alpha\text{-oxoglutarate + NADH + } NH_4^+ \xleftrightarrow{\text{GLDH}} \text{glutamate + } NAD^+ + H_2O$$

In case of injured hepatocytes, GLDH is released from mitochondria to the cytosol and finally to systemic circulation. Consequently, increased GLDH levels in plasma have

been associated with mitochondrial injury. The assay (Randox, Spain) involves the determination of absorbance at 340 nm after incubation at 25 ^{0}C of the serum with the adequate co-factors for 3 and 5 minutes (absorbance A1 and A2 respectively) and after the addition of the substrate (A3) and the incubation for 5 minutes (A4). The final result was calculated firstly as: (A3-A4)-(A1-A2) = ΔA / 5 minutes and then the final result was reported as: 197* ΔA / 5 minutes (U/l).

3.6.3. Lipid peroxidation assay

The formation of malondialdehyde (MDA) has been associated with lipid peroxidation induced by ROS and has been used as an indirect measurement of the oxidative injury. Hepatic tissue has been homogenized in 1 ml of Tris-base buffer (pH=7) and we quantify the concentration of the proteins by the method of Bradford. When protein molecules bind to the Bradford reagent, the absorption maximum of the reagent shifts from 465 to 595 nm. The bovine serum albumin (BSA) is used as a protein standard in the range of 5.26 - 60µg/ml. BSA standard stock is prepared in water at 6 mg/ml and the absorbance of the blank, standard curve and the samples are measured at 595 nm in a total volume of 160 µl. Later, 250 µl of the homogenized solution are added to 250 µl of trichloroacetic acid (TCA) so than the proteins can be precipitated. Then, vortex and centrifugation at 3000 rpm during 15 min at 4 ^{0}C follows. We mixed the supernatant with 250 µl of thiobarbituric acid (TBA) and incubated it at 100 ^{0}C for 30 minutes, where a pink chromogen compound was formed and its absorbance at 540 nm was then measured. The standard curve for MDA was prepared by dissolving 120 µl of 1,1,3,3-tetraethoxypropane in 50 ml of (hydrochloric acid) HCL 0.1 M and heating at 50 ^{0}C for 1 hour and then adequate dilutions up in the range of 1.25 – 80 nmoles/ml were obtained. The final result was expressed as nmoles/ mg protein.

3.6.4. SIRT1 activity

First of all, we homogenized 100 mg of liver tissue in 1 ml of a mild lysis buffer (50 mM Tris–HCl pH 8, 125 mM NaCl, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 10% glycerol and 0.1% NP40). Then proteins have been quantified with the method of Brandford and then samples were further diluted with the homogenization buffer in order to

obtain equal quantity of proteins (10 µg/µl approximately). SIRT1 activity was measured using a deacetylase fluorometric assay kit (CY-1151; CycLex, MBL International Corp.), which provides an acetylated peptide as substrate for SIRT1 and a protease (lysylendopeptidase) that cuts the peptide only when it is deacetylated. Moreover, a fluorophore and quencher are coupled to amino- and carboxyl- terminal of the peptide and fluorescence is not emitted in absence of deacetylase action. When SIRT1 performs deacetylation of the peptide, protease will cut the peptide and consequently quencher will separate from fluorophore and thus fluorescence will be emitted. In this way, deacetylase enzyme activity is measured by determining this fluorescence activity. A total of 25 µl of assay buffer (50 mM Tris-HCL, pH=8.8, 0.5 mM DTT, 0.25 mAU/ml Lysylendopeptidase, 1 µM Trichostatin A and 20 µM Fluoro-Substrate Peptide in 50 µl of reaction mixture) and 5 µl of protein extracts were added to all wells. The fluorescence intensity was monitored every 2 min for 1 hour at 25 ^{o}C using the fluorescence plate reader Spectramax Gemini, applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results were expressed as the rate of reaction for the first 30 min, when a linear correlation between the fluorescence and this period of time was observed.

3.7. Molecular Biology techniques

3.7.1. Protein extraction

In order to extract proteins from a tissue, we firstly prepare the extraction buffer (Hepes or Ripa, as indicated to each study), then we add 1ml of buffer to 200 mg of hepatic tissue and we start the homogenization; in this way the cells are lysed and the proteins can be liberated in the extracellular environment. The samples are incubated for 30 min on ice and then are centrifugated in 4 ºC 12000 rpm, for 30 min, so as the proteins (supernatant) and the rest of the cell components (precipitate) are separated. After the collection of the supernatant, follows the quantification of the concentration of the proteins by the method of Bradford.

3.7.2. Western Blot

The Western Blot technique detects, by using a specific antibody, a protein between a sample of proteins that have been separated by electrophoresis and transfered to a nitrocellulose or PVDF membrane.

The first step consists of an electrophoresis in a polyacrylamide gel, which is composed by two parts: the upper or stacking gel at pH 6.8 and the lower or separating gel at pH 8.8. Sample proteins are loaded at the wells located on the stacking gel and under the impact of an electric field can migrate towards the separating gel. The different pH between these two parts makes the proteins to be concentrated at the interphase before entering to the separating gel. In this way, thinner bands can be obtained instead of diffuse bands, so the separation of the proteins will be better.

As the protein concentration of each sample has been known, appropriate dilutions are made, in order to load the same quantity of proteins (50 µg) for each sample. In each sample, we add a mix of laemli buffer (2x, Bio-rad Laboratories) and β-mercaptoethanol (Bio-rad Laboratories). Laemli buffer contains glycerol, sodium dodecul sulphate (SDS) and bromoethanol blue. Bromoethanol blue is a colorant that allows us to observe the running point of the samples in the gel. Due to glycerol, the density of the samples increases, facilitating their movement in the gel. Both β-mercaptoethanol and SDS result in the loss of the tridimensional structure and the unfolding of the proteins, as the former cleaves the disulfide bonds (S-S) of the cysteins, and the latter disrupts the non-covalent bonds. Also, SDS, as it is an anionic surfactant, charges negatively the proteins. Consequently, the denaturated proteins are able to be separated in the gel by molecular weight once an electric field is applied. Heating at 95 ºC for 5 minutes is followed to accelerate the process of denaturation. A protein marker (1,5 µl, abcam) is also to be loaded to determine by comparison the molecular weight of our protein. Electrophoresis buffer (Tris-HCl 25mM, pH=8.8, glycine 250 mM) is added on to the electrophoresis chamber and electrophoresis is performed at a constant current of 120V for 1,30 h approximately.

Once the electrophoresis is finished, stacking gel is removed and proteins in the separating gel are transfered to a PVDF membrane. The phase of transference is realized in semi-dry blot (Bio-rad Laboratories) and the transfer sandwich contains a sponge, the PVDF membrane, the gel and another sponge. The sponges have been submersed previously in transfer buffer (Tris-base 25 mM, pH=8.8, glycine 250 mM and methanol). The transfer is performed at 18 V for 30 min in Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-rad Laboratories). In this way, the proteins move from within the gel onto the membrane while maintaining the organization they had within the gel.

After the transfer, the next steps for the detection of the proteins are followed: i) blocking of non-specific binding, by placing the membrane in a blocking buffer of 5% w/v dry milk in Tris-Buffered Saline (TBS, Tris 200 mM, pH=7.5, NaCl 1.5 M, Tween 0.05%) and incubation in room temperature for 1 h. The milk attaches to all places where proteins have not attached, eliminating false positives and leading to clearer results, ii)incubation overnight at 4 ºC with the primary antibody that binds specifically to the protein of interest, iii) hybridization to the secondary antibody for 1 hour in room temperature. From one hand, the secondary antibody binds to the primary antibody that comes from the same animal source. On the other hand, it is linked to horseradish peroxidase. The horseradish peroxidase catalyses the oxidation of luminol, a reaction accompanied by emission of low-intensity light at 428 nm. For this reason, the membrane is incubated for 5 min in a solution of luminol and enhancer (Bio-rad Laboratories/Advansta) and the produced light, which is proportionate to the amount of the protein, can be detected. In order to visualize the chemiluminescent signal, we use a photographic film, where the bands of the proteins appear. Finally, the film is scanned and the generated bands are quantified by using the Quantity One program (Bio-rad Laboratories). The values obtained for each protein are then divided by the correspondent values of β-actin in order to reduce experimental variability between samples, due to incorrect loading or transference.

3.7.3. Analysis of RNA

3.7.3.a. RNA extraction

In order to extract RNA from a tissue, the next steps were followed: i) place a piece of tissue in Trizol (1 ml), so as the plasmatic membrane be destroyed, proteins, like the RNAases, be desnaturated and the RNA be seperated from the ribosomes, ii) incubation with chloroform (200 µl, 2-3 min, room temperature) and centrifugation (12000 g, 15 min, 4 ºC) for the formation and separation of aqueous phase which contains the RNA and organic phase of lipids of the destroyed membrane, iii) rejection of the organic phase and continuation with the aqueous phase, iv) incubation with isopropanol (500 µl, 10 min, room temperature) so as the RNA be dehydrated, and centrifugation (12000 g, 10 min, 4 ºC) for the precipitation of RNA, v) rejection of the aqueous phase and continuation with the precipitated RNA, vi) centrifugation with 75% ethanol: The salts are eliminated as they are soluble in 25% of water, while the RNA is precipitated in 75% ethanol, vii)rejection of ethanol and let the precipitated RNA dry, viii) dissolving the RNA in distilled water (200 µl), and ix) quantification of the concentration of RNA: spectrometric measurement of the samples; the nucleotides absorb at 260 nm as the ring of ribose absorbs in this region of spectrum. Also, the ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as “adequate” for RNA.

3.7.3.b. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The RT-PCR (Reverse Transcription-PCR) is a molecular technique where a sample of RNA is converted into cDNA, using reverse transcriptase, an enzyme that contributes to the incorporation of dNTPs to the new synthesized polimer of DNA, without RNase H activity. We used the iScript cDNA synthesis kit (Biorad). After we had prepared samples of RNA of the same quantity (1 µg), the followed incubations are carried out: i) at 25 ºC for 5 minutes, ii) at 42 ºC for 30 minutes and iii) at 85 ºC for 5 minutes. Then, we adjust the samples at a final volume of 80 µl. The low quantity of cDNA is suitable for the next procedure (Q-PCR).

3.7.3.c.Real-Time polymerase chain reaction, or Quantitative real time polymerase chain (Q-PCR)

PCR is a technique used to amplify a single or a few copies of a piece of DNA and is based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. The PCR reaction requires the following components: i) DNA template: the sample DNA that contains the target sequence, ii) DNA polymerase: enzyme that generates new strands of DNA, complementary to the target sequence and primers, iii) Primers: short pieces of single-stranded DNA, complementary to the target sequence, whose 3′-OH end is the starting point of polymerase.

The Quantitive real-time polymerase chain (Q-PCR), is a type of PCR that measures the amplified PCR product at each cycle. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes. The reaction mix contains 37,5 ng of sample cDNA, 500 nm of forward primer and 500 nm of reverse primer that bind to the gene of interest and finally 5 ul of Sso Advanced SYBR Green supermix (Bio-rad Laboratories) in a total volume of 10 μl. The Sso Advanced supermix contains all the components that are necessary for Q-PCR reaction, such as dNTPs, DNA polymerase, $MgCl_2$ and the SYBR Green I, a fluorescent DNA binding dye that binds all double–stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle.

The whole process consists of the repetition (40 times) of the following steps: i) heating the reaction to 95 °C for 30 seconds, ii) heating the reaction to 95°C for 5 seconds so as the hydrogen bonds between complementary bases can be disrupted, yielding single-stranded DNA molecules, iii) incubation for 30 seconds at the optimal temperature for the primer, so that the primers are to the single-stranded DNA template iv) incubation at 72°C for 15 seconds so that the polymerase is able to bind to the primer-template hybrid and begin the DNA synthesis and elongation of the new strand.

In order to achieve a precise determination of the amplified product, it is necessary to amplify a reference gene whose amplification is not influenced by the experimental procedure and its expression level remains constant in our biological system. In our case, the glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was used as reference gene.

3.8. Histology

Promptly after the extraction, liver samples were fixed at paraformaldehyde at 4 % for at least 24 hours and then the tissue was embedded into paraffin wax and cut in sections of 5 μm by an ultramicrotome. The sections were stained by hematoxylin–eosin; hematoxylin has a deep blue-purple color and stains nucleic acids and eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Liver damage was evaluated using an ordinal scale from 0 to 4 as follows: grade 0: absence of injury; grade 1: mild injury consisting in cytoplasmic vacuolation and focal nuclear pycknosis; grade 2: moderate injury with focal nuclear pycknosis; grade 3: severe necrosis with extensive nuclear pycknosis and loss of intercellular borders; and grade 4: severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration.

3.9. Statistics analysis

Statistical comparison was performed by variance analysis (ANOVA), followed by the Student– Newman–Keuls test, or the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant. Data were expressed as mean ± standard error

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4. RESULTS

4. Results

4.1. First study: SIRT1 in PC

Silent information regulator 1 protects the liver against ischemia–reperfusion injury: implications in steatotic liver ischemic preconditioning.

Summary

Introduction: IRI is a complex situation that is inherent to surgical procedures and various strategies have been proposed in order to combat its deleterious effects. Between them, PC, a surgical procedure that consists of short times of ischemia followed by short times of reperfusion before a prolonged ischemia-reperfusion, has been shown to protect livers against IRI. PC beneficial effects are owned, at last in part, in the activation of eNOS, AMPK, HSPs expression and decreased oxidative stress and apoptosis. PC has also been shown to protect steatotic livers which are more vulnerable to IRI than non-steatotic ones. SIRT1 is a deacetylase that activates or inhibits various proteins and in this way regulates various cellular processes involved in the cell stress response and cell cycle. Taking into account that SIRT1 has been associated with favorable effects in various IRI models, in the present study we aimed to evaluate whether SIRT1 is involved in the protective mechanisms of PC against IRI in fatty livers.

Experimental: Homozygous (Ob) Zucker rats aged 12 weeks were classified as follows: Group 1= Sham; Group 2= IR: Ob rats were subjected to 60 minutes of partial ischemia (70%) followed by 24-hour reperfusion; Group 3= PC : 5 minutes of partial ischemia (70%) followed by a reflow for 10 minutes and then livers were subjected to IRI as in group 2; Group 4= Sirtinol + PC : as in group 3, but treated with sirtinol, a SIRT1 inhibitor (0.9 mg/kg intravenously), 5 minutes before PC. Group 5: EX + PC. As in group 3, but treated with EX527 (5 mg/Kg i.v.), a SIRT1 inhibitor 30 min before PC. Rats were sacrificed after 24 hours of reperfusion. Liver injury (AST), oxidative stress (MDA) and SIRT1 activity were evaluated. Protein expression of SIRT1, ac-p53, p-AMPK, eNOS, HSP70, MAPKs (p-p38, p-ERK) and apoptosis parameters (Caspase 9 and 3, cytochrome c) were determined by Western blot.

Results: 1) SIRT1 protein levels and activity are enhanced in hepatic PC, 2) Inhibition of SIRT1 during PC increases liver injury and oxidative stress, 3) SIRT1 enhanced activity during PC was associated with activation of eNOS, AMPK and HSP70 and p-ERK, 4) Inhibition of SIRT1 during PC abolished the activation of the above proteins and resulted in enhanced apoptosis.

Conclusions: SIRT1 contributes to the protective mechanisms of PC against IRI in fatty livers.

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ORIGINAL ARTICLE

Silent information regulator 1 protects the liver against ischemia–reperfusion injury: implications in steatotic liver ischemic preconditioning*

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Conflicts of Interest
The authors have declared no conflict of interests.

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Abstract

Ischemia–reperfusion (IR) injury is an important problem in liver surgery especially when steatosis is present. Ischemic preconditioning (PC) is the only surgical strategy that has been applied in patients with steatotic livers undergoing warm ischemia. Silent information regulator 1 (SIRT1) is a histone deacetylase that regulates various cellular processes. This study evaluates the SIRT1 implication in PC in fatty livers. Homozygous (Ob) Zucker rats were subjected to IR and IR + PC. An additional group treated with sirtinol or EX527 (SIRT1 inhibitors) before PC was also realized. Liver injury and oxidative stress were evaluated. SIRT1 protein levels and activity, as well as other parameters involved in PC protective mechanisms (adenosine monophosphate protein kinase, eNOS, HSP70, MAP kinases, apoptosis), were also measured. We demonstrated that the protective effect of PC was due in part to SIRT1 induction, as SIRT1 inhibition resulted in increased liver injury and abolished the beneficial mechanisms of PC. In this study, we report for the first time that SIRT1 is involved in the protective mechanisms induced by hepatic PC in steatotic livers.

Introduction

Ischemia–reperfusion (IR) injury is the main cause of organ damage and initial poor function of liver grafts and is inherent to surgical procedures in liver transplantation. The shortage of organs has led to expand the criteria for the acceptance of marginal donors, including the use of steatotic grafts [1]. However, the use of fatty liver grafts

increases the rates of primary nonfunction and consequently compromises the graft viability after transplantation, exacerbating the organ shortage [2].

The high vulnerability of fatty livers against IR injury is due to the abnormal accumulation of fat within the cytoplasm of hepatocytes, resulting in increased hepatocellular volume and narrowing of sinusoid. As a consequence, hepatic flow is severely obstructed and results in important alterations in liver microcirculation that compromises the suitable graft revascularization and viability after transplantation [3]. Also, another important consequence of fat accumulation in steatotic livers is that hepatocytes are more susceptible to oxidative stress [4].

Therapeutic surgical strategies such as ischemic preconditioning (PC) diminish the high vulnerability of steatotic livers against IR injury [5–8]. The induced hepatoprotection is mediated, in part, through nitric oxide (NO) generation by endothelial nitric oxide synthase (eNOS) which interferes with the mechanisms responsible for IR damage, such as the exacerbated lipoperoxidation in steatotic livers [5]. In addition, PC promotes the activation of adenosine monophosphate protein kinase (AMPK), a fuel energy sensor that contributes to maintain cellular function and integrity [9]. In this line, we have previously demonstrated a direct relationship between AMPK and NO in the protective mechanisms of PC in rat steatotic liver transplantation [10].

Silent information regulator 1 (SIRT1) is a member of the family of class III histone deacetylases involved in stress responses including hypoxic stress, heat shock stress, and inflammation [8,11–13]. SIRT1 deacetylates both histone and nonhistone proteins in a NAD^+-dependent manner, including p53, eNOS, and AMPK. [14,15]. SIRT1 deacetylates p53 in the C-terminal Lys-382 residue and thus reduces its transcriptional activity and its ability to induce apoptosis [15,16]. Furthermore, it has been reported that SIRT1 ameliorates vascular function in endothelial cells after laminar shear stress, as enhancement of SIRT1 activity was associated with eNOS activation [17]. Moreover, various studies in cultured cells and in liver *in vivo* have shown evidence of AMPK activation by SIRT1 [18–20].

Silent information regulator 1 protects the heart from IR injury and decreases oxidative stress [21,22]. Moreover, the fact that SIRT1 downregulation under IR insult in heart was attenuated by PC suggests that SIRT1 may partly mediate the benefits induced by PC [23]. Accumulating data demonstrate the relationship between SIRT1 and AMPK/NO [17,24], both mediators of PC but no data have yet been reported in liver, regarding the involvement of SIRT1 in PC.

The role of SIRT1 in liver IR injury has been poorly investigated. For this reason, the aim of this paper is focused on the study of SIRT1 function in fatty liver IR injury, as well as to explore whether it is involved in the protective mechanisms induced in liver by PC.

Material and methods

Experimental animals

Homozygous obese (Ob) Zucker rats (Charles River, France) aged 12 weeks were used. Ob rats lack the cerebral leptin receptor and showed severe macro- and microvesicular fatty infiltration in hepatocytes (40–60% steatosis). All procedures were performed under isofluorane inhalation anesthesia. This study was performed in accordance with European Union regulations (Directive 86/609 EEC). Animal experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 396/12), University of Barcelona. Animals were randomly distributed into groups as described below.

Experimental design

Group 1: sham [$n = 6$]. Ob rats were subjected to laparatomy, and hepatic hilum vessels were dissected [25].

Group 2: IR [$n = 6$]. Ob rats were subjected to 60 min of partial (70%) ischemia by applying a microvascular clamp to the hepatic artery and the portal vein, thus blocking the hepatic inflow to the median and left lobes. Then, 24-hour reperfusion was followed [25].

Group 3: PC [$n = 6$]. To induce PC, 5 min of partial ischemia (70%) followed by a reflow for 10 min was applied in ob rats [25]. Livers were then subjected to IR as in group 2.

Group 4: sirtinol + PC [$n = 6$]. As in group 3, but treated with sirtinol (dissolved in DMSO), a SIRT1 inhibitor (0.9 mg/kg i.v.) 5 min before PC [26].

Group 5: EX + PC [$n = 6$]. As in group 3, but treated with EX527 (dissolved in DMSO/saline), a SIRT1 inhibitor (5 mg/Kg i.v.) 30 min before PC [27].

Biochemical determinations

Transaminases assay

Hepatic injury was assessed in terms of transaminases levels with commercial kits from RAL (Barcelona, Spain). Briefly, plasma extracts were collected before liver extraction and centrifuged at 4 °C for 10 min at 0.8 *g*. Then, 200 µl of the supernatant were added to the substrate provided by the commercial kit. ALT levels were determined at 365 nm with an UV spectrometer and calculated following the supplier instructions [28].

Lipid peroxidation assay

Lipid peroxidation in liver was used as an indirect measurement of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction [29]. MDA in combination with thiobarbituric acid (TBA)

forms a pink chromogen compound whose absorbance at 540 nm was measured. The result was expressed as nmols/mg protein.

SIRT1 activity assay

Silent information regulator 1 activity was determined according to the method described by Becatti *et al.* [30] with some modifications. Protein extracts were obtained using a mild lysis buffer (50 mM Tris–HCl pH 8, 125 mM NaCl, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, and 0.1% NP40). SIRT1 activity was measured using a deacetylase fluorometric assay kit (CY-1151; CycLex, MBL International Corp.), following the manufacturer's protocol. A total of 25 µl of assay buffer containing the same quantity of protein extracts (5 µl) were added to all wells, and the fluorescence intensity was monitored every 2 min for 1 h using the fluorescence plate reader Spectramax Gemini, applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results are expressed as the rate of reaction for the first 30 min, when there was a linear correlation between the fluorescence and this period of time.

Western blotting analysis

Liver tissue was homogenized in RIPA buffer (Tris–HCl pH = 7.5 50 mM, NaCl 150 mM, SDS 0.1%, $C_{24}H_{39}O_4Na$ 1%, NP-40 1%, EDTA 5 mM, Na_3VO_4 1 mM, NaF 50 mM, DTT 1 mM, 1 complete tablet/100 ml) for SIRT1 immunodetection and in Hepes buffer (NaCl 40 mM, EDTA 1 mM, Triton X 0.1%, glycerol 5%, NaP_2O_7 10 mM, b-glycerophosphate 10 mM, Na_3VO_4 1,5 mM, NaF 50 mM, 1 complete tablet/100 ml, Hepes-KOH pH = 7.4 50 mM) for the rest of proteins. Fifty µg of proteins were electrophoresed on 8–15% SDS-PAGE gels and transblotted on PVDF membranes (Bio-Rad). Membranes were then blocked with 5% (w/v) nonfat milk in TBS containing 0.1% (v/v) Tween 20 and incubated overnight at 4 °C with anti-SIRT1 (#07-131, Merck Millipore, Billerica, MA), anti-ac-p53 (ab37318, abcam, UK), anti-p-AMPK (Thr172, #2535), anti-caspase 3 (#9662), anti-cytochrome C (#4272), anti-p-p38 MAP kinase (Thr180/Tyr182, #9211), anti-p-p44/42 MAPK (Erk1/2; Thr202/Tyr204, #9101; all the above antibodies were purchased from Cell Signaling, Danvers, MA) anti-eNOS (610296), anti-HSP70 (610607; both from Transduction Laboratories, Lexington, KY), and anti-β-actin (A5316, Sigma Chemical, St. Louis, MO, USA). After washing, bound antibody was detected after incubation for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using WesternBright ECL-HRP substrate (Advansta) and were quantified using the Quantity One software for image analysis. Results were expressed as the densitometric ratio between the protein of interest and the loading control (β-actin).

Histology

To estimate the severity of hepatic injury, hematoxylin–eosin-stained sections were evaluated using an ordinal scale from 0 to 4 as follows: grade 0: absence of injury; grade 1: mild injury consisting in cytoplasmic vacuolation and focal nuclear pycknosis; grade 2: moderate injury with focal nuclear pycknosis; grade 3: severe necrosis with extensive nuclear pycknosis and loss of intercellular borders; and grade 4: severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration.

Statistics

Data are expressed as mean ± standard error and were compared statistically by the nonparametric Kruskal–Wallis test. A P value <0.05 was considered significant.

Results

SIRT1 protein expression and activity in PC

To study the implication of SIRT1 in PC, we first evaluated its protein expression pattern. As shown in Fig. 1a, the expression of SIRT1 in fatty livers subjected to IR was significantly augmented when compared with sham group. This increase was exacerbated when PC was carried out and reversed after sirtinol (a SIRT1 inhibitor) treatment. Treatment with EX527, another SIRT1 inhibitor, did not affect SIRT1 protein levels during PC. Furthermore, PC group exhibited an increased deacetylase activity compared with both IR and sham groups (Fig. 1b), and as expected, sirtinol and EX527 treatment groups during PC resulted in decreased SIRT1 activity. However, no significant differences in SIRT1 activity were observed between sham and IR groups or between the inhibitors groups. In addition to this, we analyzed the acetylation (Lys-382) of p53 (ac-p53), a direct substrate of SIRT1 (Fig. 1c). PC group was characterized by a marked decrease in ac-p53, which was reversed by treatment of both inhibitors. The increase of ac-p53 was more significant for sirtinol than EX527. Finally, the ac-p53 levels between sham and IR group were not significantly altered.

Liver injury

We next determined whether SIRT1 plays a role in the prevention of IR injury mediated by PC. As shown in Fig. 2a, IR injury increased ALT levels, which were reversed by PC. The administration of both sirtinol and EX527 resulted in

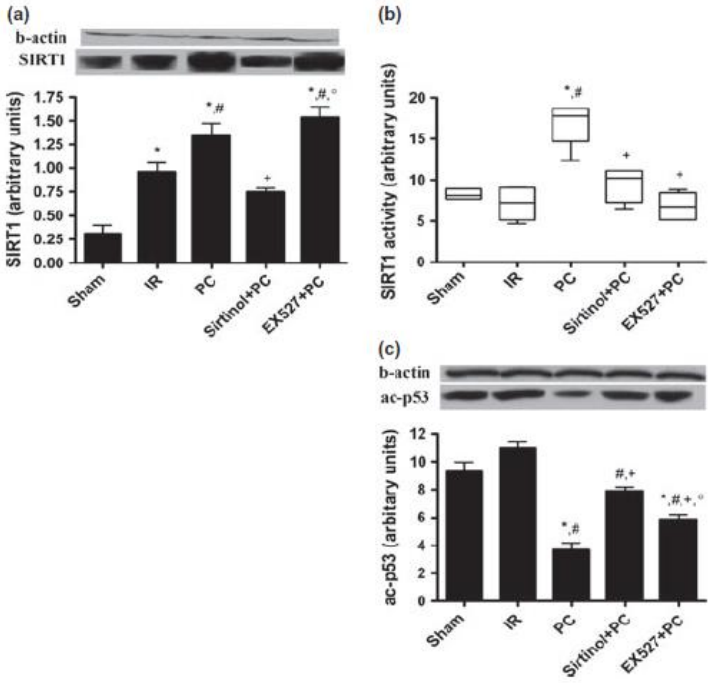

Figure 1 Role of preconditioning (PC) on SIRT1 expression and SIRT1 activity in steatotic livers subjected to ischemia–reperfusion (IR) injury. (a) Western blot and densitometric analysis of SIRT1; (b) SIRT1 enzymatic activity; (c) densitometric analysis of ac-p53. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, sirtinol + PC: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. PC $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC; $^{\circ}P < 0.05$ versus sirtinol +PC.

increased ALT levels, but sirtinol treatment provoked liver injury to a lesser extent than EX527. This result is consistent with the histological findings shown in Fig. 2b. Steatotic livers subjected to IR exhibited severe and extensive areas of coagulative necrosis with neutrophil infiltration (75%) that were significantly reduced (25%) when PC was performed. Pretreatment with sirtinol and EX527 aggravated tissue lesions as shown by extensive areas of coagulative necrosis (50% and 80% respectively), in comparison with PC group (Fig. 2c).

eNOS and AMPK activation

Given that the benefits of PC are mediated in part by NO, we explored the effects of SIRT1 on eNOS expression and AMPK activation induced by PC. As shown in Fig. 3a,b, PC potentiated IR induced eNOS expression and AMPK phosphorylation, respectively. Furthermore, the increased levels of eNOS expression/AMPK activation induced by PC were completely blocked by sirtinol and EX527 administration.

Oxidative stress and heat shock proteins

We evaluated the relevance of SIRT1 on the prevention of oxidative stress induced by IR. For this reason, we measured MDA levels in liver tissue. As indicated in Fig. 4a, the high MDA levels observed in IR group were reduced when PC was applied. SIRT1 inhibition resulted in increased lipid peroxidation and the highest MDA increase was observed when EX527 was administrated prior to PC. Furthermore, IR induced a significant increase in heat shock protein 70 (HSP70), which was further reinforced during PC. In addition, both sirtinol and EX527 reversed the HSP70 overexpression induced by PC (Fig. 4b).

MAPK kinases

We also explored the effect of SIRT1 on mitogen-activated protein kinases (MAPK) activation. As shown in Fig. 5a, PC increased extracellular signal-regulated kinase (ERK) phosphorylation, when compared with IR and sham groups. Moreover, we observed that PC reversed the

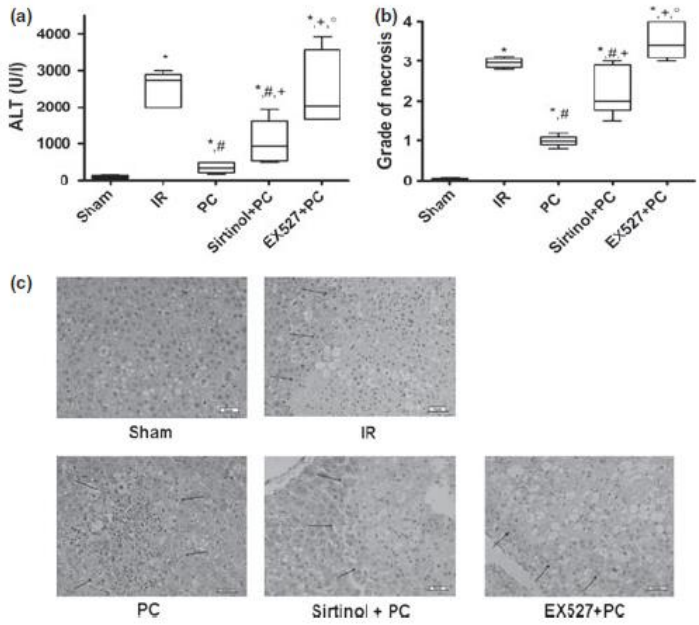

Figure 2 Effect of the inhibition of SIRT1 during preconditioning (PC) in hepatic injury. (a) Photometric analysis of alanine aminotransfe ase (ALT) levels; (b) Histological lesions in steatotic liver by hematoxylin-eosin-stained sections; (c) Grade of necrosis in the experimental groups. Sh m: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min of partial ischemia and 24 h of reperfusion, PC: IR with previous preconditio ing induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC, $^{\circ}P < 0.05$ versus sirtinol + PC.

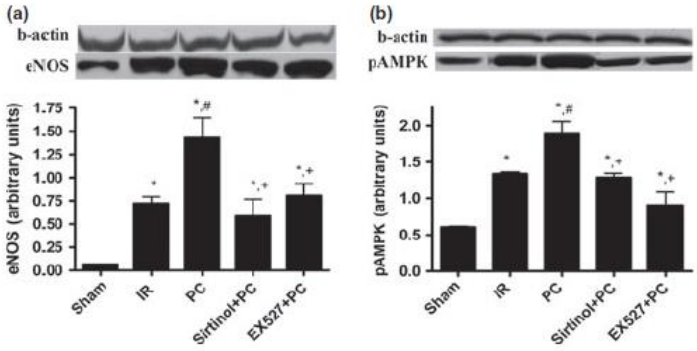

Figure 3 Implication of Silent Information Regulator 1 on eNOS expression and adenosine monophosphate protein kinase (AMPK) activation during preconditioning (PC) in steatotic livers. Western blot and densitometric analysis of eNOS and pAMPK (a and b respectively). Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC, EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

increased p-p38 protein levels caused by IR (Fig. 5b). Sirtinol and EX527 administration partially reduced the protective effects of PC on MAP kinases modulation, but no differences between both inhibitors were noted.

Apoptosis

We also evaluated the involvement of SIRT1 activation in PC and its consequences on liver apoptosis by measuring

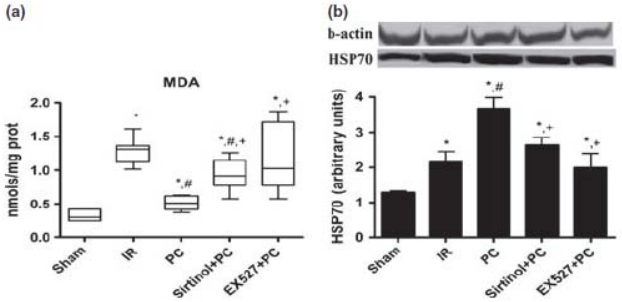

Figure 4 Effect of SIRT1 on oxidative stress and HSP70 expression during preconditioning (PC) in steatotic livers. (a) Photometric analysis of malondialdehyde levels. (b) Western blot and densitometric analysis of HSP70 protein expression. Sham: anesthesia and laparotomy, ischemia-reperfusion (IR injury: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

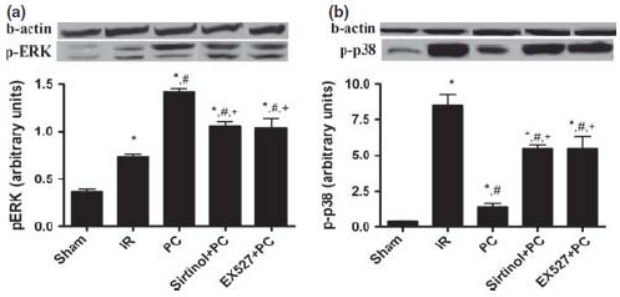

Figure 5 Modulation of MAPK expression and phosphorylation by Silent Information Regulator 1 during preconditioning (PC) in steatotic livers. Western blot and densitometric analysis of pERK (a) and p-p38 (b). Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, PC: IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

caspase-3, caspase-9 cleavage, and cytochrome c protein levels. Significant increases in the above parameters of apoptosis were seen during IR, which were then reversed when PC was applied (Fig. 6). SIRT1 inhibition by both inhibitors provoked increased liver apoptosis in comparison with PC group.

Discussion

In this study, we report for the first time that SIRT1 is implicated on the prevention of fatty liver IR injury by PC. Firstly, we have evidenced a significant up-regulation of SIRT1 protein levels induced by PC, and secondly, we have demonstrated that SIRT1 inhibition reverses the benefits of PC during liver damage. Furthermore, high SIRT1 deacetylase activity was observed in PC group, which was significantly decreased when SIRT1 was inhibited during PC by either sirtinol or EX527. The diminished levels of ac-p53 (a direct substrate of SIRT1) during PC were consistent with the high deacetylase activity, and this effect was reversed by both SIRT1 inhibitors. These results are in accordance with previous reported data in heart [23,31,32] and brain [15,33,34] where SIRT1 confers protection to those tissues against IR injury.

In addition, our findings support the fact that an overexpression of SIRT1 occurs in fatty livers subjected to IR. This observation agrees with previous *in vivo* and *in vitro* investigations in heart, where the SIRT1 levels were up-regulated by certain stresses, including IR injury, suggesting that SIRT1 could act as a self-compensatory mechanism for preventing tissue damage [21,22,30]. However, we observed that SIRT1 activity, as well as ac-p53 protein levels, was not

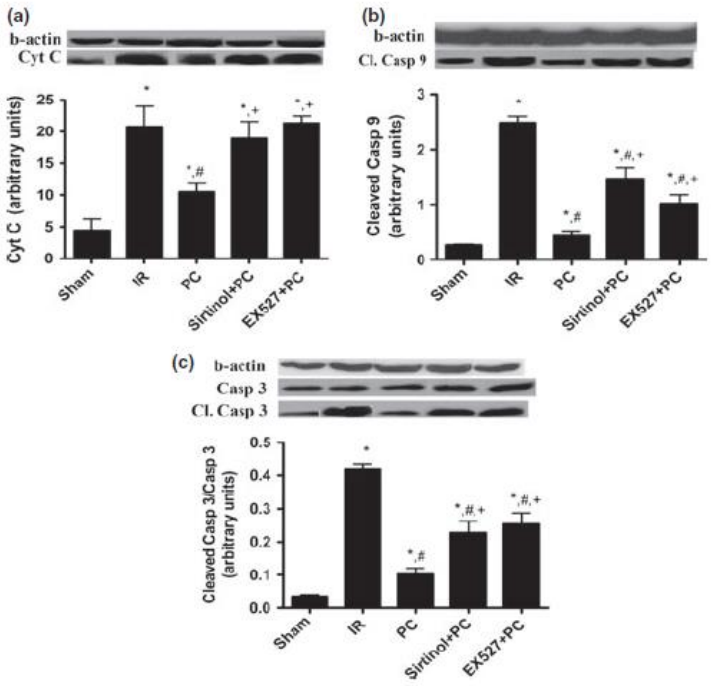

Figure 6 Effect of SIRT1 on liver apoptosis. Western blot and densitometric analysis of cytochrome C (a), cleaved caspase 9 (b) and cleaved caspase3/caspase 3 (c). Sham: anesthesia and laparotomy, ischemia–reperfusion (IR) injury: 60 min partial ischemia and 24 h of reperfusion, preconditioning (PC): IR with previous preconditioning induced by 5 min of ischemia and 10 min of reperfusion, PC + sirtinol: administration of sirtinol 5 min before PC. EX527 + PC: administration of EX527 30 min before PC. $^{*}P < 0.05$ versus sham, $^{\#}P < 0.05$ versus IR, $^{\dagger}P < 0.05$ versus PC.

altered during IR, which implicates that various factors can affect its activity. For example, in a similar study, it was observed that the activity of liver histone deacetylases is decreased only in short times of reperfusion, whereas it remains unchanged after 24 h of reperfusion [35].

Recent investigations in rodent aortic and human endothelial cells reported the relevance of SIRT1 in eNOS activation; SIRT1 interacts with eNOS, resulting in the activation of the enzyme [36–40]. In our study, SIRT1 up-regulation during PC was well correlated with the expression of eNOS which was inhibited after sirtinol or EX527 administration. This result suggests that SIRT1 is involved in PC hepatoprotection that is mediated by NO, counterbalancing the exacerbated microcirculation in fatty livers [5,41].

Protective PC mechanisms are associated with the activation of AMPK, as we have previously reported [9,10]. Once activated, AMPK phosphorylates various substrates to conserve ATP levels and switch on metabolic pathways that generate ATP [9]. The present study demonstrated that SIRT1 inhibition abolished the activation of AMPK during PC, suggesting a potential link between SIRT1 and AMPK signaling in liver PC. Our results are in agreement with reported investigations in hepatic cultured cells and mouse liver *in vivo*, showing that SIRT1 activates AMPK through LKB1 deacetylation [18–20]. In addition, we have previously reported that AMPK and eNOS activation are involved in the benefits of PC in a model of rat steatotic liver transplantation [10]. The fact that SIRT1 inhibition completely abrogated the activation of AMPK and eNOS suggests a potential relationship between SIRT1 and the above factors.

Results reported here also confirm that the overexpression of SIRT1 in PC is responsible for the attenuation of oxidative stress caused by PC. Indeed, SIRT1 inhibition reduced the prevention of lipoperoxidation induced by PC. A similar effect was observed in heart, where the overexpression of SIRT1 also attenuated oxidative stress through the stimulation of FoxO1 transcription factor, thus enhancing antioxidant enzymes like manganese superoxide dismutase [23].

It is well established that stressful conditions such as IR can induce besides ROS, the heat shock transcription response [42]. In this line, we previously provided evidence that HSP70 is activated during PC and protected against IR

injury [43]. Here, we demonstrate that SIRT1 is involved in the regulation of heat shock proteins expression in fatty liver PC, as confirmed by the decrease in HSP70 expression when SIRT1 was inhibited. These data agree with other studies in HeLa cells, demonstrating that SIRT1 enhances HSP70 expression through the regulation of HSF1 transcriptional activity. [44].

Moreover, the oxidative stress can activate MAPK by dual phosphorylation on tyrosine and threonine residues [45,46]. Given that PC affects the MAPK pathways [43,47,48], we examined whether SIRT1 regulated these kinases. We observed that SIRT1 inhibition decreased the expression of p-ERK and augmented p-p38 protein levels. ERK activation during PC protects against IR injury, by inhibiting apoptosis [49], whereas treatment with a p38 activator resulted in increased liver injury when PC was performed on steatotic livers [43]. It has also been reported that SIRT1 modulated MAPK pathways in an experimental model of IR using cardiomyocytes [30].

A variety of stressors, such as DNA damage and ROS, can activate a cascade of mediators, leading to increased apoptosis during IR injury [50]. This is accompanied by the release of cytochrome c, which promotes caspase 9 activation, which in turn activates caspase 3 and the final steps of apoptosis [51]. In our study, decreased levels of apoptotic parameters (caspase 3, caspase 9, and cytochrome c) were observed in the PC group when compared with the IR group, whereas inhibition of SIRT1 during PC promoted the increase in fatty liver apoptosis and would be in accordance with the concomitant p-ERK expression diminution, as previously commented. In addition, as it occurs with MAPK kinases, both inhibitors, sirtinol and EX527, partially reversed the protective effect of PC on apoptosis, suggesting that additional mechanisms can be involved in the beneficial effects of fatty liver PC.

Sirtinol and EX527 are both inhibitors of SIRT1 activity. However, it has been reported that sirtinol can also inhibit human SIRT2 activity *in vitro* [52], whereas EX527 has been described as a more specific inhibitor of SIRT1 and with a lower efficiency for SIRT2 inhibition [53]. In our model, treatment with either sirtinol or EX527 during PC resulted in increased liver injury. The fact that treatment with EX527 dramatically reduced the protective effect of PC, confirm our hypothesis that SIRT1 is involved in the beneficial effects of PC against IR. On the other hand, sirtinol is less potent to prevent the protection provided by PC. This fact may be attributed to its additional inhibitory effect on SIRT2; given that in recent studies, inhibition of SIRT2 has been found to be protective [54,55], the results obtained after sirtinol treatment might be the consequence of the inhibition of both SIRT2 (possible protective effect) and SIRT1 (detrimental effect).

Moreover, it has been shown that inhibition of SIRT1 by sirtinol contributes to the expression of inflammatory cytokines, through the acetylation of NF-κB [56]. However, more recent studies provided data showing that administration of sirtinol in rats subjected to trauma-hemorrhage decreased hepatic/lung injury and production of pro-inflammatory mediators [26,57]. As in our study, the PC+sirtinol group resulted in increased hepatic injury compared with PC group and the administration of sirtinol and EX527 in a sham group provoked no significant changes in the parameters studied (data not shown), a possible protective role of sirtinol should be ruled out. Furthermore, we observed that treatment with sirtinol diminished SIRT1 levels, and a similar effect has been observed in other experimental model, but the underlying mechanisms are to be investigated [58].

In summary, our study demonstrates that SIRT1 is involved in the protective effects of PC against IR injury in fatty livers. More concretely, SIRT1 is associated with the activation of eNOS and AMPK, the attenuation of oxidative stress, and apoptosis (Fig. 7). Therefore, the application of SIRT1 activators, such as resveratrol, could be a potential pharmacological treatment of patients with steatotic livers submitted to liver transplantation. Indeed, it has already been shown that resveratrol prolongs allograft survival after liver transplantation in rats [59].

In conclusion, the data reported here provide new insights into the liver protection, suggesting that SIRT1 is a

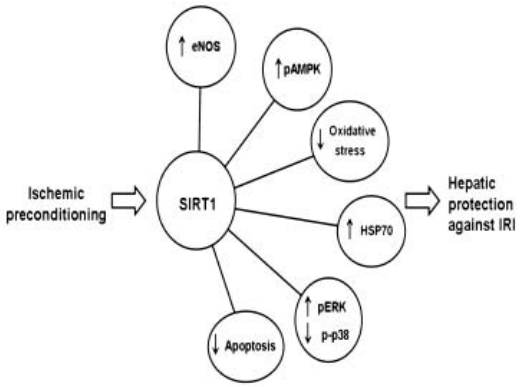

Figure 7 Effects of Silent Information Regulator 1 (SIRT1) in liver ischemic preconditioning. Ischemic preconditioning in fatty livers induces SIRT1 upregulation and enhancement of its deacetylase activity that leads from one hand, to the enhancement of cytoprotective pathways, including eNOS, HSP70, pERK expression and adenosine monophosphate protein kinase activation and from the other hand to downregulation of p-p38 and apoptosis and to decreased oxidative stress. The final result is an enhanced protection of fatty liver against ischemia–reperfusion (IR) injury.

promising pharmacological target to increase the fatty liver tolerance against IR injury.

Authorship

EP and MB: carried out the experimental work. EP, MAZ, and EF-P: provided protocols and analyzed data. MAZ and MB: established the animal experimental model. AS: carried out the histological study. VP, NDeV, EF-P, HBA, and AR: contributed to the critical analyses of the data. EP, MAZ, and JR-C: designed the study, coordinate the experiments, and wrote the paper.

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4.2. Second study: SIRT1 in OLT

Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach.

Summary

Introduction: During liver transplantation, grafts are submitted to cold ischemia through their conservation in a preservation solution and warm reperfusion after the revascularization to the recipient. Thus, IRI is an inevitable situation and the composition of the preservation solution is a crucial factor for the graft viability. SIRT1 is a NAD+-dependent deacetylase that regulates cellular stress responses, including autophagy processes. Besides this, the role of sirtuins in IRI associated to liver transplantation has not been investigated. In this work, we aim to study the SIRT1 implication in rat orthotopic liver transplantation (OTL) and its relationship with autophagy, when IGL-1 preservation solution supplemented or not with TMZ (an anti-ischemic drug) was used.

Experimental: Livers from Sprague-Dawley male rats (200-250 gr) were preserved for 8 hours in IGL-1 solution enriched or not with TMZ (10-6 M) and then subjected to OLT (Kamada´s technique). After 24 hours of reperfusion, rats were sacrificed and blood and liver tissue samples were collected for analyzing liver injury (ALT), mitochondrial damage (GLDH) and oxidative stress (MDA). Then, we examined the protein expression pattern of SIRT1, ac-p53 and ac-FoxO1 (its direct substrates), as well as the levels of NAD^+, the co-factor necessary for SIRT1 activity and the expression of NAMPT, the precursor of NAD^+. Moreover, the protein expression of p-AMPK, p-mTOR and p-p70s6K, MAPKs (p-p38 and p-ERK) and of the autophagy parameters (beclin-1, LCB3), were also determined by western blot.

Results: The presence of TMZ in IGL-1 solution reduced liver injury and increased SIRT1 protein expression levels. Moreover, TMZ presence enhanced SIRT1 deacetylase activity, as evidenced by the augmented NAD^+ levels and the decreased expression of ac-p53 and ac-FoxO1. SIRT1 overexpression was accompanied by a significant increase in p-AMPK levels, an inhibition of p-m-TOR and the subsequent inactivation of p70S6K. These findings were consistent with an important activation of autophagy parameters

(beclin-1 and LC3B). In the same group, MAPKs protein expression was also regulated. Moreover, TMZ addition in IGL-1 solution prevented the oxidative stress and the mitochondrial damage.

Conclusion: The use of a modified IGL-1 preservation solution enriched with TMZ resulted in SIRT1 overexpression and enhancement of autophagy. We evidenced by the first time the implication of SIRT1 in hepatic IRI associated to OLT.

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ORIGINAL ARTICLE

Basic Study

Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach

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Abstract

AIM: To investigate the possible involvement of Sirtuin 1 (SIRT1) in rat orthotopic liver transplantation (OLT), when Institute Georges Lopez 1 (IGL-1) preservation solution is enriched with trimetazidine (TMZ).

METHODS: Male Sprague-Dawley rats were used as donors and recipients. Livers were stored in IGL-1 preservation solution for 8h at 4 ℃, and then underwent OLT according to Kamada's cuff technique without arterialization. In another group, livers were stored in IGL-1 preservation solution supplemented with TMZ, at 10^{-6} mol/L, for 8 h at 4 ℃ and then underwent OLT. Rats were sacrificed 24 h after reperfusion, and liver and plasma samples were collected. Liver injury (transaminase levels), mitochondrial damage (glutamate dehydrogenase activity) oxidative stress (malondialdehyde levels), and nicotinamide adenine dinucleotide (NAD^+), the co-factor necessary for SIRT1 activity, were determined by biochemical methods. SIRT1 and its substrates (ac-FoxO1, ac-p53), the precursor of NAD^+, nicotinamide phosphoribosyltransferase (NAMPT), as well as the phosphorylation of adenosine monophosphate activated protein kinase (AMPK), p-mTOR, p-p70S6K (direct substrate of mTOR), autophagy parameters (beclin-1, LC3B) and MAP kinases (p-p38 and p-ERK) were determined by Western blot.

RESULTS: Liver grafts preserved in IGL-1 solution enriched with TMZ presented reduced liver injury and mitochondrial damage compared with those preserved

in IGL-1 solution alone. In addition, livers preserved in IGL-1 + TMZ presented reduced levels of oxidative stress. This was consistent with enhanced SIRT1 protein expression and elevated SIRT1 activity, as indicated by decreased acetylation of p53 and FoxO1. The elevated SIRT1 activity in presence of TMZ can be attributed to the enhanced NAMPT protein and NAD^+/NADH levels. Up-regulation of SIRT1 was consistent with activation of AMPK and inhibition of phosphorylation of mTOR and its direct substrate (p-p70S6K). As a consequence, autophagy mediators (beclin-1 and LC3B) were over-expressed. Furthermore, MAP kinases were regulated in livers preserved with IGL-1 + TMZ, as they were characterized by enhanced p-ERK and decreased p-p38 protein expression.

CONCLUSION: Our study shows that IGL-1 preservation solution enriched with TMZ protects liver grafts from the IRI associated with OLT, through SIRT1 up-regulation.

Key words: Sirtuin 1; Ischemia-reperfusion injury; Liver transplantation; IGL-1 preservation solution; Trimetazidine

Core tip: Sirtuin 1 (SIRT1) has been implicated in pathways associated with ischemia-reperfusion injury (IRI), but its role in rat orthotopic liver transplantation has not yet been established. In our study, SIRT1 protein expression levels and activity increased when Institut Georges Lopez 1 (IGL-1) preservation solution was supplemented with trimetazidine, which was associated with less hepatic injury and mitochondrial damage. The increased deacetylation of FoxO1 by SIRT1 agreed with less oxidative stress and the activation of the autophagy pathway. These findings support the notion that SIRT1 up-regulation may be an effective strategy for reducing IRI and improving liver transplantation outcome.

Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Varela AT, Rolo AP, Palmeira CM, Roselló-Catafau J. Sirtuin 1 in rat orthotopic liver transplantation: An IGL-1 preservation solution approach. *World J Gastroenterol* 2015; 21(6): 1765-1774 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i6/1765.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i6.1765

INTRODUCTION

Liver ischemia-reperfusion injury (IRI) can cause primary graft non-function and may lead to organ failure[1,2]. The oxygen deprivation during ischemia provokes depletion of cellular energy, whereas the subsequent re-oxygenation during reperfusion initiates a cascade of complex pathways, including the production of reactive oxygen species (ROS), which in part are responsible for the subsequent induction of hepatocellular injury. Given the complexity of IRI pathophysiology, a more profound knowledge of the underlying mechanisms is needed in order to design new therapeutic strategies able to minimize its adverse effects.

SIRT1 is a histone deacetylase that either activates or suppresses the transcription activities of various non-histone proteins through its nicotinamide adenine dinucleotide (NAD^+)-dependent activity. SIRT1 has been associated with the pathophysiology of IRI in several organs[3]. In fact, SIRT1 is involved in a wide variety of cellular processes, including apoptosis, cellular stress and autophagy[4-7]. It has been reported that SIRT1 deacetylates p53, thus reducing its transcriptional activity and its ability to induce apoptosis[8]. Forkhead box-containing protein O 1 (FoxO1) is also a target for SIRT1, and its deacetylation has been implicated in the detoxification of ROS and the promotion of autophagy[9]. Furthermore, we have recently shown that SIRT1 activation contributes, in part, to the protective effects of liver ischemic preconditioning against IRI[10].

Adequate liver preservation is vital for the success of transplantation, in order to maintain graft quality after cold storage. At present, University of Wisconsin (UW) solution is the most widely used preservation solution. Recent studies have demonstrated that Institute Georges Lopez 1 (IGL-1) preservation solution is a valuable alternative for liver grafts in orthotopic liver transplantation (OLT)[11,12]. Moreover, supplementation of IGL-1 with trimetazidine (TMZ) has been shown to increase the preservation of both steatotic and non-steatotic liver grafts in an isolated and perfused "*ex vivo*" model[13]. However, the role of SIRT1 in rat OLT when IGL-1 solutions are used has not been assessed to date.

Given that TMZ is a promising additive for increasing liver graft preservation and since SIRT1 exerts a protective role against warm IRI in the liver, the aim of this study is to investigate the potential role of SIRT1 in rat OLT when TMZ-enriched IGL-1 preservation solution is used.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) were used as donors and recipients. Throughout the study, animals were housed in conventional animal facilities where temperature and humidity were controlled with a 12 h light/dark cycle. All animals had free access to water and a standard laboratory diet. All procedures were performed under isofluorane inhalation anesthesia. The experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 400/12), University of Barcelona and all procedures complied with European Union regulations for animal experiments (EU guideline 86/609/EEC). Rats were randomly distributed into groups as de-

scribed below.

Experimental design

The following experimental groups were created: (1) Sham (n = 6): Animals underwent transverse laparotomy and received silk ligatures in the right suprarenal vein, diaphragmatic vein, and hepatic artery; (2) IGL-1 (n = 6): Livers were flushed and stored in IGL-1 preservation solution for 8h at 4 ℃, and then underwent OLT according to Kamada's cuff technique without arterialization. Rats were sacrificed 24 h after reperfusion for liver and plasma sample collection; and (3) IGL-1 + TMZ (n = 6): Same as group 2, but livers were preserved in IGL-1 solution supplemented with trimetazidine (TMZ) at 10^{-6} mol/L.

Transaminase assay

Hepatic injury was assessed in terms of alanine aminotransferase (ALT) levels with commercial kits from RAL (Barcelona, Spain). Briefly, plasma extracts were collected before liver extraction and centrifuged at 4 ℃ for 10 min at 0.8 g. Then, 200 μL of the supernatant was added to the substrate provided by the commercial kit. ALT levels were determined at 365 nm with a UV spectrometer (DU 800, Beckman Coulter) and calculated following the supplier's instructions[14].

Glutamate dehydrogenase activity

Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme that catalyses the conversion of glutamate to 2-oxoglutarate. It was used as an indirect marker of mitochondrial damage; it was measured in plasma, as described previously[15].

Lipid peroxidation assay

Lipid peroxidation in the liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction[16]. Liver samples were homogenized in Tris-HCL pH = 7 and 250 μL of trichloroacetic acid (TCA) were added to 250 μL of liver homogenates. Then, the samples were centrifugated in 3000 rpm at 4 ℃ for 15 min. Then, 250 μL of thiobarbituric acid (TBA) were added to the supernatant and were heated at 100 ℃ for 30 min. MDA reacted with TBA to form a pink chromogenic compound whose absorbance at 540 nm was measured. The result was expressed as nmols/mg protein.

NAD^+/NADH determination

NAD^+/NADH from liver were quantified with a commercially available kit (MAK037, Sigma Chemical, St. Louis, MO, United States) according to the manufacturer's instructions.

Western blot analysis

Liver tissue was homogenized in HEPES buffer as previously described[10]. Fifty μg of proteins was separated on 8%-15% SDS-PAGE gels and transblotted on PVDF membranes (Bio-Rad). Membranes were then blocked for one hour with 5% (w/v) nonfat milk in T-TBS and incubated overnight at 4 ℃ with antibody against SIRT1 (#07-131), p-mTOR (Ser2481, #09-343), mTOR (#04-385), all purchased from Merck Millipore, Billerica, MA; ac-p53 (ab37318, abcam, United kingdom); ac-FoxO1 (D-19, sc-49437), BECN1 (H-300, sc-11427), both purchased from Santa Cruz Biotechnology Inc, CA, United States; p-AMPK (Thr172, #2535), p-p38 mitogen activated protein (MAP) kinase (Thr180/Tyr182,#9211), p-70S6K (Thr389, #9205), LC3B (#2775), p-p44/42 MAPK (Erk1/2, Thr202/Tyr204), #9101, all from Cell Signaling, Danvers, MA, NAMPT (AP22021SU, Acris Antibodies GmbH, Germany), HSP70 (610607, Transduction Laboratories, Lexington, KY) and b-actin (A5316, Sigma Chemical, St. Louis, MO, United States). Membranes were then incubated for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using WesternBright ECL-HRP substrate (Advansta, Barcelona, Spain) and were quantified using the Quantity One software for image analysis. Results were expressed as the densitometric ratio between the protein of interest and the loading control (b-actin).

Statistical analysis

Data are expressed as mean ± SE. Statistical comparison was performed by variance analysis, followed by the Student-Newman-Keuls test (Graft Pad prism software). $P < 0.05$ was considered significant.

RESULTS

Liver injury and mitochondrial damage

We first determined liver and mitochondrial injury through transaminase and GLDH levels respectively in the different experimental groups. Livers preserved in IGL-1 and subjected to OLT showed the highest ALT and GLDH levels, whereas addition of TMZ to the IGL-1 solution resulted in a significant decrease in liver and mitochondrial injury in comparison with IGL-1 solution alone (Figure 1).

SIRT1, NAMPT, ac-p53 ac-FoxO1 proteins expression and NAD^+ levels

In order to explore the potential involvement of SIRT1 in the protective effects of TMZ on rat OLT, we first determined its protein expression pattern. SIRT1 protein expression was significantly increased when livers were preserved in IGL-1 solution compared with the Sham group (Figure 2C). Interestingly, the addition of TMZ to IGL-1 solution clearly enhanced SIRT1 expression compared with IGL-1 solution alone (Figure 2C). In view of the altered SIRT1 protein levels, we then investigated parameters associated with SIRT1 activity, such as the protein expression of

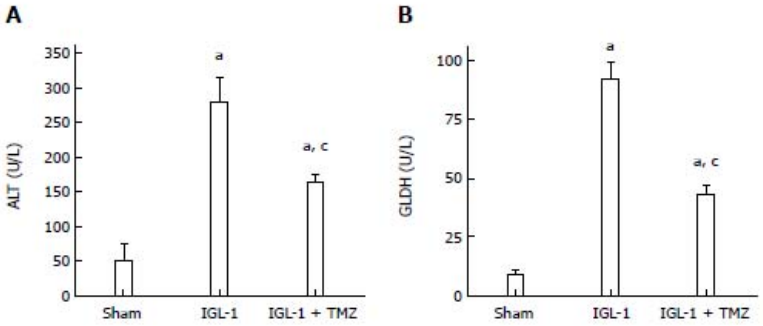

Figure 1 Alanine aminotransferase levels (A) and hepatic glutamate dehydrogenase (B) in plasma after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10-6 M Trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1. ALT: Alanine aminotransferase; GLDH: Glutamate dehydrogenase.

nicotinamide phosphoribosyltransferase (NAMPT), the NAD^+/NADH levels, as well as the acetylation state of two direct substrates, p53 and FoxO1. As shown in Figure 2A, liver graft preservation in IGL-1 solution led to high NAMPT protein expression, which was further enhanced in case of IGL-1 supplemented with TMZ. Furthermore, NAD^+/NADH levels were significantly reduced in both IGL-1 groups in comparison to non-treated animals (Figure 2B). However, the presence of TMZ in IGL-1 resulted in a better preservation of NAD^+/NADH levels than the IGL-1 alone (Figure 2B). This was consistent with decreased acetylated FoxO1 and p53 protein levels in IGL-1 + TMZ group compared with IGL-1 (Figure 2D and Figure 2E respectively). These results suggest an increase in SIRT1 activity in the IGL-1 + TMZ group; therefore, the protective effect of TMZ is exerted, at least in part, through the induction of both SIRT1 expression and activation.

Oxidative stress and HSP70 protein expression

Next, we evaluated lipid peroxidation as an indicator of oxidative stress in OLT. Livers preserved in IGL-1 solution showed significantly increased MDA compared with Sham (Figure 3A). This increase was prevented by the addition of TMZ in IGL-1 solution. Moreover, livers preserved in IGL-1 solution up-regulated the levels of the cytoprotective heat shock protein 70 (HSP70), which was further enhanced in the presence of TMZ (Figure 3B).

p-AMPK and p-mTOR activation

It is known that both AMP-activated protein kinase (AMPK) and SIRT1 regulate each other and share many common target molecules[17]. We therefore assessed the possible involvement of AMPK activation in the protective effects of TMZ. As shown in Figure 4A, livers preserved with IGL-1 solution showed increased phosphorylated AMPK (p-AMPK). However, this AMPK activation was further enhanced in the presence of TMZ. Given that the mammalian target of rapamycin (mTOR) activity is regulated by AMPK, we next evaluated both phosphorylated mTOR (p-mTOR) and mTOR protein levels as well as the phosphorylation levels of its direct substrate, p-p70S6K. As shown in Figure 4B and C, the addition of TMZ to IGL-1 solution significantly reduced p-mTOR/mTOR and p-p70S6k protein levels compared with IGL-1 preservation solution alone.

Autophagy: Beclin-1 and LC3B protein levels

Autophagy is a conserved cellular process that is activated under conditions of nutrient stress to promote cell survival. Given the importance of the mTOR signaling pathway in the suppression of autophagy[18] and the fact that IGL-1 preservation solution enriched with TMZ was characterized by inactivation of both mTOR and p70S6k protein, we sought to test whether TMZ-induced SIRT1 protective effects might be mediated through the activation of autophagy. To do so, we explored the expression of beclin-1 and LC3B, two well-known proteins involved in the autophagic pathway. Both proteins presented significant up-regulation in the IGL-1 + TMZ group compared with both Sham and IGL-1 group (Figure 5A, B).

MAP kinases

Finally, we explored the modulation of MAP kinases by TMZ. Here we observed increased phosphorylation of extracellular signal-regulated kinase (ERK) in IGL-1 preserved livers, which was further enhanced when TMZ was added to the preservation solution (Figure 6A). Moreover, the presence of TMZ in IGL-1 solution reversed the increased phosphorylation of p38 protein levels detected in the livers preserved in IGL-1 solution (Figures 6B and 7).

DISCUSSION

The present study provides the first evidence that SIRT1 up-regulation in a rat model of OLT contributes to a better preservation of liver grafts against IRI. SIRT1 over-expression is attributed to the use of a modified IGL-1 preservation solution enriched with

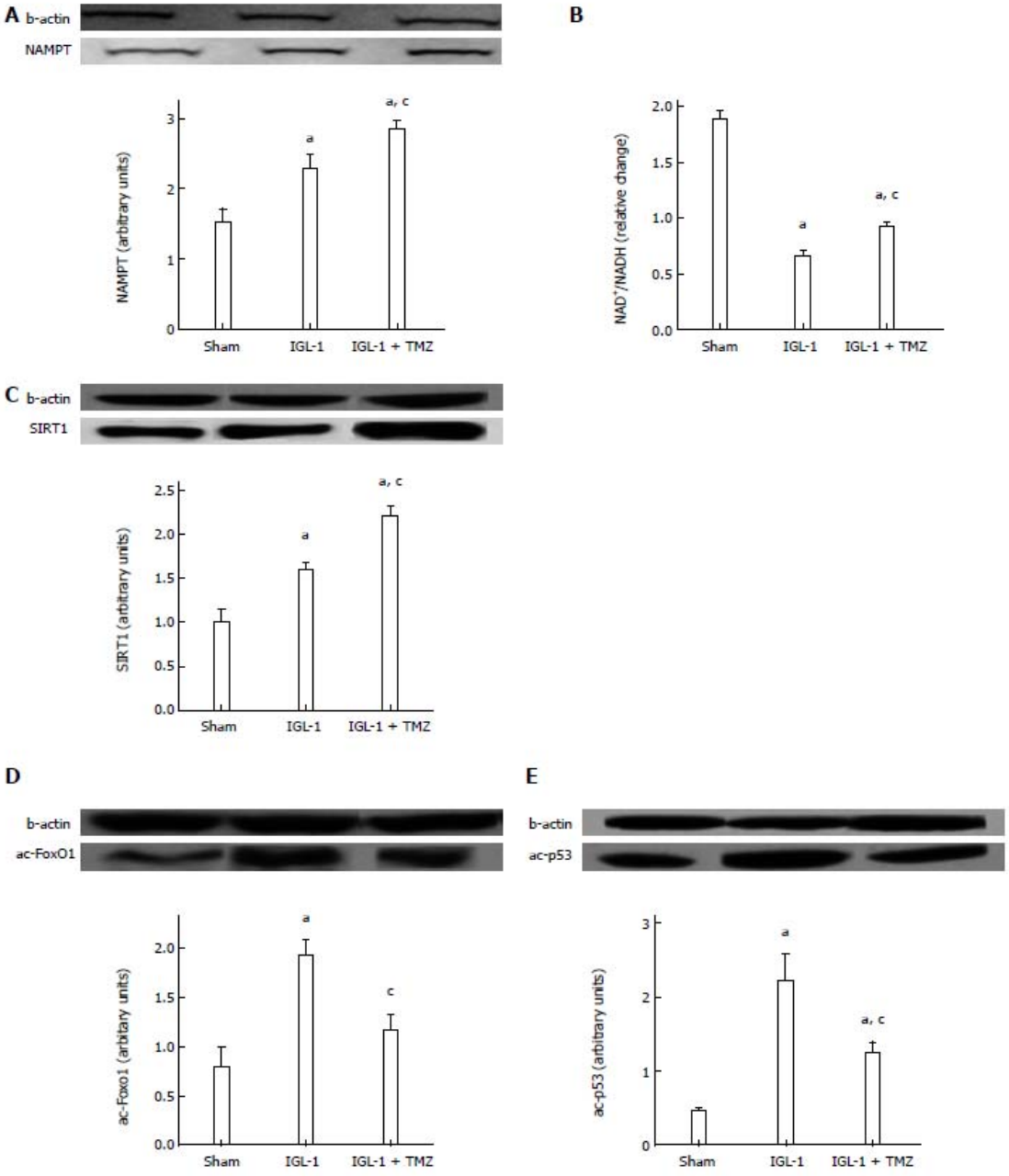

Figure 2 NAMPT protein expression (A), NAD⁺/NADH levels (B), SIRT1 (C), ac-FoxO1 (D) and ac-p53 (E) protein expression in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation, IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-6} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1. NAMPT: Nicotinamide phosphoribosyltransferase; NAD⁺: Nicotinamide adenine dinucleotide; SIRT1: Sirtuin 1; FoxO1: Forkhead box-containing protein O 1; NADH: Nicotinamide adenine dinucleotide.

TMZ, an anti-ischemic drug administered for the treatment of angina pectoris[19]. In earlier work with an isolated perfused rat liver model, we already showed that TMZ addition to IGL-1 solution promotes cytoprotective markers that are induced during ischemia, such as hypoxia inducible factor-1α through nitric oxide generation, as well as those promoted during reperfusion, such as heme oxygenase-1[13]. Here, we report that the addition of TMZ in IGL-1 preservation solution favors the activation of SIRT1 in rat OLT.

The increases observed in SIRT1 protein levels

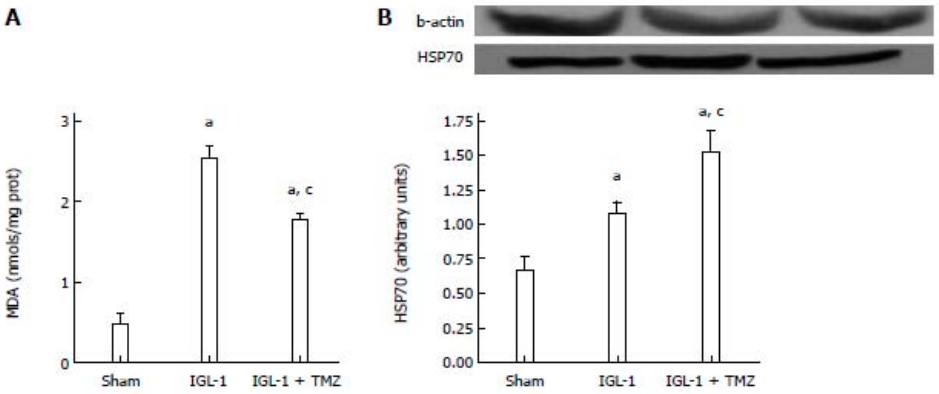

Figure 3 Malondialdehyde (A) and HSP70 protein levels (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-5} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1. MDA: Malondialdehyde.

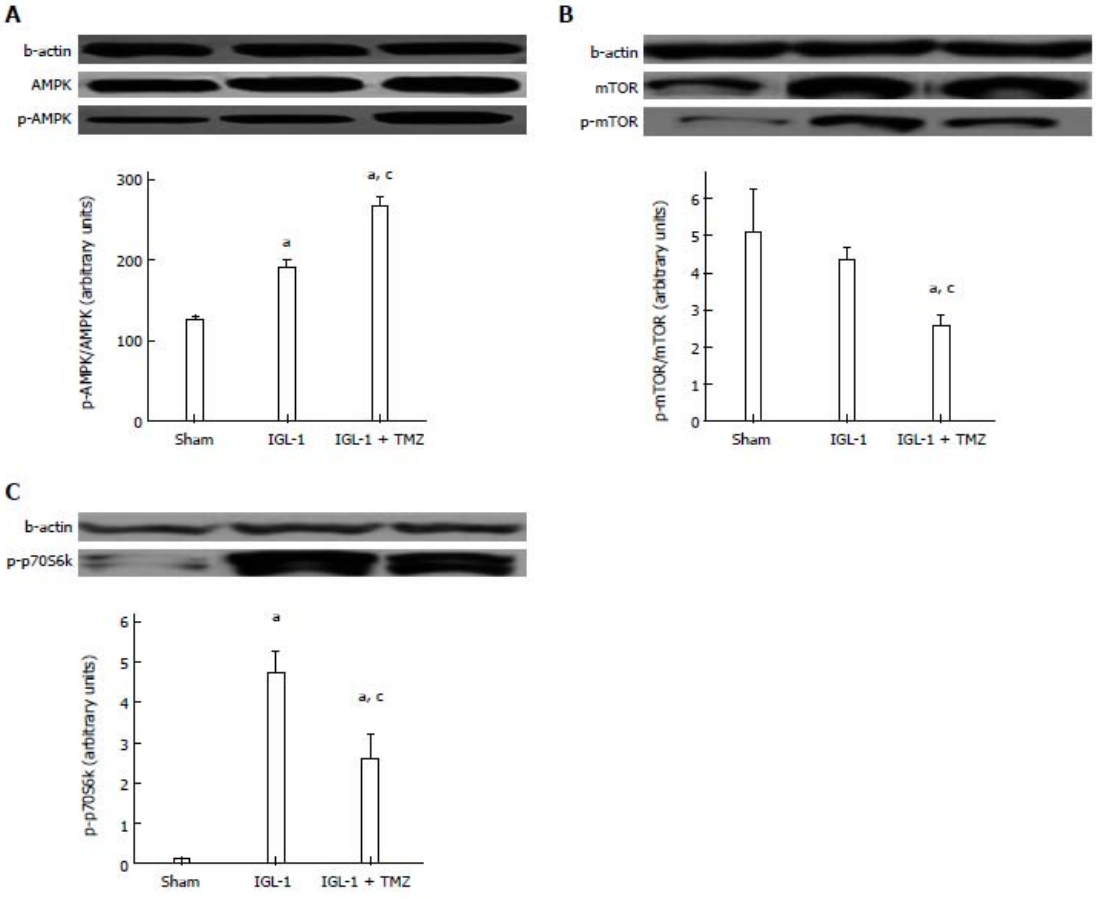

Figure 4 Protein levels of p-AMPK (A), p-mTOR/mTOR (B) and p-p70S6k (C) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-5} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

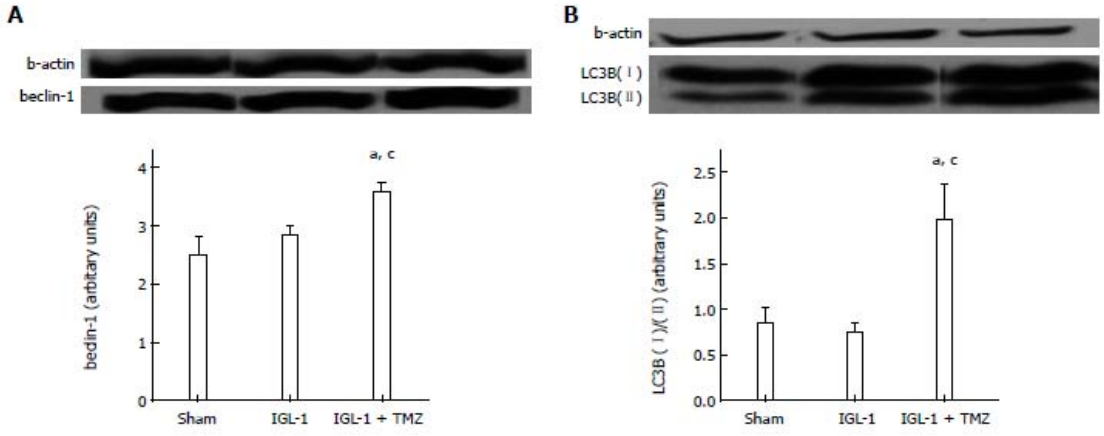

Figure 5 Protein expression of beclin-1 (A) and LC3B (Ⅱ)/(Ⅰ) (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-4} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

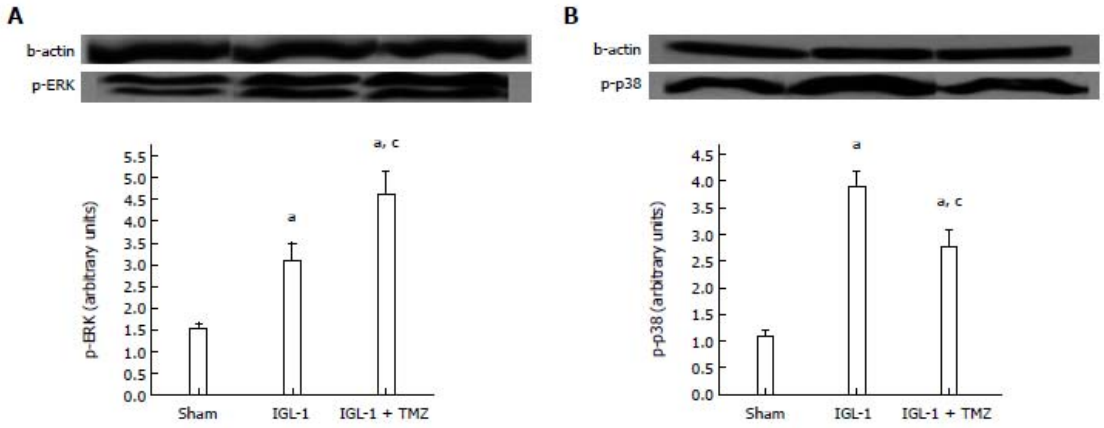

Figure 6 Protein levels of pERK (A) and p-p38 (B) in livers after 24 h of reperfusion. Sham: Liver harvested without transplantation; IGL-1: Liver transplanted after 8 h of cold storage in IGL-1 solution; IGL-1 + TMZ: Liver transplanted after 8 h of cold storage in IGL-1 solution with 10^{-4} mol/L trimetazidine (TMZ). $^{a}P < 0.05$ *vs* Sham; $^{c}P < 0.05$ *vs* IGL-1.

were consistent with an effective prevention of liver injury and mitochondrial damage. We previously reported the protective effect of TMZ on liver damage after graft preservation, and showed that its addition to UW solution reduces liver injury and improves liver function[20]. Our current data confirm that SIRT1 up-regulation in OLT due to TMZ can be considered as a protective cellular signaling response against IRI. This finding corroborates previous reports of a protective effect for SIRT1 against IRI in different organs such as heart, kidney and brain[21-23].

SIRT1 over-expression in liver grafts preserved in IGL-1 + TMZ was concomitant with increased levels of both NAMPT and NAD^+. NAD^+ is a cofactor required for SIRT1 enzymatic activity. Furthermore, it has been shown that NAMPT is the rate-limiting enzyme in the NAD^+ biosynthetic pathway and directly regulates SIRT1 activity in mammalian cells[24]. NAMPT up-regulation has been shown to be beneficial against cardiac IRI through preservation of NAD^+ levels and SIRT1 regulation[25]. Taking this into account, we suggest that in the IGL-1 + TMZ group the increased NAMPT protein levels promote NAD^+ production, which in turn contributes to the high SIRT1 deacetylase activity. Indeed, in the same group we observed decreased levels of acetylated p53 and FoxO1, two direct substrates of SIRT1, which have been reported to be SIRT1 activity markers[26,27]. Therefore, the TMZ protective effects observed are mediated, at least in part, by enhanced SIRT1 activity.

SIRT1 plays an important role in cellular stress, including the oxidative stress associated with IRI. FoxO1 deacetylation by SIRT1 has been linked with the capacity of FoxO1 to enhance the transcription

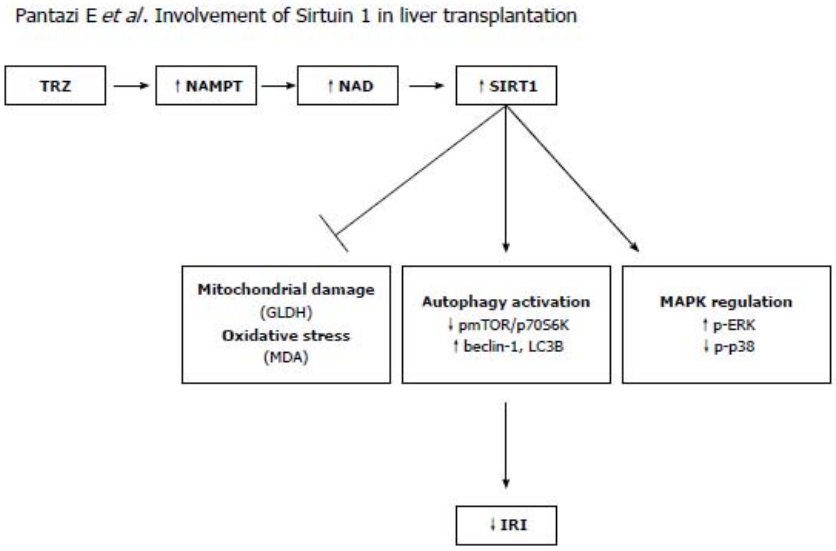

Figure 7 Schematic model of the protective effect of sirtuin 1 against ischemia-reperfusion injury associated to rat orthotopic liver transplantation. Trimetazidine (TMZ) addition to IGL-1 promotes NAMPT expression and enhances NAD+ levels, which in turn provokes SIRT1 up-regulation. SIRT1 contributes to decreased hepatic injury by inhibiting mitochondrial damage and oxidative stress, activating autophagic pathway and by enhancing p-ERK and decreasing p-p38 protein expression. SIRT1: Sirtuin 1; IRI: Ischemia-reperfusion injury; OLT: Orthotopic liver transplantation.

of anti-oxidant enzymes, thus contributing to the resistance against oxidative stress[4,5,21]. For these reasons, we decided to explore the potential relationship between SIRT1, ac-FoxO1 and the lipid peroxidation that occurs in OLT. In the present study, we found that the significant SIRT1 induction and FoxO1 deacetylation were accompanied by marked decreases in lipid peroxidation when TMZ was added to IGL-1. Moreover, SIRT1 up-regulation in OLT may contribute to reducing hepatic vulnerability against oxidative stress and to improving the mitochondrial status during oxidative stress conditions. Our results corroborate those of Ou *et al*[28] who demonstrated that over-expression of SIRT1 under oxidative stress conditions enhanced mitochondrial function in embryonic stem cells. In addition, Hsu *et al*[21] demonstrated that hearts over-expressing SIRT1 were more resistant to oxidative stress in response to IRI, due, in part, to the effective FoxO1 deacetylation by SIRT1.

It is well known that increases in heat shock protein expression are involved in the protection against oxidative stress[29]. Our results also demonstrate strong HSP70 protein expression in the IGL-1 + TMZ group. This direct connection between SIRT1 and HSP70 during liver IRI was recently reported by our group in liver ischemic preconditioning[10].

Autophagy plays an important role in both sensing oxidative stress and removing oxidative damaged proteins and organelles[30]. This process involves the inhibition of p-mTOR (mammalian TOR) and the subsequent inactivation of its direct substrate, p70S6k[31]. During autophagy, specific proteins such as beclin-1 (in the initial stages of autophagosome formation) and LC3B (during autophagosome expansion) are activated[32,33]. AMPK activation results in mTOR inhibition and subsequent autophagy activation[31]. In addition, SIRT1 deacetylase activity has been associated with activation of AMPK, and SIRT1- mediated deacetylation of FoxO1 has also been implicated in increased autophagy[34-36]. In our study, SIRT1 overexpression and the enhanced activity in the IGL-1 + TMZ group was concomitant with AMPK activation, reduced levels of p-mTOR/p-p70S6k, and increased autophagy (beclin-1 and LC3B). Our findings are in agreement with other investigations carried out in liver[37-39] and kidney[40] which have shown the protective effect of autophagy against IRI. Autophagy activation through a NAMPT/SIRT1-dependent mechanism has also been shown to be protective against cerebral ischemia[41].

However, the role of autophagy in IRI has been controversial, as various studies have evidenced either a beneficial or detrimental role. For example Jiang *et al*[40] have reported a renoprotective role of autophagy against IRI; during ischemia, autophagy can contribute to the provision of nutrients, whereas during reperfusion can eliminate damaged proteins and organelles. Similarly, Matsui *et al*[42] in cardiac IRI showed that autophagy was protective during ischemia, but beclin-1 dependent autophagy activation during reperfusion was associated with cell death. Autophagy is stimulated by various factors and it still remains unknown which steps determine the decision for the survival or death. Between them, the time of ischemia seems to influence the autophagy outcome, as a prolonged ischemia time can result in excessive activation of autophagy and subsequently to cell death[43]. In our case, autophagy is associated with decreased graft injury and thus we may suppose that eight hours of cold preservation is not sufficient

time for an excessive and detrimental activation of autophagy. More profound investigations are required in order to define the regulatory mechanisms of autophagy.

In a recent publication, we reported that SIRT1 modulates (MAP) kinases, whose activation is a consequence of oxidative stress generation associated to IRI[16]. Here, we show that TMZ addition to IGL-1 enhances p-ERK protein levels and reduces p-p38 protein levels in comparison to IGL-1 alone. As a result, the increased SIRT1 over-expression in OLT coincided with the modulation of the MAP kinases, as reported in other studies[44].

Taken together, our results show that TMZ exerts its protective role against IRI associated with OLT, in part, through the induction of SIRT1 protein expression and activity. We found that SIRT1 up-regulation prevented liver injury and oxidative stress and promoted liver autophagy (see Figure 7). Our findings support the benefits of pharmacological activation of SIRT1, a new therapeutic strategy for improving liver graft preservation.

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COMMENTS

Background

Ischemia-reperfusion injury (IRI) is a complex but unavoidable situation during liver transplantation, which contributes to organ failure. Sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that regulates several cellular pathways associated with IRI, including oxidative stress and autophagy. Institut Georges Lopez 1 (IGL-1) preservation solution has been proposed as a good alternative to UW solution for the preservation of liver grafts. Moreover, the addition of trimetazidine (TMZ), an anti-ischemic drug, to both preservation solutions has been shown to improve liver graft preservation. In this study, the authors demonstrate that TMZ addition in IGL-1 solution reduces IRI associated with rat orthotopic liver transplantation (OLT) through increases in both SIRT1 protein expression and activity.

Research frontiers

SIRT1 plays an important role in several processes, including IRI. Here we report for the first time that the presence of TMZ in IGL-1 provoked elevated FoxO1 deacetylation, oxidative stress diminution and augmented autophagy and was associated with SIRT1 activation.

Innovations and breakthroughs

SIRT1 exerts a protective effect against IRI in several organs through a variety of mechanisms. However, SIRT1 involvement in models of transplantation has not been determined to date. The present study evaluated the potential role of SIRT1 in a rat OLT model. SIRT1 was up-regulated when livers were stored in IGL + TMZ preservation solution and helped to improve the protection of liver grafts against IRI, as reflected by decreases in hepatic injury, mitochondrial damage, and oxidative stress.

Applications

Pharmacological treatment in order to enhance SIRT1 activity is a promising tool for reducing the detrimental effects of IRI associated with liver transplantation.

Peer-review

This is a relevant manuscript on to date important issue as IRI in organ transplantation as well as in other clinical condition. The manuscript is well written and the study is well conducted. The beneficial effect of adding trimetazidine on preservation solution is documented in several ways ranging from hepatic enzyme dosage to all the biological cellular expression of damage (SIRT1, ac-p53 ac-Fox O1 protein expression, to oxidative stress and HSP70 protein expression.

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4.3. Third study: SIRT1 in ROLT

Losartan activates sirtuin 1 in rat reduced-size orthotopic liver transplantation.

(under revision in World Journal of Gastroenterology)

Summary

Introduction: SIRT1 is a histone deacetylase that has been associated with protective mechanisms against IRI, but its role in liver transplantation has been poorly investigated. Angiotensin II, the main effector of the renin-angiotensin system, has been associated with increased hepatic injury and its inhibition with beneficial effects against IRI. Although both angiotensin II and SIRT1 are involved in common processes related to IRI, a potential link between them has not yet been reported in a model of LT. The purpose of this study is to evaluate the possible SIRT1 implication in the rat ROLT, as well as to examine a potential relationship between SIRT1 and losartan, an antagonist of angiotensin II type I receptor (AT1R).

Experimental: Livers of male Sprague-Dawley rats (200-250gr) were preserved in University of Wisconsin (UW) storage solution for 1 hour at 4 ^{0}C and then subjected to ROLT (Kamadas´technique). In an additional group, losartan was orally administered (5 mg/kg) 24 hours and 1 hour before the surgical procedure to both the donor and the recipient rats. Liver injury (transaminases), SIRT1 protein levels and activity, SIRT3 protein and mRNA expression, ERS parameters (GRP78, IRE1a and p-eIf2), heat shock proteins (HO-1, HSP70) expression and apoptosis parameters (Caspase 12 and 3) were measured 24 hours after reperfusion.

Results: The present study demonstrated that losartan pretreatment diminished hepatic injury in ROLT, which was consistent with induction of both SIRT1 protein expression and activity. Losartan administration provoked also enhanced NAD^{+} levels, the co-factor necessary for SIRT1 activity. Furthermore, SIRT1 induction by losartan pre-treatment coincided with decreases in the ERS parameters and in liver apoptosis. Losartan administration also modulated HSPs expression. In addition, both mRNA and protein levels of SIRT3 were comparable in ROLT and losartan + ROLT group.

Conclusions: We evidenced that SIRT1 is induced upon losartan pretreatment in ROLT and can be considered as an emerging therapeutic strategy in order to diminish hepatic IRI associated to ROLT.

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Basic study

Losartan activates Sirtuin 1 in rat reduced-size orthotopic liver transplantation

Pantazi E *et al.* SIRT1 and losartan in liver transplantation

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Author contributions: Pantazi E carried out the experimental work; Pantazi E, Bejaoui M, Folch-Puy E provided protocols and analyzed data; Zaouali MA; Bejaoui M, Panisello A established the animal experimental model and injury parameters; Rolo AP, Palmeira CM determinated NAD^+, NAMPT levels and contributed to critical analyzes of the data and discussion; Pantazi E, Folch-Puy E and Roselló-Catafau J designed the study, coordinate the experiments and wrote the paper.

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Ethics approval: The present study does not involve human beings/samples.

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 400/12), University of Barcelona and all procedures complied with European Union regulations for animal experiments (EU guideline 86/609/EEC).

Biostatistics: Data are expressed as mean ± standard error. Statistical comparison was performed by variance analysis, followed by the Student– Newman–Keuls test, using the GraphPad Prism software. $P < 0.05$ was considered statistically significant.

Conflict-of-interest: The authors declare that they have no conflict of interest or any financial interests.

Data sharing: Any further information related to technical appendix, statistical code and dataset are available from the corresponding author at jrcbam@iibb.csic.es. The authors gave informed consent for data sharing.

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Abstract

AIM: To investigate a possible association between losartan and sirtuin 1 (SIRT1) in reduced-size orthotopic liver transplantation (ROLT) in rats.

METHODS: Livers of male Sprague-Dawley rats (200-250 gr) were preserved in University of Wisconsin preservation solution for 1 hour at 4 ^{0}C prior to ROLT. In an additional group, an antagonist of angiotensin II type 1 receptor (AT1R; losartan), was orally administered (5 mg/kg) 24 hours and 1 hour before the surgical procedure to both the donors and the recipients. Transaminase (as an indicator of liver injury), SIRT1 activity, and nicotinamide adenine dinucleotide (NAD+, a co-factor necessary for SIRT1 activity) levels were determined by biochemical methods. Protein expression of SIRT1, acetylated FoxO1 (ac-FoxO1), NAMPT (the precursor of NAD+), heat shock proteins (HSP70, HO-1) expression, endoplasmic reticulum stress (GRP78, IRE1α, p-eIF2) and apoptosis (caspase 12 and caspase 3) parameters were determined by Western blot. Possible alterations in protein expression of mitogen activated protein kinases (MAPK), such as p-p38 and p-ERK, were also evaluated. Furthermore, the SIRT3 protein expression and mRNA levels were examined.

RESULTS: The present study demonstrated that losartan administration led to diminished liver injury when compared to ROLT group, as evidenced by the significant decreases in ALT (358,3 ± 133,44 vs 206 ± 33,61, P< 0.05) and AST levels (893,57 ± 397,69 vs 500,85 ± 118,07, P< 0.05). The lessened hepatic injury in case of losartan was associated with enhanced SIRT1 protein expression and activity (5,27 ± 0,32 vs 6,08 ± 0,30, P<0.05). This was concomitant with increased levels of NAD^{+} (0,87 ± 0,22 vs 1,195 ± 0,144, P<0.05) the co-factor necessary for SIRT1 activity, as well as with decreases in ac-FoxO1 expression. Losartan treatment also provoked significant attenuation of endoplasmic reticulum stress parameters (GRP78, IRE1a, p-eIF2) which was consistent with reduced levels of both caspase 12 and caspase 3. Furthermore, losartan administration stimulated HSP70 protein expression and attenuated HO-1 expression. However, no changes were observed in protein or mRNA expression of SIRT3**.** Finally, the protein expression pattern of p-ERK and p-p38 were not altered upon losartan administration.

CONCLUSION: The present study reports that losartan induces SIRT1 expression and activity, and that it reduces hepatic injury in a ROLT model.

Keywords: losartan, sirtuin 1, endoplasmic reticulum stress, liver ischemia reperfusion injury, angiotensin II

Core tip

Losartan is an angiotensin II type 1 receptor (AT1R) antagonist known to protect livers against ischemia-reperfusion injury (IRI). However, the mechanisms underlying this hepatoprotective effect are not fully understood, especially in case of reduced-size orthotopic liver transplantation (ROLT). SIRT1 has recently emerged as an important target to modulate for alleviating IRI. In our study, we describe that AT1R antagonism enhances SIRT1 activity and prevents endoplasmic reticulum stress and liver apoptosis in a rat model of ROLT. Consequently, losartan increases the resistance of ROLT grafts against IRI.

Pantazi E, Bejaoui M, Zaouali MA, Folch-Puy E, Pinto Rolo A, Panisello A, Palmeira CM and Roselló-Catafau J. Losartan activates SIRT1 in rat reduced-size orthotopic liver transplantation. *World J Gastroenterol* 2015; In press

INTRODUCTION

Ischemia-reperfusion injury (IRI) is an important obstacle during liver transplantation, contributing to a significant loss of graft function. It is characterized by a cascade of deleterious cellular responses that lead to inflammation, cell death, and ultimately, organ failure [246]. These complications are increased in case of reduced-size liver grafts compared with standard liver transplant operations [247, 248]. Thus, further investigation is required to explore new therapeutic strategies to counteract IRI.

Various reports have associated the renin-angiotensin system (RAS) with liver IRI [90, 249]. The main effector of RAS is angiotensin II, which is produced via angiotensin converting enzyme (ACE) from angiotensin I. It exerts its biological actions through two receptor subtypes: angiotensin II type I receptor (AT1R) and angiotensin II type II receptor (AT2R) [82]. Angiotensin II has been associated with increased inflammation and oxidative stress in liver IRI, and various studies have evidenced that AT1R antagonists, such as losartan, efficiently protected livers against IRI in both warm ischemia and transplantation models [87, 89, 250, 251].

Sirtuins are deacetylases dependent on nicotinamide adenine dinucleotide (NAD)+ that either activate or suppress various proteins. Thus, they are implicated in various cellular pathways, including metabolic processes, apoptosis and oxidative stress [165]. Sirtuin 1 (SIRT1) and the mitochondrial sirtuin 3 (SIRT3) are the most studied sirtuins and represent interesting targets for counteracting IRI in various organs [252, 253]. SIRT1 has been shown to be involved in a wide range of cellular processes related to cell cycle and the cellular response to stresses, including the endoplasmic reticulum stress (ERS) [164, 227, 234, 254].

IRI is known to promote ERS which finally induces cellular death [76]. In addition, we have previously shown that inhibiting ERS can be a useful strategy against IRI [255]. In a model of partial hepatectomy with ischemia-reperfusion in steatotic and non-steatotic rat livers, ERS inhibition ameliorated hepatic damage by reducing inflammation and apoptosis [255]. Therefore, we may hypothesize that preventing ERS might be useful for ameliorating the negative outcomes of reduced-size orthotopic liver transplantation (ROLT).

There is little evidence about a potential relationship between SIRT1 and amgiotensin II antagonists. Miyazaki *et al.* have reported that SIRT1 overexpression suppresses AT1R in cultured vascular smooth muscle cells [256]. In addition, a recent study in primary cultures of adipocytes evidenced a mutual interaction between RAS and SIRT1, with an association with metabolic homeostasis. [257]. Conversely, there are no reports concerning a relationship between SIRT1 and angiotensin II antagonists in liver transplantation. Given that both are involved in common processes related to IRI, ERS, and apoptosis [258, 259], we hypothesized

that SIRT1 may be implicated in the protective effects of an AT1R antagonist against hepatic IRI following ROLT.

The present study therefore aimed to assess whether an AT1R antagonist, losartan, could be effective in protecting reduced-size liver grafts from IRI and to examine the possible underlying mechanisms involved. Furthermore, a potential relationship between losartan and SIRT1 was explored.

MATERIALS AND METHODS

Experimental animals

Male Sprague–Dawley rats (200–250 g) were used as donors and recipients. Animals were housed in conventional temperature- and humidity-controlled facilities with a 12-hour light/dark cycle. All animals had free access to water and a standard laboratory diet. All procedures were performed under isoflurane inhalation anesthesia. Animal experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 400/12), University of Barcelona and all procedures complied with European Union regulations for animal experiments (EU guideline 86/609/EEC). Rats were randomly distributed into groups as described below.

Experimental design

The following experimental groups were created:

1) Sham (n=6): Animals were subjected to transverse laparotomy and silk ligatures were located in the right suprarenal vein, diaphragmatic vein, and hepatic artery. After 24 hours, animals were sacrificed and blood and liver samples were collected and stored at -20 ^{0}C and -80 ^{0}C respectively, for further investigation.

2) ROLT (n =12, 6 transplants): ROLT was performed according to the Kamada's cuff technique, without hepatic artery reconstruction [242]. During the donor surgery, the right suprarenal vein, diaphragmatic vein, and hepatic artery were ligated and the bile duct was cannulated. Then, the reduction of the liver was carried out. Liver reduction was achieved by removing the left lateral lobe and the two caudate lobes just before harvesting the liver, resulting in a 40% reduction of the liver mass. The pedicle of the left lateral lobe was ligated with 5.0 silk ligature, and the lobe was removed. The two caudate lobes were removed separately with the ligation[243]. Then, the donor livers were flushed and preserved with cold (4 ^{0}C) University of Wisconsin (UW) solution for 1 hour and then implanted to the receptor. Receptors were killed 24 hours after transplantation and blood and liver samples were collected and stored at -20 ^{0}C and -80 ^{0}C respectively for further investigation.

3) Losartan + ROLT (n=12, 6 transplants): We used the same protocol as for group 2, but an AT1R antagonist (losartan) was orally administered (5 mg/kg) at 24 hours and 1 hour before the donor and the recipient surgery [89].

Transaminase assay

Hepatic injury was assessed in terms of transaminase levels with commercial kits from RAL (Barcelona, Spain). Briefly, plasma extracts were collected before liver extraction and centrifuged at 4 °C for 10 min at 0.8xg. Then, 200 µL of the supernatant were added to the substrate provided by the commercial kit. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined at 365 nm with an ultraviolet spectrometer and calculated according to the manufacturer´s instructions [260].

NAD^+/NADH determination

Liver NAD^+ / NADH levels were quantified with a commercially available kit (MAK037, Sigma Chemical, St. Louis, MO, USA) according to the manufacturer's instructions.

Western blot analysis

Liver tissue was homogenized in a HEPES ((N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer as previously described [261]. Then, 50 µg of proteins were separated on 8%–15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and trans-blotted on PVDF (polyvinylidene difluoride) membranes (Bio-Rad). Membranes were then blocked for one hour with 5% (w/v) non-fat milk in T-TBS (tween-tris-buffered saline) and incubated overnight at 4 °C with the corresponding primary antibody: SIRT1 (#07-131), purchased from Merck Millipore, Billerica, MA; ac-FoxO1 (D-19, sc-49437) and GRP78 (GRP78, H-129, sc-13968), both purchased from Santa Cruz Biotechnology Inc, CA, USA); SIRT3 (#2627), cleaved caspase-3 (Asp175, #9664), p-eIF2a (Ser51, #9721), IRE1α (#3294), caspase-12 (#2202), p-p38 Thr180/Tyr182, #9211), p-p44/42 (Erk1/2, Thr202/Tyr204, #9101) purchased from Cell Signaling, Danvers, MA; HSP70 (610607, Transduction Laboratories, Lexington, KY); Heme Oxygenase-1 (H4535), NAMPT (AP22021SU, Acris Antibodies GmbH, Germany); and b-actin (A5316, Sigma Chemical, St. Louis, MO, USA). Membranes were then incubated for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using WesternBright ECL-HRP substrate (Advansta, Barcelona, Spain) and quantified via the Quantity One software for image analysis. Results were expressed as the densitometric ratio between the protein of interest and the loading control (b-actin).

Real-time quantitative reverse-transcription polymerase chain reaction

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed. Total liver RNA was isolated using a TRIzol reagent (Invitrogen). Reverse transcription was realized on a 1 μg RNA sample using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The reaction included incubation at 25 ^{0}C (5 min), at 42 ^{0}C (30 min) and 85 ^{0}C (5 min) and then cDNA was stored at -80 ^{0}C. Subsequent PCR amplification was conducted in an iCycler iQ Multi-Color Real-Time PCR device (Bio-Rad Laboratories) using SsoAdvanced™ Universal SYBR Green Supermix (Bio-rad Laboratories) and the following rat primers for SIRT3: forward, 5′-tagtccagggtgtggaaagg-3′ and reverse, 3′-ccgcaggtgaagaagtaagc-5′. Reactions were performed in duplicate and threshold cycle values were normalized to GAPDH gene expression. The ratio of SIRT3 relative expression to GAPDH was calculated by the ΔCt formula.

Statistical analysis

Data are expressed as mean ± standard error. Statistical comparison was performed by variance analysis, followed by the Student– Newman–Keuls test, using the GraphPad Prism software. $P < 0.05$ was considered statistically significant.

RESULTS

Hepatic injury

We first examined whether treatment with losartan affected hepatic injury in our experimental model. As shown in Table 1, increased ALT and AST levels were observed when rats were submitted to ROLT in comparison with the sham group. However, treatment with losartan significantly reduced the transaminase levels in the ROLT group.

Losartan-induced SIRT1 expression and activity

To investigate the possible interaction of SIRT1 with angiotensin II, we investigated the activity and the protein expression pattern of SIRT1. Animals subjected to ROLT showed augmented SIRT1 protein expression levels, which were further enhanced when losartan was administered (Fig. 1A). In addition, losartan administration prior to the ROLT procedure significantly increased SIRT1 activity compared with both the ROLT and sham groups (Fig. 1B). However, no significant differences were observed between the sham and ROLT groups.

In addition, we examined the levels of NAD^+/NADH, the co-factor necessary for SIRT1 activity and nicotinamide phosphoribosyltransferase (NAMPT) protein expression, which is the major precursor for NAD^+ biosynthesis. Figure 1C demonstrates that NAD+ levels were high in the sham group, but decreased in the ROLT and losartan + ROLT groups; however, losartan pre-

treatment contributed to elevated NAD^+ levels compared with ROLT alone. NAMPT protein was significantly augmented in both the ROLT and losartan + ROLT group in comparison to sham (Fig. 1D).

Further, the forkheadbox (FoxO) transcription factors subfamily have been shown to mediate some of the effects of sirtuins. Given that FoxO1 is a direct substrate of SIRT1, we therefore determined its acetylation (Fig. 1E). Animals subjected to ROLT showed elevated ac-FoxO1 protein levels compared with the sham group. By contrast, the augmented SIRT1 activity found when losartan was administered was consistent with a decrease in the ac-FoxO1 protein levels.

Losartan acted independently of SIRT3 expression

Because SIRT1 appeared to be modulated, we explored the role of SIRT3. We observed that SIRT3 mRNA levels were significantly downregulated in both ROLT and losartan + ROLT groups when compared with the sham group (Fig. 2A). The same pattern was observed for SIRT3 protein levels, with significant decreases in animals subjected to ROLT and losartan + ROLT (Fig. 2B).

Angiotensin II inhibition attenuated ERS

To identify other potential molecular mechanisms involved in the hepatoprotective effect of losartan against IRI, we examined different ERS parameters, including GRP78, IRE1α, and p-eIF2. As indicated in Figure 3, important increases of all ERS parameters occurred following ROLT but not the sham operation. Losartan pre-treatment also restored the ERS parameters.

Losartan affected heat shock protein expression

Because heat shock proteins are implicated in liver IRI, we determined the protein expression pattern of heme homoxygenase 1 (HO-1) and of the heat shock protein 70 (HSP70). As it is shown in Figure 4, enhanced HO-1 and HSP70 protein levels were found in animals subjected to ROLT. However, Losartan treatment decreased HO-1 protein levels and increased HSP70 protein levels.

Angiotensin II inhibition reduced liver apoptosis

Liver IRI is characterized by increased hepatic apoptosis, so we determined the protein levels of caspase-12 and caspase-3, which are known to promote apoptosis. Figure 5 shows that increased levels of both proteins in animals undergoing ROLT were diminished by losartan pre-treatment.

MAPK regulation

The mitogen activated protein kinases (MAPKs) are serine/threonine protein kinases that mediate intracellular signal transduction events associated with IRI. Therefore, we determined the activation of extracellular signal-regulated kinase (ERK) and p38. Figure 6A shows that animals undergoing ROLT had increased levels of p-ERK, but that losartan pre-treatment did not enhance ERK activation compared with ROLT alone. Moreover, the content of p-p38 was decreased in both the ROLT and losartan + ROLT groups. Losartan pre-treatment did not alter p-p38 content when compared to ROLT alone (Fig. 6B).

DISCUSSION

This study demonstrated that inhibition of AT1R lessens hepatic injury in ROLT. Specifically, we provide new insights into losartan-mediated hepatoprotection in rats undergoing ROLT, including the induction of SIRT1 and the attenuation of ERS.

The protective effects of losartan against IRI were associated with increased SIRT1 activation and protein expression. SIRT1 up-regulation and angiotensin II blockade have been separately reported as therapeutic strategies against IRI in various organs [90, 252, 262, 263]. Enhancement of SIRT1 has also been associated with decreased hepatic injury in rat orthotopic liver transplantation [264]. In our experimental rat ROLT model, SIRT1 protein expression was upregulated, but we observed no differences in its activity. Furthermore, FoxO1 deacetylation was inhibited in the ROLT group. SIRT1 overexpression and failure to augment its activity during IRI has also been reported in a recent work by our group [261]. In addition, losartan administration not only enhanced SIRT1 expression but also significantly increased both SIRT1 activity and FoxO1 deacetylation in comparison with the ROLT group. Further, losartan-induced increases in SIRT1 activity can be attributed to the enhanced NAD^+ levels, which are indispensable for sirtuin activity. In turn, the NAD+ levels may be attributed to the NAMPT levels, which were slightly, but not significantly, increased after losartan treatment. Moreover, enhanced deacetylation of FoxO1 was related with NAMPT/NAD^+ increases in rat orthotopic liver transplantation [264].The present data demonstrate the existence of an angiotensin II/SIRT1 axis in liver transplantation, and that the benefits of angiotensin II inhibition against liver IRI are mediated, at least in part, through SIRT1 activation. This is consistent with a recent study in rat skeletal muscle, in which angiotensin II administration decreased SIRT1 expression [265].

Next, we speculated that SIRT3 might be affected by ROLT and losartan treatment. Real-time qRT-PCR and Western blot analysis revealed that SIRT3 mRNA and protein levels were significantly decreased in both the ROLT and losartan + ROLT groups compared with the sham group. This may be attributed to the mitochondrial disturbances that commonly take place

during IRI [204]. SIRT3 is the major mitochondrial deacetylase implicated in metabolism, oxidative stress responses, and cardiac IRI [195, 253, 266, 267]. The fact that SIRT3 mRNA and protein levels were comparable between the ROLT and losartan + ROLT groups suggests that the protective effect of losartan was independent of the SIRT3 pathway.

The endoplasmic reticulum is an organelle responsible for protein folding. Under stress conditions, the homeostasis of the endoplasmic reticulum is disturbed, leading to accumulation of unfolded proteins. In this case, an adaptive unfolded protein response (UPR) is activated to lessen the effects of ERS; however, when the insult is exaggerated in IRI, the ERS response can lead to cell death [73]. The UPR has three core branches: an inositol-requiring enzyme 1α (IRE1α) that induces the cleavage of the mRNA encoding X-box-binding protein 1 (XBP-1); a PKR-like endoplasmic reticulum kinase (PERK) that phosphorylates the eukaryotic translation initiation factor 2 (eIF2a); and an activating transcription factor (ATF6). Under stress conditions, IRE1α, PERK, and ATF6 are released from their binding with the 78-kD glucose-regulated/binding immunoglobulin protein (GRP78) and become activated [268]. In a liver transplantation model, we have previously seen that activation of these UPR branches is associated with cell death and is a determinant factor of liver injury [76]. In this study, we observed that ROLT triggered the activation of GRP78 and the subsequent activation of the IRE1α and p-eIF2 pathways. Moreover, losartan pre-treatment abolished the activation of all ERS parameters. This is consistent with a recent study in human islets, which revealed that losartan exerted its protective effects against glucotoxicity by reducing ERS [269].

Losartan treatment was also accompanied by significant regulation of HSP70 and HO-1. The chaperone activity of HSP70 has been associated with cellular attempts to maintain proteins in an accurately folded state [73]. In our study, losartan pre-treatment induced HSP70 overexpression, which could have contributed to a decreased accumulation of unfolded proteins and therefore less ERS. Furthermore, because a direct relationship has previously been reported between SIRT1 and HSP70 in hepatic IRI, SIRT1 might contribute to HSP70 enhancement [261]. The increased ERS levels observed in the ROLT group were consistent with enhanced HO-1 protein expression that probably occurred due to an adaptive cell mechanism to prevent stress, as previously proposed by Liu *et al.* [270]. In this sense, HO-1 expression was decreased when losartan pre-treatment diminished ERS.

Apoptosis is one of the most significant events in the pathophysiology of liver IRI. Aiming to mitigate the effects of ERS-mediated apoptosis could be an effective strategy for minimize IRI. It is known that IRE1α provokes caspase 12 cleavage, which in turn activates caspase 9 and then caspase 3 to stimulate apoptosis [271, 272]. In our study, the induction of ERS in the ROLT group led to increased cell death, as reflected by the enhanced caspase 12 and caspase 3 protein

levels. Further, the decrease in ERS in the losartan + ROLT group coincided with decreases in the levels of these caspases.

MAPKs are linked with cell cycle, liver regeneration, apoptosis, and oxidative stress pathways. The ERK cascade is closely connected with the regulation of cell growth and differentiation, whereas p38 is involved in cellular responses to environmental stress [112]. It has been reported that active p38 MAPK is present in the quiescent liver, and that it is dephosphorylated in the regenerating liver [273, 274]. ERK phosphorylation is also involved in the signaling pathways of liver regeneration [19]. Therefore, the lowered p-p38 and increased p-ERK levels observed in the ROLT and losartan + ROLT groups could be associated with enhanced liver regeneration. In a previous study, our group reported that losartan pre-treatment did not enhance liver regeneration after ROLT [275]. Thus, losartan pre-treatment did not provide an additional increase in liver regeneration, resulting in no differences in p-p38/ERK activation between the two ROLT groups. Consequently, we can assume that SIRT1 activation by losartan treatment is not associated with liver regeneration in a ROLT model. Losartan administration decreased significantly hepatic injury and affected signaling processes related to IRI, such ERS and apoptosis. However, it could not further enhance liver regeneration, an essential processes for the success of transplantation with reduced-size liver grafts. Further studies will be required to elucidate the mechanisms by which losartan improves hepatic injury after ROLT.

Furthermore, angiotensin II is known to exert vasoconstrictor effects [276-278] and angiotensin II blockers, such as losartan, have been reported to decrease arterial pressure and act as effective antihypertensive agents [279, 280]. A potential hypotensive effect of losartan was out of the scope of the present study, whereas prolonged time treatments with losartan are usually applied in order to evaluate blood pressure changes [281].

In conclusion, the present results indicate that SIRT1 is implicated in the protective effects of AT1R inhibition by losartan against IRI following ROLT. Losartan pre-treatment markedly attenuates liver injury by regulating signaling pathways that are involved in the pathophysiology of IRI, including heat shock protein, ERS, and liver apoptosis pathways. Moreover, it is evidenced that SIRT1 is a downstream target of angiotensin II in a rat ROLT model. Further studies are required to identify whether other angiotensin peptides (i.e., 1–7) can also modulate SIRT1.

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COMMENTS

Background

Ischemia-reperfusion injury (IRI) is a complex pathophysiological process inherent to liver transplantation. Endoplasmic reticulum stress (ERS) and apoptosis are common features of liver IRI in this context. Angiotensin II is a basic constituent of the renin-angiotensin system and has been shown to worsen IRI. Angiotensin II acts by binding to angiotensin II type I receptors (AT1R) and angiotensin II type II receptors (AT2R). Of note, antagonists of these receptors have been found to protect against liver IRI. In addition, sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that modulates various cellular pathways associated to IRI, but its relationship with angiotensin II in liver IRI has not been studied. In this study, we demonstrate that administration of losartan, an antagonist of AT1R, significantly reduced liver injury in a rat model of reduced-size orthotopic liver transplantation (ROLT) by activating SIRT1 and decreasing ERS and liver apoptosis.

Research frontiers

Angiotensin II has been associated with inflammatory responses and oxidative stress in liver IRI. Inhibition of its action with AT1R antagonists, such as losartan, results in decreased hepatic injury by attenuating pro-inflammatory responses, activating HIF-1a and peroxisome proliferator-activated receptor gamma (PPAR-γ) in various hepatic IRI models. Here we report that the hepatoprotective effects of losartan against IRI associated with ROLT are mediated through SIRT1 enhancement, HSP70 overexpression, and attenuation of ERS and liver apoptosis.

Innovations and breakthroughs

The role of SIRT1 in a ROLT model has not yet been determined, nor has the potential link between angiotensin II and SIRT1 or ERS in liver IRI. The present study evaluated the potential role of losartan administration on SIRT1 expression and activity and on ERS activation in a rat ROLT model. We demonstrated that angiotensin II inhibition led to SIRT1 up-regulation and a subsequent decrease in ERS that contributed to reduced hepatic injury following ROLT.

Applications

Pharmacological activation of SIRT1 by losartan might be a promising therapeutic tool for ameliorating the detrimental effects of IRI following ROLT in rat models.

Peer review

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FIGURE LEGENDS

Table 1: Effect of losartan administration in liver injury after ROLT. Alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST) in plasma after 24h of reperfusion. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham, #P<0.05 vs. ROLT

Figure 1: Effect of losartan treatment in SIRT1 protein expression and SIRT1 activity parameters. (A) SIRT1 protein expression, (B) SIRT1 activity, (C) NAD+/NADH levels, (D) NAMPT and (E) ac-FoxO1 protein expression in livers after 24 hours of reperfusion. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham, #P<0.05 vs. ROLT

Figure 2: Implication of losartan administration in (A) mRNA and (B) protein levels of SIRT3. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: Same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham

Figure 3: Role of losartan pretreatment in endoplasmic reticulum stress parameters. (A) GRP78, (B) IRE1a, (C) p-eIf2 protein levels. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham, #P<0.05 vs. ROLT

Figure 4: losartan administration regulates heat shock proteins expression. (A) HO-1 and (B) HSP70 protein levels. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: Same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham, #P<0.05 vs. ROLT

Figure 5: Liver apoptosis in ROLT after losartan treatment. Protein levels of (A) Caspase 12 and (B) Cleaved Caspase 3. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University

of Wisconsin solution; losartan + ROLT: Same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham, #P<0.05 vs. ROLT

Figure 6: MAP kinases modulation by losartan administration. Effect of losartan in (A) p-ERK and (B) p-p38 protein expression. Sham: liver harvested without transplantation, ROLT: liver subjected to reduced-size orthotopic liver transplantation after 1h of cold storage in University of Wisconsin solution; losartan + ROLT: same as ROLT group, but with further administration of losartan 24 hours and 1 hour before the surgical procedure to both the donor and the recipient. *P<0.05 vs. Sham

FIGURES

	sham	ROLT	Losartan + ROLT
ALT (U/L)	48,8 ± 2,58	358,3 ± 133,44 *	206 ± 33,61*,#
AST (U/L)	88,2 ± 4,65	893,57 ± 397,69*	500,85 ± 118,07*,#

Table 1

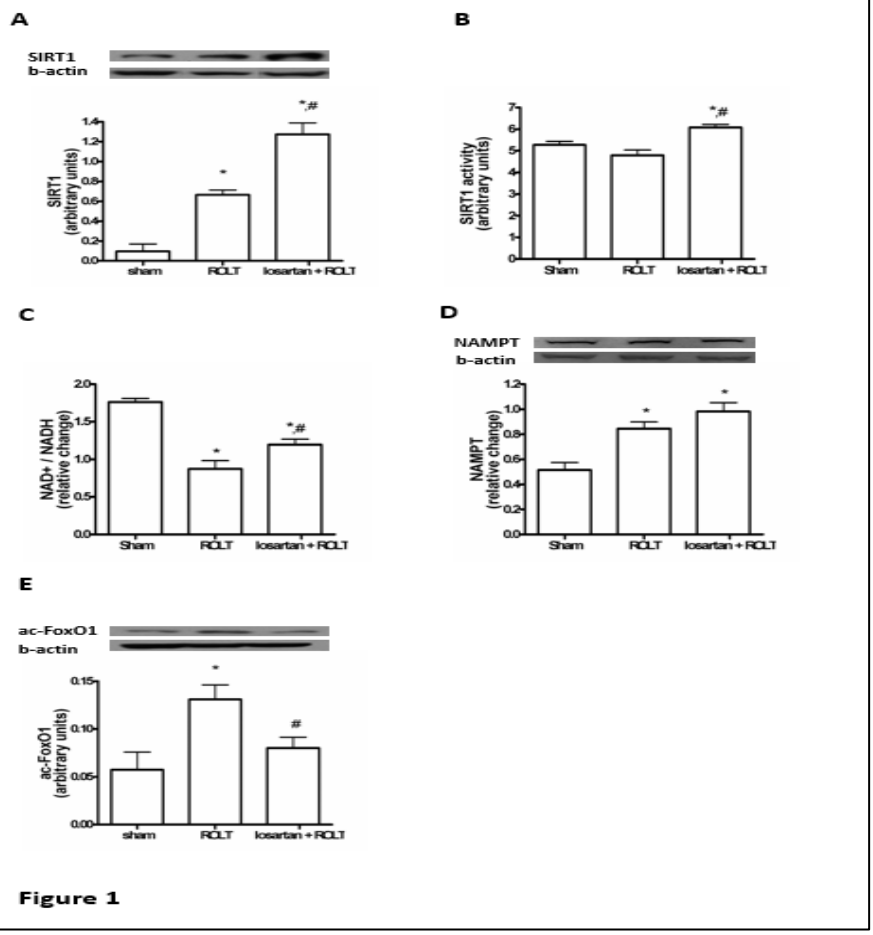

Figure 1

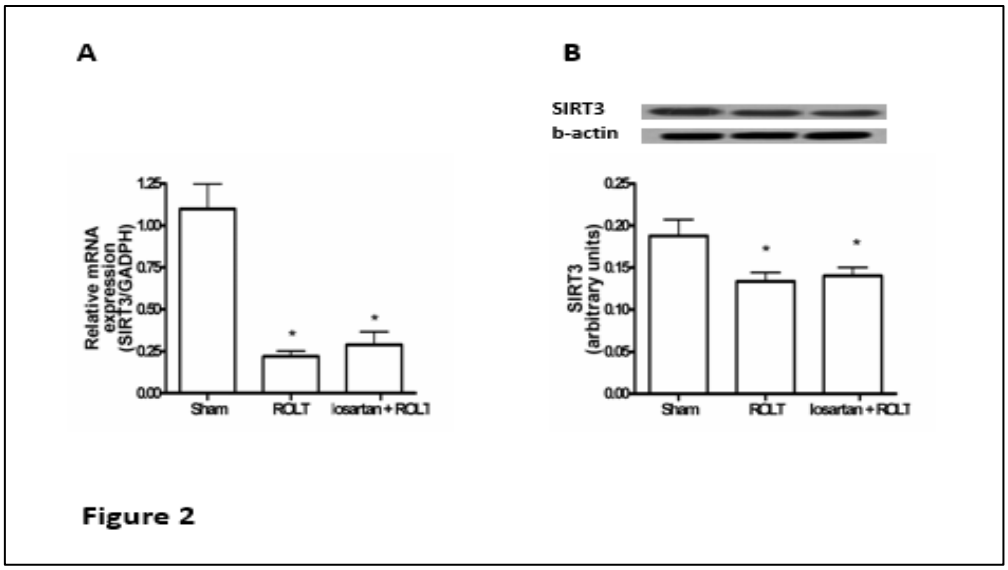

Figure 2

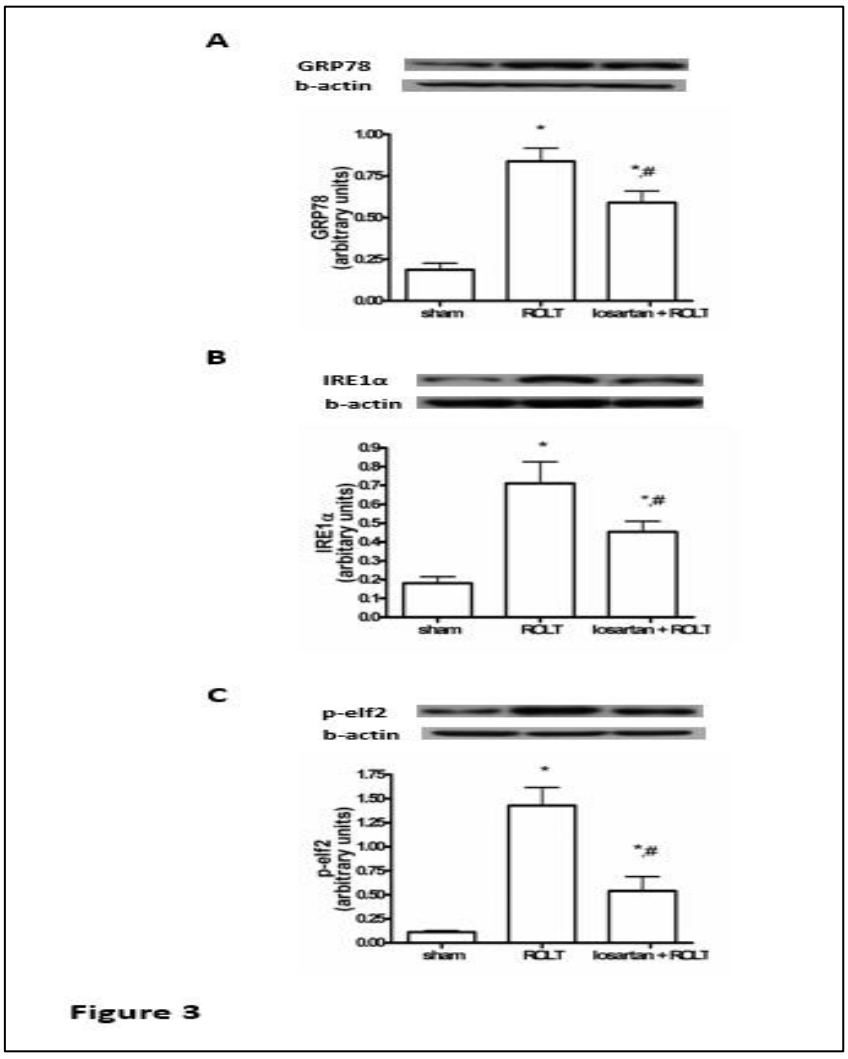

Figure 3

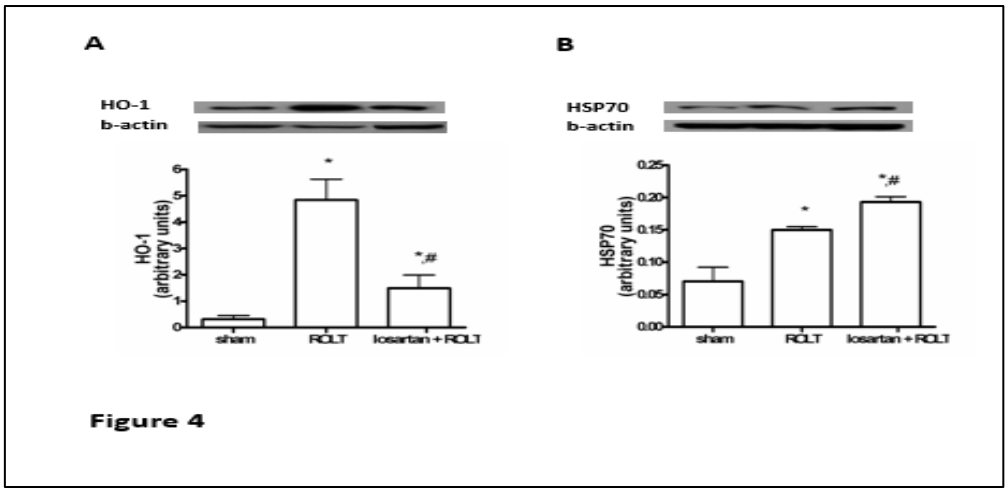

Figure 4

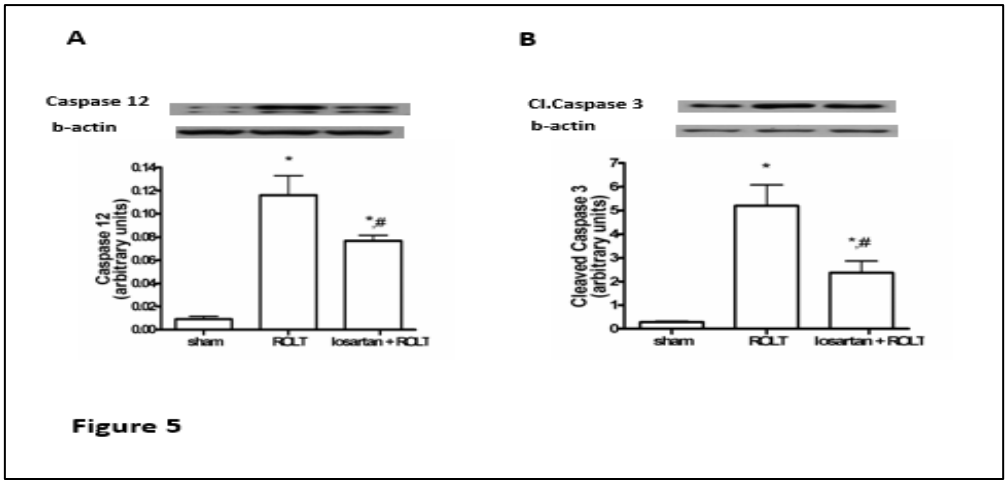

Figure 5

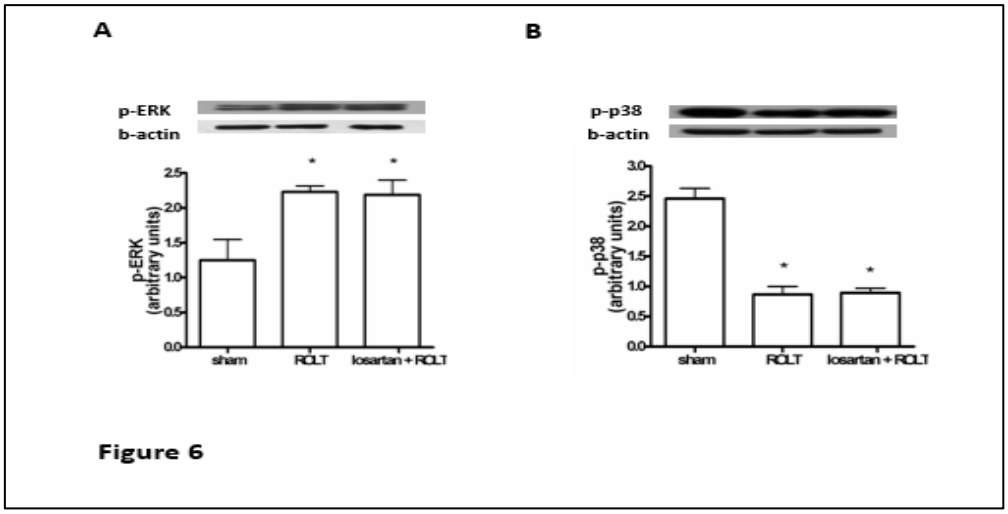

Figure 6

5. DISCUSSION

5. DISCUSSION

A wide range of pathological processes contribute to IRI. Particularly during organ transplantation, IRI contributes to early graft dysfunction. For this reason, it is important to gain additional mechanistic insight into the molecular mechanisms underlying this injury. Recently, SIRT1 has emerged as a critical modulator of various cellular processes, including those that contribute to the pathogenesis of IRI. Considering the poor evidence of SIRT1 in liver IRI and organ transplantation, here we aimed to identify the potential impact of SIRT1 modulation in various situations of liver IRI, including PC, IRI associated with rat OLT or with ROLT.

5.1. Ischemic preconditioning

In the first study, we explored the role of SIRT1 after 1-hour of warm partial ischemia (70%) followed by 24 hours of reperfusion in steatotic livers. In fact, hepatic steatosis is a major risk factor after liver surgery because steatotic livers show poor tolerance to IRI [282]. Moreover, it has been reported that operative mortality associated with steatosis exceeds 14% after major resection compared to 2% for healthy livers [283, 284]. Therefore, developing protective strategies to minimize the adverse effects of IRI in steatotic livers is an urgent need. In our study, we observed that SIRT1 expression is significantly enhanced after 1 hour of ischemia followed by 24 hours of reperfusion. However, neither its deacetylase activity nor the levels of acetylated p53 have been altered. Various factors seem to be able to modify the activity of the histone deacetylases, including SIRT1, which are not fully clarified. For example, in a similar model of mice warm IRI, it has been reported that the liver histone deacetylase activity decreases after 1 hour of ischemia and after 1 hour of ischemia followed by 1 hour reperfusion. However, it remains unchanged after prolonged times of reperfusion [285]. Thus, we can suggest that the duration of ischemia and reperfusion can affect the activity of histone deacetylases and the conditions of our model (24 hours of reperfusion) cannot favor SIRT1 deacetylation, although the protein levels of SIRT1 are significantly enhanced. Various studies in heart have mentioned the up-regulation of SIRT1 protein expression in various conditions of stress, like oxidative stress, aging and hypoxia/reoxygenation which has been

correlated with protection against damage [186, 286] . Thus, in our case we can suggest that enhancement of SIRT1 protein expression could act as a self-compensatory mechanism in order to provide resistance against injury.

As PC is a surgical strategy applied to diminish hepatic IRI, we sought to investigate whether SIRT1 is involved in the protective effect of PC after warm ischemia-reperfusion. For this reason, not only we assessed PC prior to ischemia reperfusion, but also we administrated SIRT1 inhibitors, either sirtinol or EX527, prior to PC. Significant increases in SIRT1 deacetylase activity and expression, as well as decreased ac-p53 levels were evident in PC group, which were abolished when SIRT1 inhibitors were applied. Further, application of SIRT1 inhibitors was associated with increased liver injury in comparison to PC group. . Thus, we demonstrated that enhancement of SIRT1 activity is an additional molecular mechanism by which PC diminishes hepatic IRI in fatty livers. However, it remains to be elucidated how PC promotes the deacetylase activity of SIRT1.

Then, we examined the possible mechanisms by which SIRT1 could exert its beneficial effect in PC. PC provoked eNOS and AMPK activation, which were reversed by SIRT1 inhibition. AMPK activation is responsible for maintenance of energy levels during sustained ischemia and eNOS activation contributes to alleviating the microcirculation disturbances which are more exacerbated in fatty livers [110] [103]. Consequently, our study demonstrates that AMPK and eNOS activation are mediated by SIRT1 in fatty liver PC. SIRT1 may activate AMPK through deacetylation of serine-threonine liver kinase B1 (LKB1), provoking its translocation from nucleus to cytoplasm, where it enhances the phosphorylation of Thr172 on the α-subunit of AMPK [287]. Furthermore, it has been shown that SIRT1 and eNOS colocalize in endothelial cells, SIRT1 deacetylates eNOS and thus promotes eNOS activity, which results in enhanced production of NO [213]. Besides this, it has been shown that AMPK can augment SIRT1 activity by augmenting NAD^+/NADH levels and that eNOS activation has been correlated with SIRT1 increases, providing thus evidence of the existence of a strong correlation between SIRT1-AMPK and SIRT1-eNOS [211, 287, 288]. In addition, eNOS activation by AMPK during PC has also been described in rat LT [110]. In our

case, it has been demonstrated that SIRT1 is upstream of AMPK and eNOS in hepatic PC.

Production of ROS and oxidative stress consist the most important hallmarks of reperfusion injury. In addition, steatotic livers are more vulnerable to oxidative stress due to their reduced antioxidant defenses and augmented generation of ROS [289]. In our study, SIRT1 overexpression and activity during fatty liver PC was associated with diminished oxidative stress. This agrees with previous published studies that have described the effect of SIRT1 against oxidative stress; SIRT1 can deacetylate the FoxO factors, like FoxO1 and subsequently promote their transcriptional activity, resulting in the expression of antioxidants enzymes, like catalase, MnSOD and Trx [188] [290].

In addition, SIRT1 has also been correlated with HSPs; we have shown that inhibition of SIRT1 abolished the HSP70 expression increases during PC. HSP70 gene expression is regulated by the transcription factor heat shock factor 1 (HSF1). Normally, HSF1 is localized in the cytoplasm, but in presence of stress, like ischemia or hypoxia, HSF1 is phosphorylated and translocates to nucleus in order to induce HSP70 transcription [291]. Besides phosphorylation, recently it has been shown that HSF1 transciption activity can also be enhanced by SIRT1 deacetylation [292]. In this way, SIRT1 could favor HSP70 expression and contribute to increase the tolerance of the liver against IRI.

High oxidative stress environments, such as those occuring during ischemia-reperfusion, stimulate p38 activation. Phosphorylated p38 has been shown to initiate inflammatory pathways and administration of p38 inhibitor in steatotic livers subjected to ischemia-reperfusion attenuated liver injury [115] [116]. In our case, PC through SIRT1 activation decreased p38 activation, attenuating thus its detrimental effects. In addition to p38, ERK ½ is another MAPK involved in IRI, mainly with anti-apoptotic effect [293]. Activation of ERK was observed after ischemia-reperfusion, which was enhanced when PC was applied. SIRT1 inhibition abolished ERK activation during PC, demonstrating thus that SIRT1 regulates MAPKs activation during PC. However, the exact mechanism by which SIRT1 affects MAPKs has not yet been clarified.

The anti-apoptotic effect of ERK during PC was confirmed by the decreased anti-apoptotic markers CytC, Caspase 9 and Caspase 3. SIRT1 inhibition resulted in augmented liver apoptosis, providing thus evidence that SIRT1 contributes to the anti-apoptotic effect of hepatic PC. This fact agrees with various reports, where SIRT1 downregulates apoptosis and protects against IRI, through p53 inhibition [245, 294] [188].

In our attempt to investigate the role of SIRT1 in liver PC, we administrated separately two SIRT1 inhibitors, sirtinol and EX527, where EX527 has been shown to be a more specific inhibitor for SIRT1 [295]. In addition, it has been reported that sirtinol can also inhibit human SIRT2 activity in vitro [296] and that inhibition of SIRT2 has been found to be protective [297] [298]. Consequently, the fact that sirtinol treatment failed to reduce completely the protective effect of PC in hepatic injury might be attributed to its combined effect on SIRT2 (potential protective effect) and SIRT1 (detrimental effect). Moreover, the fact that sirtinol administration in a sham group did not provoke any diference in transaminase levels when compared to sham group, discard any other potential positive effect of sirtinol. In addition, the fact that both inhibitors partially reversed the impact of PC in MAPK kinases and apoptosis implies that additional mechanisms may be involved in the beneficial effects exerted by SIRT1 in fatty liver PC.

5.2. Transplantation models

As we observed that SIRT1 is implicated in the warm liver IRI, we then speculated to investigate the possible SIRT1 alterations in models of transplantation. We observed that in both OLT and ROLT, SIRT1 protein expression is upregulated in comparison to sham group. Besides this, in both cases, the acetylation of FoxO1 was increased, suggesting that SIRT1 deacetylase activity is attenuated. However, measurement of deacetylase activity in the ROLT model showed no changes in SIRT1 activity. These results are similar to those obtained in warm ischemia-reperfusion, providing thus evidence that either warm or cold ischemia-reperfusion result in enhancement of SIRT1 protein expression, but not in activity.

5.2.1. Orthotopic liver transplantation

Our study of SIRT1 implication in OLT was also associated with our examination of whether an IGL-1 preservation solution enriched with TMZ could enhance liver graft viability. First of all, we observed that SIRT1 protein expression levels were significantly augmented in IGL-1+TMZ group in comparison to both untreated and IGL-1 preserved livers. Further, TMZ addition in IGL-1 solution in OLT enhanced SIRT1 activity, as evidenced by significant decreases of both acetylated FoxO1 and p53. TMZ can enhance SIRT1 activity by stimulating NAMPT protein expression and consequently augmenting NAD^+ levels; NAMPT constitutes an important step in NAD^+ biosynthesis from nicotinamide and NAD^+ is essential for SIRT1 activity. TMZ protective effects against IRI have been associated with better preservation of mitochondria and energy metabolism and diminution of oxidative stress and microcirculation disturbances in an "ex-vivo" liver perfusion model [299]. In our case, TMZ addition in the preservation solution diminished mitochondrial damage and oxidative stress and was concomitant with SIRT1 activation; SIRT1 deacetylated FoxO1 and in this way might enhance anti-oxidant mechanisms. We also demonstrated that TMZ-mediated SIRT1 activation was correlated with stress-related signaling pathways, such as increases in HSP70 and p-ERK protein expression and decreases in p38 activation. Besides this, in order to verify the above effects of SIRT1 in OLT, the addition of a SIRT1 inhibitor/activator should be provided in a future study.

In our study, we related the decreased oxidative stress and the FoxO1 deacetylation with the activation of autophagy. It has been described that SIRT1 can promote autophagy by deacetylating and thus activating FoxO1, which results to increased expression of autophagy-related genes [300]. Autophagy is closely linked with oxidative stress, impaired mitochondrial function and accumulation of protein aggregates [301]. Autophagy can be a way for the elimination of mitochondria that release ROS and apoptotic factors, preventing thus the damage to neighboring mitochondria and the entire cell [302]. Thus, we could suppose that autophagy enhancement due to the presence of TMZ could contribute to decrease the detrimental effects of oxidative stress and maintain cellular homeostasis. Moreover, the addition of an autophagy inhibitor and the study of its potential effect on oxidative

stress and mitochondrial integrity could be essential to clarify the relationship between autophagy and oxidative stress in OLT. Furthermore, as the role of autophagy in the phase of reperfusion remains controversial and the duration of ischemia appears to be a determinant factor for autophagy outcome [303], future studies could examine various times of ischemia and its relationship with the autophagy. In addition, as activation of autophagy can be a cell response for the elimination of unfolded proteins and accumulation of unfolded proteins can provoke ERS [304], it could also be interesting to explore whether autophagy activation in TMZ-mediated activation of SIRT1 was concomitant with ERS decreases.

5.2.2. Reduced size orthotopic liver transplantation

In the third study of our work, we aimed to investigate whether SIRT1 is altered in a model of ROLT, as well as its potential link with angiotensin II and ERS. Various stimuli, such as ischemia, can potentially cause ER dysfunction/stress, which is characterized by a marked up-regulation of ER chaperones such as GRP78. When ERS is excess and/or prolonged, the initiation of the apoptotic processes is promoted by the activation of caspase-12–dependent pathways; Caspase-12 is located on the ER membrane and is activated only by ER stress [305, 306]. In our study, we observed that inhibition of AT1R resulted in attenuation of ERS and liver apoptosis. This fact agrees with other studies in pancreas and heart demonstrating that the administration of losartan or others AT1R antagonists protect against ERS [307] [269, 308]. Although the intracellular signaling pathway by which angiotensin II induces ERS is not fully elucidated, it has been supposed that AngII might enhance protein synthesis [305]. In our study, we observed that Losartan induced a significant increase of SIRT1 protein expression and activity, and taking into account that SIRT1 has been associated with ERS [309] [254], we can suppose that SIRT1 activation might be an intermediate mechanism between angiotensin II and ER. In addition, SIRT1 could also contribute to diminished apoptosis, as the anti-apoptotic effect of SIRT1 has been demonstrated in our first study related to PC. Besides this, the unchanged mRNA and protein levels of SIRT3 between the animals submitted to ROLT and those pretreated with Losartan prior to ROLT revealed that SIRT3 is not involved in Losartan protective effects.

It is well known that HSPs play essential roles as molecular chaperones facilitating the folding and the intercellular transport of cellular proteins [310]. In addition, due to the fact that various stressful stimuli, such as IRI, increase the intercellular synthesis of HSPs, we then speculated whether HSP70 and HO-1 protein expression could be modified in our model. Indeed, the decreased ERS in case of losartan + ROLT group was concomitant with increased HSP70 protein levels, suggesting thus that HSP70 may contribute to the attenuation of misfolded proteins and thus to reduced ERS. Further, similarly to our previous studies in PC and OLT, HSP70 up-regulation corroborated with SIRT1 increased activity. On the other hand, HO-1 protein levels were enhanced in case of ROLT group, but decreased significantly after losartan treatment. As HO-1 expression has been reported to be attenuated upon stress decreases [311], we may suppose that in case of ROLT, HO-1 levels augmented in a cell attempt to enhance cytoprotective mechanisms and diminish injury, whereas the losartan protective action abolished its activation.

We next investigated whether SIRT1 up-regulation by losartan is concomitant with MAPKs alterations, as occurred in the previous studied models. We observed that pERK and p-p38 protein expression is enhanced and decreased respectively in the same way in both transplant groups. Although MAPKs have been mainly associated with stress responses and apoptosis, they also participate in the intracellular kinase cascades that drive cell cycle progression during liver regeneration. ERK phosphorylation has been clearly shown to lead to DNA replication and promote hepatocyte proliferation [19]. On the other hand, it still remains controversial the effect and the timing of p38 activation [274, 312, 313]. Between the various studies, it has been proposed that p38 is dephosphorylated in the regenerating liver [273]. Moreover, in the same experimental conditions, we have previously observed that losartan does not further enhance liver regeneration when compared to ROLT group [275]. Consequently, we can assume that MAPKs are not regulated in our model, as liver regeneration is not altered. This fact could also lead us to the conclusion that SIRT1 is not related with hepatic regeneration in a ROLT model.

5.3. New perspectives

The present tesis demonstrated that SIRT1 is implicated in the pathophysiology of hepatic IRI and surgical or pharmacological strategies able to enhance its activity can be effective strategies to mitigate the IRI detrimental effects. Besides this, there is poor evidence in the literature concerning the potential role of other members of sirtuins family in IRI. Between them, SIRT3 could be an ideal candidate to explore in future studies, taking into account its correlation with the anti-oxidative defense mechanisms of mitochondria. Indeed, it has been recently reported that deficiency of SIRT3 augments the susceptibility of heart to IRI [253]. Thus, it remains to be proved whether a similar effect can be achieved in liver, as well as the potential underlying mechanisms.

One of the most remarkable foundings demonstrated here is that SIRT1 contributes to increase the resistance of steatotic livers towards IRI. Steatotic livers have been the main point of various investigations, due to the increased rate of obesity in our days, as well as to the shortage of organs for transplantation. For this reason, it is an urgent need the development of strategies that modulate fatty acid metabolism and thus contribute to decrease the susceptibility of steatotic livers towards IRI. In this context, activating SIRT1 and SIRT3 might be a potential strategy to be explored. It has been reported that deletion of hepatic SIRT1 resulted in hepatic steatosis, hepatic inflammation and endoplasmatic reticulum stress [314]. The relevance of SIRT3 in the hepatic metabolism has also been confirmed in a study showing that its overexpression in hepatocytes decreased the accumulation of lipids via AMPK activation [315]. Thus, it would be interesting to examine the role of SIRT1 and SIRT3 in the context of steatotic LT.

6. CONCLUSIONS

6. Conclusions

The conclusions of the present thesis are the following:

- SIRT1 is involved in the protective effects of PC in fatty livers against IRI. SIRT1 activity is enhanced during PC and contributes to increase the activation of eNOS and AMPK, to enhanced HSP70 and p-ERK protein expression and to decreases in p-p38 expression, oxidative stress and liver apoptosis.

- SIRT1 is implicated in OLT, as its enhanced activity has been associated with the beneficial mechanisms of TMZ addition to IGL-1 preservation solution. These include the decreases in mitochondrial damage and oxidative stress, the activation of AMPK, ERK and autophagy and the p-p38 decreases

- SIRT1 is a downstream target of AngII and forms part of the hepato-protective mechanisms exerted by losartan administration in ROLT. SIRT1 enhanced activity by losartan was associated with increased HSP70 expression, decreases in ERS and HO-1 protein expression and in liver apoptosis.

7. BIBLIOGRAPHY

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8. ANNEX

Here there are reported the publications related directly or indirectly with the present thesis:

- Role of sirtuins in ischemia-reperfusion injury. Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Roselló-Catafau J. World J Gastroenterol. 2013;19(43):7594-602. doi: 10.3748/wjg.v19.i43.7594. Review
- Advances in treatment strategies for ischemia reperfusion injury. Eirini Pantazi, Mohamed Bejaoui, Emma-Folch-Puy, René Adam and Joan Roselló-Catafau. Submitted to Expert Opinion on Pharmacotherapy
- Emerging concepts in liver graft preservation. Bejaoui M, Pantazi E, Folch-Puy E, Baptista PM, García-Gil A, Adam R, Roselló-Catafau J. World J Gastroenterol. 2015 Jan 14;21(2):396-407. doi: 10.3748/wjg.v21.i2.396.
- Polyethylene glycol rinse solution: an effective way to prevent ischemia-reperfusion injury. Zaouali MA, Bejaoui M, Calvo M, Folch-Puy E, Pantazi E, Pasut G, Rimola A, Ben Abdennebi H, Adam R, Roselló-Catafau J. World J Gastroenterol. 2014 Nov 21;20(43):16203-14. doi: 10.3748/wjg.v20.i43.16203.
- Bortezomib enhances fatty liver preservation in Institut George Lopez-1 solution through adenosine monophosphate activated protein kinase and Akt/mTOR pathways. Bejaoui M, Zaouali MA, Folch-Puy E, Pantazi E, Bardag-Gorce F, Carbonell T, Oliva J, Rimola A, Abdennebi HB, Roselló-Catafau J. J Pharm Pharmacol. 2014 Jan;66(1):62-72. doi: 10.1111/jphp.12154.
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REVIEW

Role of sirtuins in ischemia-reperfusion injury

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Abstract

Ischemia-reperfusion injury (IRI) remains an unresolved and complicated situation in clinical practice, especially in the case of organ transplantation. Several factors contribute to its complexity; the depletion of energy during ischemia and the induction of oxidative stress during reperfusion initiate a cascade of pathways that lead to cell death and finally to severe organ injury. Recently, the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases has gained increasing attention from researchers, due to their involvement in the modulation of a wide variety of cellular functions. There are seven mammalian sirtuins and, among them, the nuclear/cytoplasmic sirtuin 1 (SIRT1) and the mitochondrial sirtuin 3 (SIRT3) are ubiquitously expressed in many tissue types. Sirtuins are known to play major roles in protecting against cellular stress and in controlling metabolic pathways, which are key processes during IRI. In this review, we mainly focus on SIRT1 and SIRT3 and examine their role in modulating pathways against energy depletion during ischemia and their involvement in oxidative stress, apoptosis, microcirculatory stress and inflammation during reperfusion. We present evidence of the beneficial effects of sirtuins against IRI and emphasize the importance of developing new strategies by enhancing their action.

Key words: Sirtuin 1; Sirtuin 3; Ischemia-reperfusion injury; Oxidative stress; Apoptosis

Core tip: Sirtuins are responsible for the regulation of protein activation by deacetylating a range of proteins that play important roles in the pathophysiology of various diseases. The present review summarizes the beneficial effects of sirtuins 1 and 3, the two most prominent sirtuins involved in mammalian energy homeostasis and oxidative stress. We conclude that both sirtuins might be attractive targets for counteracting the detrimental effects of ischemia-reperfusion injury.

Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Roselló-Catafau J. Role of sirtuins in ischemia-reperfusion injury. *World J Gastroenterol* 2013; 19(43): 7594-7602 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7594.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7594

INTRODUCTION

Sirtuins belong to the highly conserved class III histone

deacetylases with homology to the yeast silent information regulator 2. To date, seven sirtuins have been described in mammals. They posses nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase activity, with the exception of sirtuin 4 (SIRT4) which has only ADP-ribosyltransferase activity, and SIRT1 and SIRT6 which have not only deacetylase activity but also relatively weak ADP-ribosyltransferase activity[1]. Their enzymatic activity depends on their protein expression levels, the availability of NAD^+ and the presence of proteins that modulate sirtuin enzymatic activity. For instance, SIRT1 expression increases during starvation or when cells are exposed to conditions of oxidative stress and DNA damage[2,3].

Sirtuins are found in several subcellular locations, including the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2), and mitochondria (SIRT3-SIRT5). In some studies, however, SIRT1 has been found to possess cytosolic activity, and SIRT2 has been found to be associated with nuclear proteins[4].

Several recent studies have shown that sirtuins regulate a wide variety of cellular processes, such as gene transcription, metabolism and cellular stress response[5-7]. SIRT1, the most studied member of the family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis[8,9]. SIRT3 has recently been reported to have a considerable impact on mitochondrial energy metabolism and function[10,11]. In this review, we will focus mainly on SIRT1 and SIRT3 functions in ischemia-reperfusion injury (IRI).

IRI is one of the most significant problems in graft injury, contributing to primary graft dysfunction or nonfunction after organ transplantation[12-14]. Many factors contribute to IRI. First of all, the loss of oxygen supply during ischemia results in the reduction of adenosine triphosphate (ATP) synthesis and subsequent changes in ion influx, acidosis and cell swelling which may eventually lead to cell death. The restoration of blood flow is followed by an excessive acute inflammatory response triggering the reperfusion injury. Although the ischemic insult causes significant damage in cells, the tissue injury generated during reperfusion is much more severe. On reperfusion, oxygen is suddenly available, and metabolism proceeds rapidly, resulting in a sudden production of reactive oxygen species (ROS), cytokines and chemokines which increase the accumulation of inflammatory cells (monocytes, dendritic cells and granulocytes). In combination with excessive nitric oxide (NO), ROS are able to induce DNA damage and activate various types of cell death pathways[15-17].

Understanding the mechanisms involved in the pathogenesis of IRI is the first step to mitigate its adverse effects. Sirtuins are known to regulate many important processes in cell physiology, including those affecting IRI, such as cellular metabolism and stress response. This makes them potentially appealing targets for therapeutic interventions against IR-induced injury.

ROLE OF SIRTUINS IN ISCHEMIA

The low energy state during ischemia results in activation of adenosine monophosphate protein kinase (AMPK), a fuel-sensing enzyme that is positively regulated by an increased ratio of adenosine monophosphate to ATP. When AMPK is activated, it stimulates processes that restore ATP levels (*e.g.*, fatty acid oxidation) and inhibits other processes that consume ATP (*e.g.*, protein synthesis)[18]. The activity of sirtuins is directly related to the metabolic state of the cell due to their dependence on NAD^+. Suchankova and collaborators found that glucose-induced changes in AMPK are linked to alterations in the NAD^+/reduced nicotinamide adenine dinucleotide ratio and SIRT1 abundance and activity[19]. These results may suggest a possible interaction between AMPK and SIRT1 in ischemic conditions. Indeed, an activator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, has been found to improve IRI and increase SIRT1 expression in the rat kidney[20]. Furthermore, enhancing the activity of SIRT1 through the application of resveratrol, a SIRT1 activator, has been demonstrated to protect against cerebral ischemia[21].

Another element that plays an essential role in triggering cellular protection and preventing metabolic alterations caused by oxygen deprivation is hypoxia-inducible factors (HIFs). Mammals possess three isoforms of HIFα, of which HIF1α and HIF2α are the most structurally similar and the best characterized. During hypoxia, protein levels of HIF2α increase slightly, but it presents significant activation, which suggests that its activity is regulated by additional post-translational mechanisms. One of these post-translational modulations may be deacetylation, since in hypoxic Hep3B cells SIRT1 deacetylates lysine residues in the HIF2α protein, enhancing its transcriptional activity[22].

Additionally, SIRT1 interacts with HIF1α, but in this case SIRT1 represses HIF1α transcriptional activity[23]. Under hypoxic stress, decreased cellular NAD^+ downregulates SIRT1, increases HIF1α acetylation, and thereby promotes the expression of *HIF1α* target genes[23]. Interestingly, other studies have shown that HIF2α compete with HIF1α for binding to SIRT1[24]. Moreover, it has been demonstrated that SIRT6 is also linked to HIF1α by repressing the transcription of *HIF1α* target genes[25].

Likewise, the effects of SIRT3 appear to be protective in the context of hypoxic stress in human cancer cells. SIRT3 overexpression resulted in decreased ROS production, impediment of HIF1α stabilization and subsequent suppression of tumorigenesis[26,27]. However, the effect of SIRT3 in HIF1α stabilization in IRI has not been reported to date.

One of the most important factors involved in the metabolic control regulated by SIRT1 is peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), a transcriptional co-activator of many nuclear receptors and transcriptional factors. SIRT1 functionally interacts with PGC1α and deacetylates it, thus inducing the expression of mitochondrial proteins involved in ATP-generating pathways[28]. Increased PGC1α activity is also associated with lower levels of oxidative damage during

ischemia, as shown by the decrease ROS scavenging in rodents lacking PGC1α subjected to global ischemia[29]. Furthermore, the uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, regulates the proton electrochemical gradient and in neuronal cells PGC1α is required for the induction of UCP2 and subsequent protection against oxidative stress[30]. It has also been shown that enhanced activity of SIRT1 during ischemic preconditioning (IPC) or resveratrol preconditioning confers protection against cerebral ischemia by reducing UCP2 levels, which results in increased ATP levels[21]. However, a more recent study associated the protective effect of resveratrol against oxidative stress in cerebral ischemia with increased levels of SIRT1/PGC1α and UCP2[31]. Moreover, the exact role of UCP2 during ischemia is not fully understood, as studies of its effects have produced conflicting results[32-35].

ROLE OF SIRTUINS IN REPERFUSION

Deprivation of oxygen to the grafts during ischemia induces severe lesions, but the most important damage is caused during reperfusion, when oxygen entry to the organ is restored. During reperfusion, the cellular metabolism returns to aerobic pathways, which results in the generation of a wide variety of ROS, including superoxide, hydrogen peroxide and reactive nitrogen species, such as peroxynitrite. ROS are mainly produced in mitochondria and trigger several phenomena, including accumulation of Ca^{2+}, caspase activation, cytokine upregulation, lipid, protein and DNA damage[36-38]. ROS can be eliminated by enzymatic pathways including manganese superoxide dismutase (MnSOD), catalase (Cat) and peroxidases. Imbalance between ROS generation and elimination produces oxidative stress[15,16].

Various reports in cardiomyocytes have demonstrated the protective role of SIRT1 against oxidative stress[39,40]. Hearts overexpressing SIRT1 were more resistant to oxidative stress in response to IRI, as SIRT1 upregulated the expression of anti-oxidants like MnSOD and thioredoxin 1[41]. SIRT1 also deacetylated Forkhead box-containing protein O (FoxO) 1 transcription factor, inducing its nuclear translocation and subsequent transcription of anti-oxidant molecules[41,42]. Moreover, the question of whether SIRT1 can induce the transcription of other FoxO transcription factors, like FoxO3α, has not yet been investigated. However, the levels of SIRT1 activation are decisive for its protective role, as very high cardiac SIRT1 expression induces mitochondrial dysfunction and increases oxidative stress[39]. Furthermore, in a model of kidney IRI, the protective effect of SIRT1 against oxidative stress has also been demonstrated since SIRT1 upregulated Cat levels and maintained peroxisome number and function[43].

Although mitochondrial sirtuins (SIRT3-SIRT5) have not been studied as extensively as SIRT1, an increasing body of evidence indicates the importance of SIRT3 in mitochondrial biology and function. Lombard *et al*[44] demonstrated that SIRT3 is the dominant mitochondrial deacetylase, as a significant number of mitochondrial proteins are hyperacetylated in $SIRT3^{-/-}$ mice. SIRT3 deacetylates and thus enhances the activity of various proteins that appear to be an important part of the anti-oxidative defense mechanisms of mitochondria, such as MnSOD[45,46], regulatory proteins of the glutathione[47-49] and thioredoxin system[50].

Transcriptional upregulation of the antioxidant enzymes MnSOD, Cat and peroxiredoxin can also be achieved by FoxO3α transcription factor, which is translocated to the nucleus after being deacetylated by SIRT3[51,52]. Furthermore, SIRT3 is necessary for the enhanced expression of cytochrome c, which presents peroxidase- and superoxidase-scavenging capacity[47,49,53]. However, a similar anti-oxidant effect of SIRT3 in models of IRI has not yet been established.

A wide array of functional alterations develop in mitochondria during reperfusion injury[36,54]. In healthy cells, their primary function is the provision of ATP through oxidative phosphorylation in order to meet the high energy demands. There is increasing evidence of the involvement of a multi-protein complex called the mitochondrial permeability transition pore (mPTP) in the decline in mitochondrial function, which is a common finding during reperfusion injury[55-57]. SIRT3 is known to deacetylate the regulatory component of the mPTP, cyclophilin D, and thereby reduce its activity and the subsequent mitochondrial swelling in the heart[58]. It has also been shown that SIRT4 interacts with the adenine nucleotide translocator, another component of mPTP, and that SIRT5 deacetylates cytochrome c, but the physiological importance of these interactions has not yet been established[59,60], especially in models of IRI.

Microcirculatory alterations play an important part in IRI. During the ischemic period, vascular hypoxia can cause increased vascular permeability. After reperfusion, complement system activation, leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation further aggravate microvascular dysfunction[61].

NO produced by endothelial NO synthase (eNOS) is a key regulator of endothelial function, as it opposes the vasoconstrictive actions of endothelins and provokes vasodilatation. Thus, it can abrogate the microcirculatory stress generated during reperfusion[62]. However, NO produced by inducible NO synthase (iNOS) exacerbates IRI through the NOS-derived superoxide production or the generation of peroxynitrite[12]. There is a large body of evidence in favor of the relationship between eNOS and SIRT1; SIRT1 interacts and modifies the acetylation state of eNOS, resulting in the activation of the enzyme[63-65]. In $SIRT1^{+/+}$ hearts subjected to IRI SIRT1 was associated with eNOS activation[66]. SIRT1 activation by resveratrol protected against subacute intestinal IRI by reducing the NO production through iNOS[67] Moreover, various experimental models showed that resveratrol inhibits endothelin-1 levels, providing better regulation of vascular tone[68-70]. However, a recent study in human umbilical vein endothelial cells

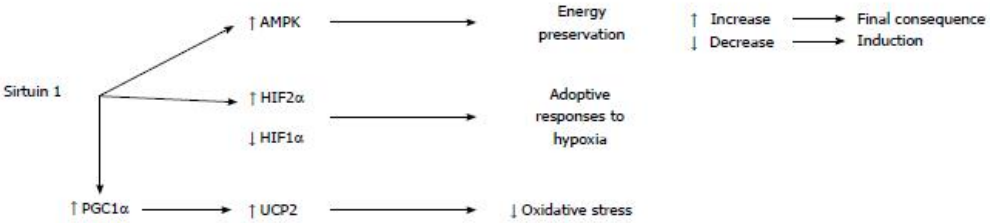

Figure 1 Protective role of sirtuin 1 during ischemia. Sirtuin 1 (SIRT1) activates adenosine monophosphate protein kinase (AMPK) as a cell response to counteract the energy deficiency. SIRT1 upregulates hypoxia-inducible factor 2α (HIF2α) and downregulates HIF1α to increase their transcriptional activity. SIRT1 upregulates peroxisome proliferator-activated receptor-γ coactivator, leading to enhancement of anti-oxidant capacity of uncoupling protein 2 (UCP2). PGC1α: Peroxisome proliferator-activated receptor-γ coactivator.

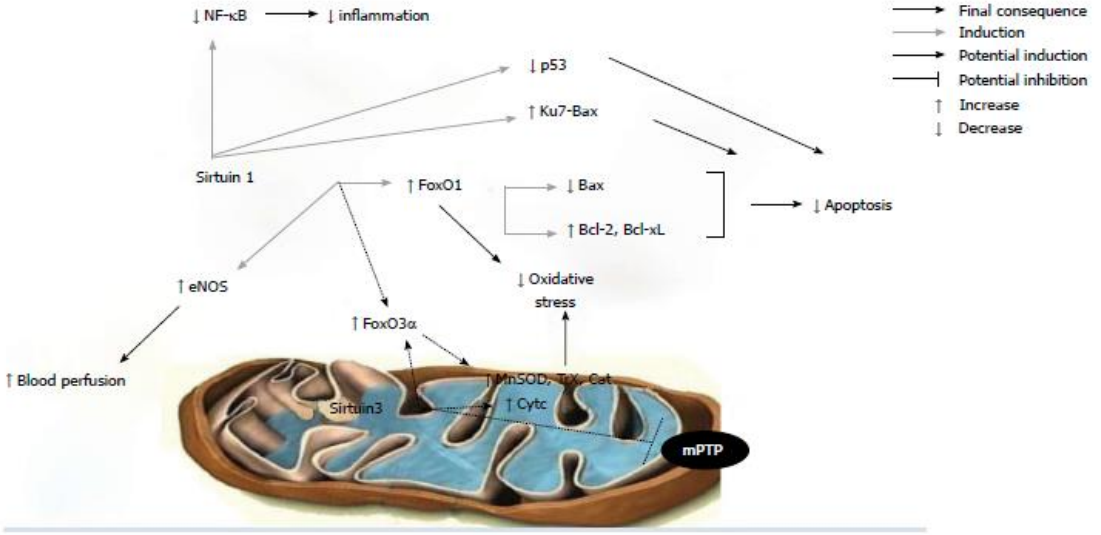

Figure 2 Protective role of sirtuin 1 and suggestive role of sirtuin 3 during reperfusion. Sirtuin 1 (SIRT1) inhibits inflammation through inhibition of nuclear factor kappa B and activates endothelial nitric oxide synthase for a better microcirculation. SIRT1 downregulates apoptosis through multiple pathways, for example, inhibiting p53 transcriptional activity or favoring the binding between Ku70 and Bax. SIRT1 also enhances forkhead box-containing protein O 1 (FoxO1) transcriptional activity, resulting in Bax downregulation and in the upregulation of B cell lymphoma-2 and Bcl-like X. Deacetylation of FoxO1 by SIRT1 also results in lessening oxidative stress, whereas the same effect may be achieved by deacetylation of forkhead box-containing protein 3 alpha (FoxO3α). Sirtuin 3 (SIRT3) is suggested to contribute to decrease in oxidative stress either by a direct interaction with mitochondrial anti-oxidant enzymes [manganese superoxide dismutase (MnSOD), thioredoxin system (Trx), cytochrome (Cyt)] or by enhancing FoxO3α to transcribe MnSOD and Cat. Mitochondrial permeability transition pore (mPTP) may also be inhibited by SIRT3 and result in less production of oxidative stress. NF-κB: Nuclear factor kappa B; eNOS: Endothelial nitric oxide synthase; Bcl-2: B cell lymphoma-2; Bcl-xL: Bcl-like X; Bax: Bcl-2-associated X; Cat: Catalase.

to exert a beneficial effect in regulating hepatic fatty acid metabolism, it would be interesting to investigate their role in the context of liver transplantation. Currently, the shortage of organs for transplantation has obliged physicians to utilize marginal grafts, including grafts with moderate steatosis. Steatotic livers exhibit a more severe inflammatory reaction and more exacerbated oxidative stress and consequently a higher vulnerability to IRI[12]. Thus, activating SIRT1 and SIRT3 might be a potential strategy to protect steatotic livers from IRI as well as to expand the donor pool for liver transplantation. In fact, in preliminary studies our group observed that SIRT1 is involved in the protective mechanisms against IRI elicited by IPC in fatty livers.

For this reason, both surgical and pharmacological strategies should be developed to enhance the activity of sirtuins and thus mitigate the detrimental effect of IRI. Recent studies have highlighted the important role of SIRT1 in IPC-mediated protection in the heart and brain; in IPC brain, SIRT1 prevents neuronal death[97], whereas during cardiac IPC, SIRT1 regulates HIF1α protein levels[98,99]. A recent review has also associated SIRT1 with the protective effects of hyperbaric oxygen preconditioning against apoptosis in the rat brain[100]. However, it is still to be established whether SIRT1 contributes to the protective effects of preconditioning through the regulation of other signalling pathways. Furthermore, its possible implication in IPC related mechanisms in other organs, including the liver or kidney, remains to be elucidated.

Nor has the potential role of sirtuins in cold ischemia

has shown that the inhibitory effects of resveratrol on endothelin-1 levels are SIRT1-independent[71].

ROLE OF SIRTUINS IN IRI-ASSOCIATED INFLAMMATION

IRI results in a profound inflammatory tissue reaction with immune cells infiltrating the tissue. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors with nuclear factor kappa B (NF-κB) being one of the key modulators of inflammation. After activation, the transcription factor migrates to the nucleus and enhances the transcription of pro-inflammatory genes potentiating the inflammatory response. This is followed by an infiltration of lymphocytes, mononuclear cells/macrophages, and granulocytes into the injured tissue[72-74].

In this way, SIRT1 plays an important role in neuroprotection against brain ischemia by deacetylation and subsequent inhibition of p53 and NF-κB pathways[75]. In $SIRT1^{+/+}$ hearts subjected to IRI SIRT1 was correlated with decreased acetylation of NF-κB and possible prevention of inflammation[66]. Moreover, the anti-inflammatory action of SIRT1 by deacetylating NF-κB and thus inhibiting the expression of endothelial adhesion molecules has also been demonstrated in human aortic endothelial cells[74].

SIRTUINS: CELL SURVIVAL OR DEATH?

Apoptotic cell death is a well known mechanism involved in IRI which occurs *via* activation of caspases that cleave DNA and other cellular components[16,17,76]. There is evidence that SIRT1 is associated with longevity in mammals and enhances mammalian cell survival under stress conditions *via* regulating the specific substrates[77-79]. In fact, several studies have mentioned the anti-apoptotic effect of SIRT1 in IRI. SIRT1 deacetylates known mediators of apoptosis, such as the tumor-suppressor p53, resulting in inhibition of its transcriptional activity[80,81]. SIRT1 also deacetylates the DNA repair factor Ku70[2,82,83], thus Ku70 prevents the translocation of Bax, a pro-apoptotic B cell lymphoma-2 (Bcl-2) family protein, to the mitochondria. In ischemic kidney and brain SIRT1 has been identified as an important survival mediator, given that increased SIRT1 was associated with reduced p53 expression and apoptosis[75,84]. SIRT1 also modulates apoptosis-related molecules through the deacetylation of the FoxO family of transcription factors. During IRI in heart-specific $SIRT1^{+/+}$ transgenic mice, SIRT1 induces nuclear translocation of FoxO1, which upregulates the anti-apoptotic factors Bcl-2 and Bcl-like X and downregulates Bax[41]. As regards other members of the FoxO family, Brunet *et al*[85] revealed a dual role of SIRT1 in the cell cycle depending on stress conditions; SIRT1 inhibited the ability of FoxO3 to induce cell death, thus promoting cell survival and, surprisingly, it also increased the ability of FoxO3 to induce cell cycle arrest and resistance to oxidative stress.

A possible pro-apoptotic role of SIRT1 in IRI has not been reported previously. However, studies in human embryonic kidney cells have revealed that SIRT1 can promote cell death by inhibiting NF-κB in response to tumor necrosis factor alpha[86]. Further investigation is required to define the conditions under which SIRT1 may promote apoptosis.

Apoptotic pathways are known to be initiated during reperfusion upon the opening of the mPTP which leads to the release of caspase-activating molecules[87,88]. Since SIRT3 is located in the mitochondria, it may be involved in anti-apoptotic pathways. In this regard, SIRT3 protects various types of cells from apoptotic cell death triggered by genotoxic or oxidative stress[89-92]. The pro-apoptotic role of SIRT3 has also been associated with tumor suppression and restraint of ROS[93]. However, SIRT3 has also been reported to contribute to Bcl-2- and JNK-related apoptotic pathways in human colorectal carcinoma cells[94]. In any case, the potential anti-apoptotic mechanisms of SIRT3 during IRI are yet to be elucidated.

CONCLUSIONS AND PERSPECTIVES

A wide range of pathological processes contribute to IRI. Particularly during organ transplantation, IRI contributes to early graft dysfunction. For this reason, it is important to gain additional mechanistic insight into the molecular mechanisms underlying this injury. In the past few years, sirtuins have emerged as critical modulators of various cellular processes, including those that contribute to the pathogenesis of IRI.

In this paper, we have reviewed the signaling pathways of SIRT1 and SIRT3 protection in IRI. SIRT1 has been shown to exert its beneficial effect against oxidative stress, hypoxic injury or inflammation associated with IRI by activating FoxO1, PGC1α and HIF2α or by inhibiting NF-κB transcription factors (Figures 1 and 2). SIRT3's protective role in IRI is mainly mediated by activating FoxO3α and mitochondrial anti-oxidant enzymes (Figure 2). Investigations that can further determine other intracellular signaling, trafficking and post-translational modifications by SIRT1 and SIRT3 in a variety of cell systems and environments will allow us to translate this knowledge into effective treatment strategies that will be applicable in multiple disorders.

Numerous studies have demonstrated key roles for SIRT1 and SIRT3 in brain, heart and kidney IRI. However, the protective effect of these sirtuins against ischemic processes in other organs such as the liver has not yet been demonstrated. The relevance of SIRT3 in the hepatic metabolism has been confirmed in a study showing that its overexpression in hepatocytes decreased the accumulation of lipids *via* AMPK activation[95]. Furthermore, deletion of hepatic SIRT1 resulted in hepatic steatosis, hepatic inflammation and endoplasmatic reticulum stress[96]. Since SIRT1 and SIRT3 have been shown

and reperfusion yet been established. In the context of liver IRI, a previous study by our group demonstrated that during normoxic reperfusion, after cold ischemia, the presence of NO favors HIF1α accumulation, also promoting the activation of other cytoprotective proteins, such as heme oxygenase-1[101]. Among these cytoprotective proteins, SIRT1 may be ideally suited to enhance the protective effect.

This review summarizes the basic mediators of IRI influenced by the action of SIRT1 and SIRT3 and highlights the importance of their regulation. Future research should aim to elucidate the complete action of all members of the sirtuins family in IRI, and to develop pharmacological strategies that can allow their action to be modulated.

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Advances in treatment strategies for ischemia reperfusion injury

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Advances in treatment strategies for ischemia reperfusion injury

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Advances in treatment strategies for ischemia reperfusion injury

Abstract

Introduction: Ischemia-reperfusion injury (IRI) is a pathophysiological process that limits the outcome of various surgical interventions. Clinical trials, based on the data of experimental models, aim to prove whether a pharmacological/technical approach could be suitable for providing a beneficial effect in humans. Due to the complexity of IRI, few pharmacological treatments have reached to be investigated in clinical phase III.

Areas covered: In this review we report the clinical trials of phase III associated with IRI, which are mainly focused in brain and heart clinical situations. Future clinical trials of phase III might be focused on the advances of those of phase II, which include the administration of matrix metalloproteinases or caspases or P-selectin inhibitors and of anti-oxidant agents.

Expert opinion: More efforts to standardize the tools for evaluating the grade of organ function, as well as the application of techniques suitable for organs with increased vulnerability to IRI could be the objectives for future clinical trials.

Key words: clinical trial, inflammation, ischemia-reperfusion injury, suboptimal grafts

Abbreviations: IRI: ischemia-reperfusion injury; ATP: adenosine triphosphate; NHE: Na^+/H^+ exchanger; mPTP: mitochondrial permeability transition pore; CABG: coronary artery bypass graft; MI: myocardial infarction; PLP: Pyridoxal 5-phosphate; LV: left ventricular; ECCM: extracellular collagen matrix; MMPs: matrix

metalloproteinases; NAC: N-Acetylcysteine; rPSGL-Ig: recombinant P-selectin glycoprotein ligand IgG; MP: machine perfusion

1. Introduction

Ischemia-reperfusion injury (IRI) is an inevitable situation in a wide range of surgical settings and has an important impact on the clinical outcome [1].It occurs when the blood supply is interrupted for a prolonged period of time and then it is restored. Although the involved IRI mechanisms have been the subject of extensive investigations, the effective strategies for its prevention, are still lacking.

It is well known that during the ischemic period, tissues are deprived of oxygen and nutrients leading to cellular metabolism breakdown. Besides this, a more severe damage is provoked to the ischemic tissue when blood flow is restored (reperfusion) which extend inflammation and necrotic and apoptotic events leading finally to the organ failure [2, 3]. IRI mechanisms are highly complex and involve the interaction between different molecular pathways which justify further clinical investigations for the development of therapeutic assays in order to combat IRI detrimental effects.

It is well described that clinical trials aim to examine whether a medical strategy could present a beneficial impact without adverse effects in humans. For safety purposes, clinical trials are carried out firstly with small groups of patients (phase I) to discard any potential harm. Furthermore, important aspects related to the design of the protocol, such as the doses, route or timing of administration are established. In the clinical trial of phase II, the number of participants is augmented and the main objective is to determine whether the drug has sufficient biological activity against the disease and evaluate its safety. If the treatment complies with the predefined clinical end points, further investigation is accomplished in a clinical trial of phase III. In this case, a large group of patients (including thousands) participate in order to confirm its effectiveness, examine the side effects and compare the obtaining results to those for a standard

treatment. Once the drug has been marketed, further investigation is realized in order to evaluate information on the drug's effect in various populations, as well as any potential side effect associated with its long-term use (phase IV) [4]. To provide reliable results, the studies follow strict scientific standards. Clinical studies must always be approved by an Institutional Review Board; an independent committee of physicians and statisticians that assure that the study complies with the ethical laws and the rights and welfare of the participants are protected. The final result of clinical trials is the advances in medical knowledge and patient care.

The present review aims to present the last advances in clinical trials phase III for situations associated to IRI. For this reason, a general overview of the factors and mediators that contribute to IRI is firstly presented. Then, the results and the molecular basis of clinical studies of phase III are analyzed. Moreover, recent clinical studies of phase II that can potentiate the design of new clinical trials of phase III are commented. This review was only based on the published results in Pubmed website considering the selective search for clinical trials of phase III or II.

2. Pathophysiology of ischemia-reperfusion injury

IRI is a complex pathophysiology process that is inherent to organ surgery. Ischemia is characterized by oxygen deprivation to the organ. Energy metabolism is breakdown and adenosine triphosphate (ATP) synthesis in inhibited leading to the increased intracellular acidosis. In these conditions, the action of Na^+/H^+ exchanger (NHE) is stimulated and resulted in an increased Na^+ and Ca^{2+} influx [5].

During reperfusion, the NHE is further activated and the final result is a high intracellular increase of Ca^{2+} [6]. This Ca^{2+} accumulation and the return of intracellular pH to normal values provokes the opening of the mitochondrial permeability transition

pore (mPTP) in the inner mitochondrial membrane, allowing a free passage of protons and small molecules. This fact are responsible for the irreversible cell damage andl death through two main mechanisms. First of all, colloidal osmotic pressure is raised that causes mitochondria swelling and breakdown of the outer membrane. Mitochondrial proteins, such as cytochrome c, are released in the intermembrane space, activate caspases and further initiate apoptotic pathways. In addition, the disruption of the oxidative phosphorylation leads to the activation of degradation enzymes such as phospholipases, nucleases, and proteases [7].

Inhibition of respiratory chain can also promote a rapid and extensive production of reactive oxygen species (ROS) by mitochondria. Also, ROS are generated by xanthine oxidase, NADPH oxidase, as well as by the activated Kupffer cells and neutrophils. ROS have deleterious effects on various cellular functions; ROS damage various biomolecules, including nucleic acids, membrane lipids, enzymes, and receptors. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentalization, which can result in cell lysis. Also, lipid peroxidation and protein oxidation may contribute to the impaired cellular function and cell death [8, 9]

In addition ROS generation also promote Kupffer cells activation and production of pro-inflammatory cytokines including tumor necrosis factor-a (TNF-a) and interleukin-1b (IL-1b). These chemokines are responsible for neutrophil recruiting and their adhesion to the sinusoidal cell and hepatocytes. Neutrophils further release ROS as well as proteolytic enzymes (elastases, proteinases, and collagenases), which can degrade components of the extracellular matrix, attack cells, and inactivate various proteins such as immunoglobulins and proteins of complement [10, 11]. The augmented production of chemokines in combination with the increased translocation of P-selectin to the surface of endothelial cells and platelets promote the adherence of leukocytes to the

microvascular endothelium, contributing thus to an exacerbated inflammatory response and tissue injury. A brief summary of the most important mediators are shown in figure 1.

Given that IRI significantly limits the clinical outcome in various surgical procedures and minimizing its adverse effects could dramatically alleviate the extent of organ failure. For this reason, more clinical trials should be attempted in order to explore effective methods for improving clinical treatments for IRI. Along these lines we discuss the relevance of the different clinical trials targeting IRI in phase III, as follows.

3. Clinical trials of phase III

3.1. Heart

There are very few studies of clinical phase III concerning complex circumstances associated with IRI. Most studies have been proposed for combating cardiac damage in situations where low-flow states are followed by immediate recovery of flow, as happens in coronary artery bypass graft (CABG) surgery. CABG is a type of surgery that improves blood flow to the heart and is commonly used to treat people with severe coronary heart disease [12]. According a study realized in the decade of 90s, CABG procedure is widely used in United States (approximately 400000 per year), but 3% of patients die within 30 days of surgery [13]. CABG is usually accompanied by serious complications including myocardial infarction (MI), arrhythmias, renal insufficiency, and stroke. Many of these are attributable to the process of IRI [13, 14]. Consequently, the development of strategies able to significantly attenuate IRI could also improve the outcome of CABG.

3.1.a. Inhibitors of the complement system

Pro-inflammatory pathways, such as activation of complement, can result in thrombosis, myocardial injury and MI and subsequently increase the postoperative morbidity and mortality after CABG. The complement consists of a family of circulating proteins, such as enzymes and cofactors, as well as the receptors for complement factors such as C5a. The complement system can be activated by IgM or IgG antigen/antibody binding, the presence of microbial cell surfaces or by the binding with the glucosamine residues on bacterial cell walls. The activated complement promotes phagocytosis and inflammation in order to confer protection against the bacterial and viral invasion [15]. However, it can also provoke tissue damage [16, 17]. In addition, it has been shown that IRI induces an early activation of the complement system and subsequent initiation of inflammation [18]. In this case, C5b assemblies other molecules of the component and the resulting complex leads to the formation of a tube, which is inserted into the plasma membrane and induces the cellular lysis [19]. Apart from the cell injury, the expression of pro-inflammatory mediators and the recruitment of inflammatory cells and leukocyte adhesion to endothelium is enhanced [20].

Clinical trials have already shown the beneficial effect of pexelizumab , a C5 complement inhibitor, in the end point of death and MI in patients undergoing CABG surgery without concomitant valve surgery, but not in case of CABG with concomitant valve surgery [21, 22]. Then, pexelizumab was tested in a CABG (with or without valve replacement) surgery trial of phase 3 which involved the participation of 3,099 patients (PRIMO-CABG trial) [23]. In this trial, patients had one or more risk factors (diabetes, female gender, a history of congestive heart failure/MI /cerebral vascular accident /transient ischemic attack/urgent surgical intervention/prior CABG). Patients were randomly assigned to receive either an intravenous pexelizumab/placebo (2.0 mg/kg)

followed by a 24-hour infusion (0.05 mg/kg/hour) 10 minutes prior to initiating cardiopulmonary bypass. The investigators concluded that pexelizumab treatment could not confer a significant reduction in the composite endpoint of death or MI through day 30 in the population of CABG without valve replacement. However, pexelizumab did have a beneficial effect in the intent-to-treat population of patients undergoing CABG with or without concurrent valve surgery.

Moreover, as patients with various risk factors require a prolonged (≥90 minutes) cross-clamp time, a sub analysis of the original PRIMO-CABG trial was realized in order to investigate the potential effect of complement inhibition on post-operative myocardial injury and morbidity after prolonged aortic cross-clamp time [24]. In this phase III clinical study, inhibition of terminal complement activation with pexelizumab reduced the degree of myocardial tissue injury after prolonged ischemia. Moreover, it improved postoperative outcomes across the entire population, including the higher risk patients who were susceptible to increased postoperative morbidity and mortality after prolonged cross-clamp time.

3.1.b. Antagonists of purinergic receptors

ATP plays a central role in cellular metabolism but also can be an extracellular messenger that activates a family of cell surface receptors, the P2 receptor family, in order to promote various physiological responses. P2 receptors are found in the central nervous system and peripheral tissues and can be activated by ATP or ADP. The physiological consequences of activation of P2 receptors by ATP include contractile responses of cardiac, vascular, and smooth muscle; excitatory and inhibitory effects on neurons; stimulatory and inhibitory effects on ion, hormone, platelet, and inflammatory cell secretions [25]. In normal physiological conditions, ATP is co-released with various neurotransmitters such as acetylcholine and regulates physiological functions, such as

the contraction of the heart muscle. On the other hand, a sudden increase of ATP accompanied by a prolonged opening of P2 channels can result in the activation of a non-desensitizing channel; provoking Ca^{2+} and Na^{+} influx and K^{+} efflux. The final result could be plasma membrane depolarization, swelling and disaggregation of the cytoskeletal network and finally cell death or cardiac arrhythmia [26, 27].

Pyridoxal 5-phosphate (PLP) is the biologically active form of pyridoxine (vitamin B6) and catalyzes various biochemical reactions, including biosynthesis of amino acids and amino sugars and lipid metabolism. Further, PLP is an important coenzyme for the synthesis of neurotransmitters, such as dopamine [28]. PLP also exerts an antagonistic effect on purinergic receptors, affecting the ATP-induced increase in Ca^{2+} [29]. Consequently, the administration of a drug that acts as antagonist of purinergic receptors could be promising for limiting IRI, by preventing calcium entry into cells. This fact led to the development of MC-1, a drug that contains PLP and the investigation of its potential effects. Indeed, in a rat model of focal embolic stroke, MC-1 has been found to be neuroprotective, as significantly reduced the infarct volume and improved functional recovery [30, 31]. Further, in a randomized trial of phase II with high-risk patients for ischemic complications, treatment with MC-1 was associated with decreased myocardial injury [30, 31].

In a phase III, multicenter, randomized, double-blind, placebo-controlled clinical trial (MEND-CABG II) was evaluated the effect of MC-1, 250 mg/d, given preoperatively and for 30 days postoperatively on cardiovascular death or nonfatal MI in high-risk patients undergoing CABG surgery [32] . Briefly, as high-risk patients were persons aged of older than 65 years; smokers; persons suffered from diabetes mellitus, stroke, heart failure, renal dysfunction or MI. The study concluded that administration of MC-1 at 250 mg/d immediately before and for 30 days following CABG surgery, did not

lessen cardiovascular death or nonfatal MI in high-risk patients. However, the authors did not exclude the possibility that it may be efficient in populations of elevated risk of IRI, such as patients with acute MI, shock, prolonged valve surgery or that a different dose could provide different results.

3.1.c. Na^+/H^+ exchanger inhibitor

NHE is considered to be an important mediator of IRI, as previously mentioned. Consequently, inhibitors of NHE have been proposed to be a useful tool to combat IRI [6, 33] and a phase II clinical trial confirmed that cariporide, a NHE-1 inhibitor, is a safe drug to be used in patients undergoing CABG [34]. Thus, the EXPEDITION (for Na^+/H^+ Exchange inhibition to Prevent coronary Events in acute cardiac condition), the first clinical study phase III for evaluating the efficacy of inhibiting NHE, was assessed in order to evaluate its potential efficacy in preventing the death or the MI in patients undergoing CABG [35]. Cariporide showed to be cardioprotective,as it decreased the incidence of MI, but its neurologic effects preclude its use at the present time.

NHE inhibitors show cardioprotective effects but it seems to be necessary to discover NHE inhibitors that do not exhibit neurotoxicity. In this sense, the administration of TY-51924, a NHE inhibitor that contains an additional water-soluble functional group, has been developed and it has been shown that not only it protects heart against IRI, but also presents a low distribution to central nervous system [36]. Taking this into account, a clinical phase II study is aimed to evaluate the efficacy and safety of TY-51924 at 30 mg/kg in patients with MI undergoing primary percutaneous coronary intervention (PCI).

3.2. Brain

3.2.a. Anti-thrombotic drug

Ischemic stroke is a pathophysiology that affects about 22 million people per year, of whom 4 million come from high-income countries [37, 38]. Ischemic stroke is provoked when blood circulation is blocked in a part of the brain and subsequently results in an important loss of neurologic function. Strokes usually result from a thrombus that is traveling into the central circulation and finally occludes a blood vessel. Regulation of blood homeostasis is an important factor that can affect the sequences of stroke [39]. Fibrin and the tissue plasminogen activator (tPA) are important components of blood homeostasis. Fibrin contributes to clot formation and thus prevents blood bleeding. On the other hand, tPA converts the inactive zymogen plasminogen into the active protease plasmin, which catalyzes the digestion of fibrin and thus promotes clot dissolution. Consequently, it has been tested the efficiency of recombinant tPA (rtPA or alteplase) treatment in order to achieve the restoration of blood flow through thrombolysis (breakdown of blood clots) and thus attenuate ischemic tissue damage.

Although various studies have evidenced the beneficial use of rtPA against stoke [40, 41], its benefits are limited due to the increased incidence of intracerebral hemorrhage. Thus, these studies have proposed that in order to attenuate the risk of hemorrhage and to augment the potential for recovery, it is essential an immediate treatment (within 90-180 minutes of the onset of the symptoms) with less than 0.95 mg of t-PA per kilogram of body weight. Longer intervals from the onset of stoke have also been studied [42], which resulted in the acquisition of a licence for the use of rt-PA within 3 h of ischaemic stroke in USA (1996) and Europe (2002) [43]. However, a more recent clinical study evidenced that treatment with 0.9 mg of intravenous rTPA within 6 hours of symptom onset was not associated with enhanced brain metabolic recovery, a crucial

factor for ameliorating clinical outcome, after ischemic stroke [44]. Besides this, it still remains controversial the beneficial action of rTPA, as many doubts have been aroused concerning its final effects for the functional outcome or for people older than 80 years old, the time window for achieving a net benefit, as well as the contribution of other factors on the treatment outcome, such as stroke severity, use of antiplatelet drugs before stroke [43]. For example, it has been suggested that endovascular treatment could be combined with rTPA in order to achieve a rapid reperfusion and thus ameliorate the clinical outcome [45]. Thus, the use of recombinant human tissue plasminogen activator (t-PA) for cerebral arterial thrombolysis requires a careful evaluation of both the risks and the potential benefits.

4. Clinical trials of phase II

Following, we describe the most recent studies of phase II that can potentially be the basis for a future design of clinical studies of phase III. In this case, studies carried out in heart, liver and kidney, are reported.

4.1. Heart

4.1.a. MMPS inhibitors

A common consequence of MI is left ventricular (LV) remodeling, which contributes significantly to LV dysfunction and death. Remodeling of the extracellular collagen matrix (ECCM) plays a major role in LV remodeling. In response to MI, myocytes and their surrounding extracellular matrix reorganize resulting in progressive ventricular dilatation, wall thinning, and cardiac dysfunction. The ECCM is composed of highly organized proteins, such collagen type I and III, that form the structural scaffold of various tissues, including myocardium; thus they confer structural support and integrity to the adjoining myocytes, contributing thus to the proper LV pump [46]. Besides this,

in heart diseases, the myocardial matrix is degraded by matrix metalloproteinases (MMPs) and not only the structural support is eliminated, but also the products of ECCM degradation, such as fibronectin, laminin, and elastin enhance the migration of cells like macrophages [47, 48]. Indeed, MMPs activation has been implicated in ECCM damage and LV remodeling, as well as in cardiac IRI [49, 50]. In this sense, MMPs inhibitors have been reported to reduce reperfusion injury [51].

One of the most potent MMPs inhibitors is doxycycline, which inhibits MMP-9 and -2 , the two members MMPS that have been largely reported to play a significant role during MI and LV remodeling[48, 52-54] and various studies have associated the inhibitory effect of doxycycline in MMPs with improved LV function and attenuation of inflammation [55, 56]. Taking also into account that doxycycline belongs to tetracyclines, drugs with a well-known safety profile [57], the effects of doxycycline on LV remodelling in patients treated with acute myocardial infarction and LV dysfunction were evaluated in a randomized, phase II trial[58]. All the patients were treated with primary PCI and doxycycline was administered at 100 mg oral dose immediately after primary PCI and then every 12 h for 7 days. The time and the duration of doxycycline administration was in accordance with previous studies [55, 56], as the time of inhibiting MMPs seems to be a crucial factor for the final effect on LV remodeling; an early short-term MMPs inhibition after experimental MI provides a beneficial effect, in comparison to the unfavorable results of a prolonged MMPs inhibition [48, 59]. The study evidenced that this short-term treatment with doxycycline was efficient to lessen LV remodeling.

4.2. Liver

4.2.a. Caspase inhibitors

Studies in human liver transplantation have evidenced the presence of apoptosis after reperfusion and concluded that the intensity of apoptosis increases with hepatic injury and is related with delayed graft dysfunction [60, 61]. Consequently, inhibition of apoptosis may serve to decrease allograft reperfusion injury. In this sense, various studies investigated the potential effect of IDN-6556, an irreversible and broad-spectrum caspase inhibitor, in cold ischemia and warm reperfusion [62, 63]. IDN-6556 administration was associated with decreased hepatic apoptosis and hepatic injury. Subsequently, a clinical phase II trial in liver transplantation aimed to evaluate whether the addition of IDN-6556 in the storage and flush solutions and in combination or not with its intravenous administration could ameliorate liver injury by reducing hepatic apoptosis, as well as the appearance of any possible side effect [64]. The study concluded that IDN-6556 lessened both reperfusion-mediated apoptosis and postoperative liver injury, without provoking any complication, when it was added to the storage and flush solutions. However, an additional intravenous administration of IDN-6556 abolished the previously observed protective effects. The investigators associated this paradoxical phenomenon with the process of turnover of neutrophils. Apoptosis is essential for neutrophil turnover and limitation of inflammation; aged neutrophils or neutrophils that have migrated to inflamed sites undergo apoptosis and thus they can be recognized and eliminated by macrophages [65]. Taking this into account, inhibition of caspases activation could reduce neutrophil apoptosis and consequently exaggerate the inflammatory response [66, 67]. Indeed, in the present study postoperative intravenous administration of IDN-6556 was associated with neutrophil infiltration in the liver, confirming thus the idea that when a caspase inhibitor

is provided in the later phases of cold ischemia and warm reperfusion could intensify the inflammatory process and thus limit its beneficial effect in inhibiting the overall liver apoptosis.

4.2.b. Anti-oxidant agents

As ROS generation is one of the most considerable features of IRI, investigations have been focused on the development of drugs that could provide an anti-oxidative effect. In this case, the antioxidants should penetrate the cell membrane in order to be able to localize the ROS and scavenge them. Glutathione (GSH) is one of the most important endogenous antioxidants and changes in hepatic GSH levels can be an indicator of post-ischemic hepatic injury [68, 69]. N-Acetylcysteine (NAC) is the acetylated precursor of both the amino acid L-cysteine and reduced GSH. NAC can easily enter to intracellular compartments and its reduced thiol groups can scavenge oxygen free radicals [70]. In addition, NAC has a very good safety profile, as no side effects dependent to dose have been reported [71]. It has been demonstrated a protective effect of NAC administration against hepatic IRI; NAC prevented hepatic malfunction and inhibited the generation of free radicals and accumulation of neutrophils in the damaged hepatic tissue [72]. Furthermore, experimental animal models have evidenced that intravenous GSH administration decreased reperfusion injury after transplantation of both normal and fatty livers [73, 74].

In a recent prospective randomized study phase II, the potential clinical benefits of NAC infusions in patients with chronic liver disease undergoing liver transplantation with deceased donor livers were evaluated [71]. The protocol included the systemic NAC infusion 30 minutes before the initiation of the harvesting procedure. The study resulted in a significant amelioration of graft survival, through decreasing the risk of primary dysfunction and graft failure, after liver transplantation, even when older donors were

used. The authors supported the idea that early systemic infusion of NAC is necessary for the achievement of the protective effect, as in this way the liver can be effectively enriched with adequate reserves of GSH. Further advantages of the NAC administration were the absence of any adverse side effect and its low cost. Furthermore, melatonin, an hormone of pineal origin, can easily be diffused through the cell membrane and be distributed to all subcellular compartments, as it is soluble to both aqueous and lipid substances [75].

Various studies have evidenced the anti-oxidant capacity of melatonin and its beneficial effects against IRI in various organs [76-78]. As a result, the Melatonin Adjunct in the acute myocaRdial Infarction treated with Angioplasty (MARIA) trial, has been designed in order to examine whether an intravenous dose of melatonin (100 μM) would diminish infarct size in patients with acute MI [79].

4.2.c. P-selectin inhibitors

IRI contributes to poor early graft function or primary non-function in transplant recipients, partially through the induction of inflammatory process. One of the earliest events of IRI is the translocation of P-selectin to the surface of endothelial cells and platelets. This fact results in recruitment of polymorphonuclear leukocyte which further enhances endothelial P- and E- selectin expression and subsequently acute inflammatory changes [80-83]. Administration of monoclonal antibodies against P-selectin or studies in P-selectin-deficient mice have evidenced the protective effects of P-selectin against IRI [84-86]. Furthermore, treatment with a recombinant P-selectin antagonist, known as recombinant P-selectin glycoprotein ligand IgG (rPSGL-Ig) has been shown to be efficient against organ IRI in experimental settings; it decreased inflammatory responses and thus lessened hepatic and renal injury [80, 87, 88]. rPSGL-Ig also diminished hepatic neutrophil infiltration and improved survival and hepatic function in obese

Zucker rats that underwent liver "ex-vivo" cold ischemia followed by reperfusion or transplantation [89].

A single-center double blind placebo-controlled phase II study examined the potential efficacy of rPSGLIg in patients undergoing deceased-donor whole liver transplantation [90]. The study involved infusion of the donor liver with 20 mg rPSGL-Ig or placebo after liver graft retrieval via the portal vein and an intravenous dose of 1 mg/kg of rPSGL-Ig or placebo prior to arterial reperfusion. A beneficial impact of rPSGL-Ig on hepatic IRI and early liver graft function was evidenced, as indicated by consistently lower transaminases and bilirubin levels. Moreover, the investigators examined the transcript levels of biomarkers associated with hepatic IRI, such as the chemokine IP-10, the pro-inflammatory interleukin 6 (IL-6) and the anti-inflammatory interleukin 10 (IL-10) [91, 92]. Although the average differences were borderline statistically significant, the administration of rPSGLIg prevented IP-10 and IL-6 induction, whereas enhanced the cytoprotective biomarker IL-10 in comparison to placebo group. Furthermore, as similarly occurred in a previous clinical investigation [93], the administration of rPSGL-Ig was not accompanied with any side effect.

4.3. Kidney

4.3.a. P-selectin inhibitors

The potential efficacy of rPSGLIg was also investigated in patients undergoing deceased-donor kidney transplantation [94]. The trial involved a group of patients were rPSGLIg or placebo were administrated either intravenously only into the recipient or in both the recipient and in the donor organ flush. The study confirmed that no safety concerns were raised from using this drug in the peri-operative period. Considering the incidence of dialysis as a parameter for delayed graft function, it seems that rPSGLIg

did not provide a beneficial impact on renal graft function within the first seven post-transplant days. Moreover, the rPSGLIg administration to the donor organ flush tended to result in poorer short-term renal function than intravenously treatment alone. However, the investigators supported the notion that rPSGLIg treatment could be a favorable approach for ameliorating early renal graft function, as dialysis is not an absolutely reliable indicator for delayed graft function in the early postoperative period [95, 96], whereas decreased creatinine levels were present at day five in patients treated with rPSGLIg. Consequently, further clinical studies of phase 2 and phase 3 with more appropriate objective endpoints are needed to be established in order to evaluate the effect of rPSGLIg treatment in kidney transplantation.

5. Conclusions

There are few clinical trials that potentiate strategies able to minimize the adverse effects of IRI in various clinical situations. The majority of clinical trials of phase III has been carried out in cardiac experiments aiming to reduce MI and included inhibitors of complement system or NHE and purinergic receptors antagonists. An anti-thrombotic therapy has been proposed for ischemic stroke. In all of them, the proposed therapy failed to achieve the predetermined endpoint, whereas adverse effects like neurotoxicity or hemorrhage were evidenced in case of NHE inhibitor and anti-thrombotic drug respectively. Between the promising strategies to be examined in future trials of phase III, we can report the inhibitors of MMPs, caspases, P-selectin and anti-oxidant agents.

6. Expert opinion

Although the extensive investigations in defining the molecular mechanisms behind the IRI and the various therapeutic strategies proposed, few clinical studies at phase III achieved to provide encouraging results that ameliorate the clinical outcome. In all

cases, more clinical trials with more standardized protocols or including various risks factors are required in order to contribute to important advances in clinical therapy.

The fact that phase III clinical trials related to IRI were not accompanied by a complete success can be attributed to the complex pathophysiology of IRI. Various cell signaling pathways are activated during IRI and interact with each other successively; for example ROS enhance the inflammatory response and the activated neutrophils/Kupffer cells contribute to a more exacerbated ROS production. Clinical trials with pharmacological treatments usually aim to block a concrete target or signaling pathway. In this way they offer a partial solution to the situation, which is not always sufficient to provide the most complete protection with the best suppression of the bad side effects. With this in mind we suppose that the future investigations could try to examine the application of a combined use of drugs that could afford a multi target confrontation of IRI.

Clinical trials failed to show evidence for the success of a treatment, partially because there have not yet been established adequate biochemical determinations or biomarkers that reflect the organ function upon concrete conditions. Such is the case of rPSGLIg when administrated in patients undergoing kidney transplantation [94]. Considering the incidence of dialysis as a criterion for delayed graft function, rPSGLIg administration was not associated with improved renal function at the first seven post-transplant days, although it did decrease creatinine levels. Due to the fact that there is no consensus about the criteria for dialysis requirement, the decision for realizing dialysis in the early postoperative time depends on the protocols of each transplant center and in this way dialysis cannot be applied as an objective marker for evaluating early graft function. Thus, it is of crucial importance the choice and the establishment of appropriate parameters in order to evaluate the function/recovery of the organ.

Moreover, the appropriate route of administration may vary between the clinical trials of different phases, as patients with more serious disease aspects are evaluated in the more advanced phases of trials. For example, intravenous administration of caspase inhibitor IDN-6556 was found to be effective in a Phase I in patients with mild liver impairment [62]. However, this was not the case for patients with end-stage liver disease that needed to be submitted to transplantation when examined in a clinical phase II [64]. In this model that consisted of cold ischemia and warm reperfusion, the hepatic injury not only was not improved by the intravenous administration of IDN-6556, rather than it was exaggerated. Local organ treatment was found to be the most effective treatment. Consequently, the clinical trials should examine with more details the conditions upon which the route of administration is selected.

In addition, trials of phase III try to incorporate patients with various high risk factors so as to investigate the effect of the treatment in cases of increased vulnerability or injury. However, the predefined risk factors do not always present the same level of importance or intensity in the development of the disease. Besides this, clinical trials usually apply the same treatment for patients with different risk factors, a fact that can limit an appropriate interpretation of the results. For this reason, it would be better to examine the results by evaluating the impact of each risk factor separately.

In clinical transplantation field, the waiting lists for organ transplant are continuously growing, so physicians are obliged to use suboptimal grafts in order to increase donor's pool for transplantation. For example, suboptimal livers can be those characterized by steatosis, old donor age or grafts subjected to prolonged cold ischemia. Due to the fact that these organs are more vulnerable to IRI, they present an increased graft dysfunction and long-term survival problems after transplantation. For this reason, new clinical trials are needed in order to explore the most suitable strategies for rescuing suboptimal grafts

that are actually discarded for transplantation purposes. Between the trials analyzed, the use of anti-oxidant agents such NAC in liver transplantation provided favorable results for ameliorating graft function in older donors [71]. Antioxidant hormones as melatonin, whose levels decline with the age, could be combined with NAC and further ameliorate the organ function in older donor grafts. Other natural antioxidants as resveratrol could also be applied.

Fatty livers are characterized by an important accumulation of fat in hepatocytes which results in narrowing of the sinusoidal lumen and impaired tissue microcirculation and oxygen transfer. With this in mind, it could be of special interest the exploration of drugs that target to lessen the fat storage and subsequently diminish their vulnerability against IRI. Inhibitors of ubiquitin proteasome system, for example, could be an intriguing approach to evaluate, since a non-toxic low dose administration of bortezomib, a proteasome inhibitor, resulted in significant lipolysis in a rat experimental model [97]. In the same context, melatonin could be another promising candidate for decreasing fat accumulation, according to a recent hypothesis that melatonin might inhibit the ubiquitin proteasome system [98]. In this way, treatment with melatonin could combine both anti-oxidant and fat decreasing properties and thus with increased resistance against IRI. Apart from suboptimal grafts, clinical trials could also be centered to the development of strategies for organs highly vulnerable to IRI, including pancreas [99].

Machine perfusion (MP) techniques have recently emerged as an alternative preservation strategy for rescuing suboptimal graft [100]. For this reason, clinical transplantation trials should be focused on optimizing MP techniques. In this context, aspects concerning to the amelioration of perfusate solutions and to a better oxygenation of the graft should be attempted in the future for increasing the protection of high

vulnerable organs like pancreas and fatty livers. It also seems to be interesting the possible enrichment of perfusate solutions with drugs or natural products that could promote a better graft oxygenation and consequently ameliorate transplantation outcome.

Clinical trials are an indispensable step in order to evaluate the efficacy of a potential treatment in human populations. The choice of the appropriate biomarkers, a more profound examination of the impact of the various risks factors in the disease and the development of studies targeting to suboptimal grafts could be the goals for the future clinical trials.

Highlights

- Clinical trials for the prevention of IRI are limited, due to the multifactorial agents implicated in IRI pathophysiology
- Current clinical trials of phase III associated to IRI are mainly focused on heart and brain
- Future basis for phase III clinical trials might include MMPS/caspase/ P-selectin inhibitors and anti-oxidant agents
- The beneficial effects of drug therapy in clinical trials are limited by the potential side effects
- To the best of our knowledge, no phase III clinical trials have been established for the prevention of IRI in vulnerable organs such as pancreas and fatty liver

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Figure legends

Figure 1: Mediators involved in ischemia-reprefusion injury; the oxygen deprivation during ischemia provokes ATP decreases, elevated intracellular acidosis and activation of Na+/H+ exchanger (NHE). The oxygen supply during reperfusion promotes intracellular Ca2+ and opening of the mitochondrial transition pore (mPTP). This initiates the apoptotic cascade and the release of reactive oxygen species (ROS). ROS contribute to intensify the inflammatory response, whereas pro-inflammatory mediators also contribute to exacerbated ROS. The final result is organ injury.

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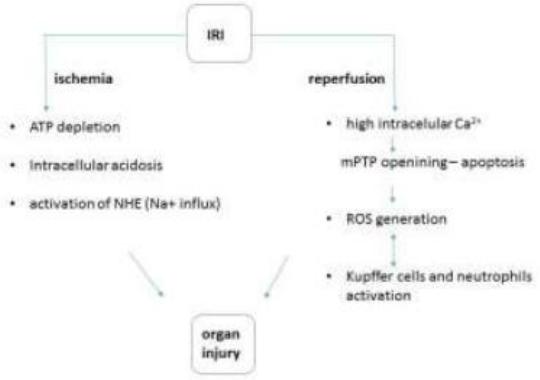

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