UNIVERSITAT DE BARCELONA

Advances in ischemia and reperfusion injury: effects on liver microcirculation and therapeutic strategies for sinusoidal protection

Diana Hide Alférez

The present doctoral thesis is focused on hepatic ischemia-reperfusion injury, a frequent pathological condition observed in surgical procedures needing vascular occlusion or during bleeding and resuscitation. This pathology is associated with vascular dysfunction and impaired perfusion, and causes a wide range of derangements in the liver, which may finally lead to hepatic failure. Despite such negative impact on the liver sinusoid, hepatocytes have been the main target for protection during ischemia-reperfusion and most of the therapies to prevent injury have been focused on this cell type. Thus, the aim of the present PhD thesis was to characterize the liver microcirculation in the setting of ischemia-reperfusion injury and evaluate possible drugs that, through an improvement in the hepatic sinusoid, could maintain a correct hepatic phenotype.

We used two experimental models of ischemia-reperfusion: an ex vivo cold ischemia model to mimic preservation for transplantation and an in vivo model of partial warm ischemia closer to hepatic resection or haemorrhagic shock. The two selected approaches to reduce liver injury were a new formulation of the human recombinant MnSOD, a potent antioxidant with improved stability and biodistribution; and simvastatin, a potent vasoprotective drug. The results of this doctoral thesis lead to the robust conclusion that ischemia-reperfusion causes acute microcirculatory dysfunction and an increase in intrahepatic vascular resistance, which lead to hepatic injury and cell death. Importantly, the maintenance of the sinusoidal phenotype using the herein analyzed vasoprotective drugs results crucial to improve organ function and viability.

Diana Hide et al., Liver Lovers

Diana Hide Alférez

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DOCTORAL THESIS

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DIRECTORS' REPORT

Barcelona, 9 March 2016

Jordi Gracia Sancho, Ramón y Cajal Researcher at the Institute of Biomedicar Research IDIBAPS, and Jaime Bosch Genover, Professor in Medicine of the University of Barcelona Medical School and Senior Consultant in Hepatology at Hospital Clínic de Barcelona,

CERTIFY:

That the doctoral thesis entitled ADVANCES IN ISCHEMIA AND REPERFUSION INJURY: EFFECTS ON LIVER MICROCIRCULATION AND THERAPEUTIC STRATEGIES FOR SINUSOIDAL PROTECTION, presented by Diana Hide Alférez for the PhD degree by the University of Barcelona has been performed under our supervision and fulfils all the requirements to be defended in front of the corresponding examining board.

Jordi Gracia-Sancho

Jaime Bosch Genover

ABBREVIATION LIST

ABBREVIATION LIST

Ang II: angiotensin II

AP-1: activator protein 1

ATP: adenosine triphosphate

BH4: tetrahydrobiopterin

CAMs: cellular adhesion molecules

cGMP: cyclic guanosine monophosphate

CNP: natriuretic peptide C

eNOS: endothelial nitric oxide synthase

ET-1: endothelin-1

GSH: glutathione

GTP: guanosine triphosphate

HIF-1: hypoxia-inducible factor 1

HMG-CoA: hydroxyl-methyl-glutaryl-coenzyme A

HO-1: heme oxygenase-1

HSC: hepatic stellate cell

iNOS: inducible nitric oxide synthase

IPC: ischemic preconditioning

IR: ischemia-reperfusion

KC: Kupffer cells

KLF2: Kruppel-like factor 2

LDL: low-density lipoprotein

LSEC: liver sinusoidal endothelial cells

NAC: N-acetylcysteine

NF-κB: nuclear factor-kappa B

NO: nitric oxide

NOx: nitrites and nitrates

O_2^-: superoxide

PKG: protein kinase G

ROS: reactive oxygen species

sGC: soluble guanylatecyclase

SOD: superoxide dismutase

TM: thrombomodulin

TNFα: tumour necrosis factor α

TXA_2: thromboxane A2

UCP-2: uncoupling protein 2

VEGF: vascular endothelial grow factor

XDH: xanthine dehydrogenase (XDH)

XOD: xanthine oxidase

INTRODUCTION

1. INTRODUCTION

1.1. ISCHEMIA-REPERFUSION INJURY

1.1.1 Definition and clinical relevance

Ischemia-reperfusion injury (IR injury) is a two-phased pathological condition characterized by an initial interruption of blood flow, its biomechanical stimulus, and oxygen (O_2) followed by the subsequent restoration of perfusion and the accompanying oxygen, nutrient supply and shear stress.

Two major types of IR injury can be distinguished[1]. Warm IR injury, which develops *in situ* during liver resection, transplantation or haemorrhagic shock, may lead to delayed liver function and failure or even multiorgan shock. Cold IR injury occurs during *ex vivo* preservation for transplantation and is usually coupled with two warm IR injury phases during organ harvesting and allograft revascularization. Although initial cellular targets of the two IR types might be different, they share common mechanisms. Generation of ROS, activation of Kupffer cells (KC) and neutrophils, increased expression of adhesion molecules and infiltration of circulation lymphocytes and monocytes are pathways present in both types of IR injury[2].

1.1.2 Ischemia

During ischemia, within a few seconds after blood flow interruption all oxygen is consumed. Oxygen interruption stops the mitochondrial respiratory chain, which conduces to the accumulation of respiratory chain intermediates and adenosine triphosphate (ATP) depletion thus leading to anaerobic glycolysis stimulation. In this situation cells are deprived from the energy necessary to maintain homeostasis, Na^+/K^+/ATPase pumps reduce their activity and Na^+ and Ca^{2+} gradients across the cell membrane are lost[3, 4]. Ca^{2+} accumulation in the cytosol activates different enzymes like phospholipases and proteases involved in inflammation. Ca^{2+} also modifies the structure of some enzymes such as xanthine dehydrogenase (XDH) to xanthine oxidase (XOD). These two enzymes catalyse the transformation of xanthine or hypoxanthine into uric acid but, instead of using NAD^+, XOD uses oxygen and

forms superoxide (O_2^-) a free radical that will accumulate during reperfusion[5, 6]. Mitochondrial hypercalcemia, secondary to cellular Ca^{2+} increase, induces mitochondrial oedema and abolishes its membrane potential and finally triggers opening of the permeability transition pore (PTP) compromising mitochondrial survival [7](Figure 1).

Finally, reduction of ATP prevents regeneration of glutathione, ascorbic acid and α-tocopherol that take place in cytosolic detoxification. Na^+ accumulation, together with the accumulation of other osmotically active particles like lactate and inorganic phosphate, lead to intracellular oedema further affecting cytoplasmic organelles and cell membrane integrity, which at the end can conduce to cell death.

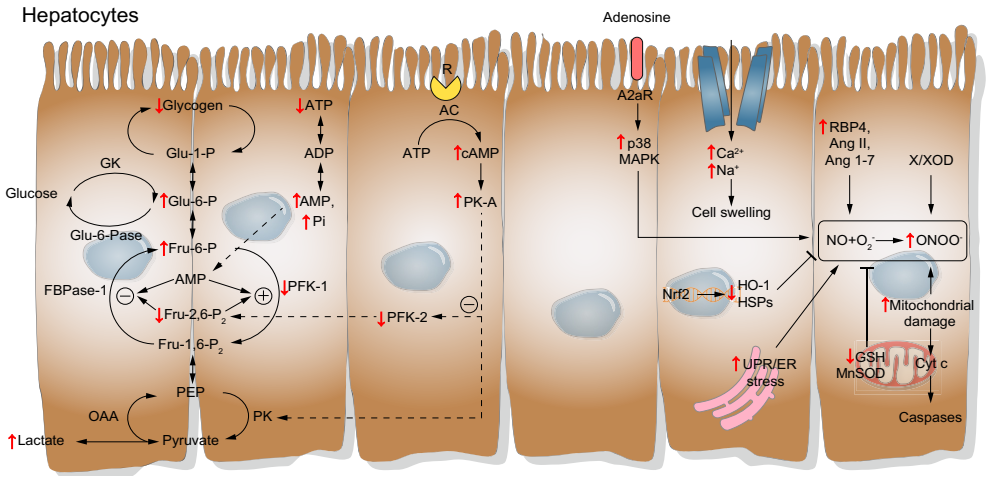

Figure 1. Hepatocyte deregulations due to ischemia-reperfusion injury. Adapted from Peralta & Gracia-Sancho. J Hepatol 2013.

1.1.3 Reperfusion

The reestablishment of blood flow restores aerobic metabolism in the ischemic liver, which suffers a series of phenomenon that can be subdivided in two phases. An early reperfusion phase occurs within the first six hours of reperfusion and is characterized by the release of reactive oxygen species (ROS) and production of inflammatory mediators (tumour necrosis factor α (TNFα) and chemokines), and a late phase, 6 to 48h after reperfusion, in which inflammatory responses caused by neutrophil and macrophage infiltration exacerbate the liver damage[8].

Upon reperfusion, toxic intracellular and extracellular ROS are generated due to O_2 reintroduction. The massive production of ROS rapidly exceeds the antioxidant capacity of the cell pushing it to an important oxidative stress. The principal sources of ROS production during reperfusion are the disruption of the respiratory chain, and the activity of NADPH oxidase and xanthine oxidase[9]. Mitochondria are one of the major sources of ROS in liver cells during reperfusion because the electrons released by the respiratory chain can be directly donated to the newly supplied oxygen[10, 11]. Xanthine oxidase is the other main generator of intracellular ROS in hepatocytes[12]. Finally, inflammatory cells such as activated KC and neutrophils are a source of extracellular ROS due to the NADPH oxidase system associated to its cell membrane[13].

Amongst their negative effects, ROS cause cell injury directly by attacking a great variety of cellular molecules, including lipids, proteins, and nucleic acids[14], and indirectly by promoting other damaging mechanisms, such as the secretion of platelet activator factor (PAF), inflammatory interleukins and TNFα. They can further activate transcription factors including hypoxia-inducible factor 1 (HIF-1), nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) leading to the synthesis of pro-inflammatory cytokines and adhesion molecules[15-17].

In the initial phase of reperfusion, changes in microcirculatory phenotype conduce to a paradoxical phenomenon called 'no reflow' characterized by increased impedance to blood flow causing delayed perfusion failure. This phenomenon, that can nowadays be defined as microcirculatory dysfunction, is characterized by an imbalance of vasodilators like nitric oxide (NO) and vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A2 (TXA_2) causing hepatic stellate cell (HSC) contraction[18]. This fact, together with liver sinusoidal endothelial cells (LSEC) and KC oedema and increased platelet adhesion cause the narrowing of sinusoidal lumen[19]. Decreased NO bioavailability is due to a reduced synthesis by endothelial nitric oxide synthase (eNOS) and an increase in NO scavenging by O_2^- [20, 21]. All these modifications observed in sinusoidal cells during ischemia-reperfusion injury are described in Figure 2.

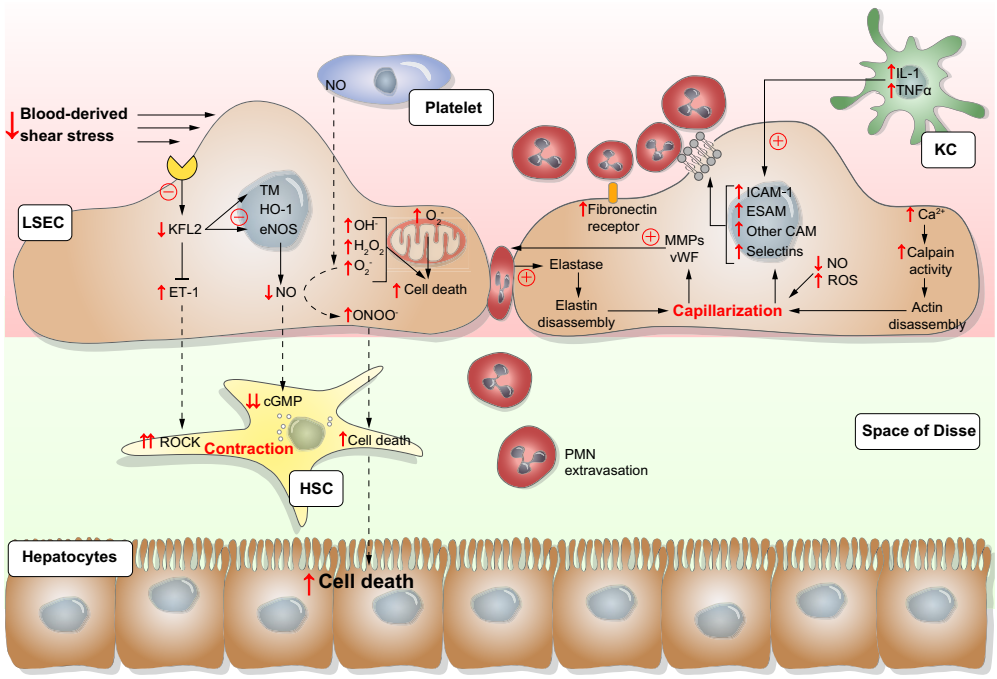

Figure 2. Liver endothelial deregulations and sinusoidal crosstalk during IR injury. Adapted from Peralta & Gracia-Sancho. J Hepatol 2013.

During reperfusion, the autoimmune response characterized by natural antibody recognition leads to activation of the complement system promoting the production of chemotactic and cytotoxic products favouring KC activation [22]. KC are indirectly activated by neighbour hepatic cells release of damage-associated molecular patterns (DAMPs). DAMPs act mainly through toll-like receptor 4 (TLR-4) and others to generate cytokines, the TLR-4 substrates activate the complement system which then induce KC activation[23, 24]. Activated KC release pro-inflammatory cytokines (TNFα, IL-1, IFNγ, IL-1 and IL-12[25, 26]) promoting neutrophil chemotaxis. IL-1 and TNFα recruit and activate CD4+ T-lymphocytes which amplify KC activation and promote neutrophil recruitment and adherence[27, 28].

In the late phase of reperfusion neutrophils play a central role in the inflammatory response. Their activation and that of the endothelium by cytokines (IL-6, TNFa, IL-1, IL-8) and pro-inflammatory mediators (PAF and others) allows narrow interaction of both cell types. There is some controversy about how neutrophils accumulate within the liver. The classical theory argues that activated neutrophils and LSEC

express cellular adhesion molecules (CAMs) on the surface leading to the close interaction and final extravasation of neutrophils. Selectins expressed on endothelial cells (P-selectin and E-selectin) and leukocytes (L-selectin) mediate weak and transitory adhesion of neutrophils known as 'rolling'[29]. Intracellular adhesion molecules (ICAM-1, VCAM-1, PECAM-1) are members of the immunoglobulin-like family. TNFa and IL-1 enhance ICAM-1 expression in endothelium, which interacts with integrins expressed in neutrophils in response to ROS and PAF[30, 31]. This interaction fastens the neutrophil to the surface of endothelial cell. Finally, extravasation is facilitated by decreased expression of cadherin, and phosphorylation of vascular endothelial cadherin and cathenin, components of the intercellular junctions[32, 33].

An alternative theory for neutrophil accumulation independent of ICAM-1 or P-selectin has been proposed[34]. This theory describes how mechanical factors such as vasoconstriction, vascular cell swelling and injury are involved in trapping these leukocytes into the sinusoids[35]. Indeed, extensive vascular injury happening during reperfusion partly eliminates LSEC barrier, thus neutrophils may have direct access to the hepatocytes[36]. When at the interstitial space, activated neutrophils cause considerable parenchymal damage. The lesions are related to the massive extracellular ROS produced that disturbs cellular homeostasis and triggers mitochondrial pore transition and cell death. Further cellular damage is caused by the release of the neutrophilic granules content including hypochloric acid and degrading enzymes such as proteases, collagenases, lipooxygenases and myeloperoxidases[37].

Another point of discussion is whether tissue injury observed in hepatic IR is due to apoptotic or necrotic cell death. Apoptosis of hepatocytes and LSEC due to IR has been described[38, 39] but other groups suggested the majority of cells undergo necrosis[40]. Although it has always been assumed that apoptosis and necrosis are completely different processes this may not be the case, hence the new term necrapoptosis has been coined to describe a process that begins with a common death signal and ends by cell lysis (necrosis) or programmed cellular resorption (apoptosis) depending on cellular ATP levels[41, 42].

1.1.4 Type of liver undergoing IR: steatotic livers

Fatty liver or steatosis, characterized by the accumulation of fat in the cytoplasm of hepatocytes, can be caused by many factors including alcohol consumption, obesity and insulin resistance. Steatotic livers are more susceptible to IR injury showing higher operative mortality rates after liver resection, moreover increased rates of primary non-function after transplantation when using steatotic donors [43].

Different hypothesis for the increased susceptibility of fatty organs to IR injury have been proposed including impaired microcirculation, ATP depletion, Kupffer cell dysfunction, increased adhesion of leukocytes and mitochondrial dysfunction[44].

Diverse authors consider the alterations in hepatic sinusoidal microcirculation present in fatty livers as one of the principal factors for the higher susceptibility to IR[45-48]. The accumulation of fat in the cytoplasm of hepatocytes causes an enlargement of these cells, which can cause sinusoidal compression and distortion, conducing to an increase of intrahepatic vascular resistance and a decrease of blood flow[49, 50]. In addition, sinusoidal microcirculatory dysfunction has been demonstrated in pre-clinical models of steatotic livers procurement for transplantation[48].

Apart from the observed microcirculatory alterations, it has been described that the parenchymal damage is higher in steatotic livers than in non-steatotic[43]. Hepatocyte de-regulation is partially explained by increased oxidative stress produced by the extra-mitochondrial fatty oxidation and the activation of the unfolded protein response and endoplasmic reticulum stress[51]. This increase in ROS causes the upregulation of mitochondrial uncoupling protein 2 (UCP2), which conduces to reduced ATP synthesis by the mitochondria[28, 52]. In fact, in steatotic livers an increase in necrotic against apoptotic cell death after IR injury has been described[53]. This fact may be explained by the low ATP production and the dysfunction of apoptosis regulators such as Bcl-2, Bcl-xL and Bax in these livers[2].

The differences in the pathophysiology comparing steatotic and non-steatotic livers mean that effective therapies in one liver type may be useless in the other type and vice versa, or the effective drug dose may differ between both liver types. Therefore, caspase inhibitors and exogenous NO donors, highly protective in non-steatotic

livers, had no beneficial or even deleterious effects in steatotic livers[43, 54] and compounds such as cerulenin, that reduce UCP-2 expression, and L-carnitine have only shown positive results in steatotic livers[55-58]. Dose wise, heme oxygenase-1 (HO-1) activators such as cobalt (III) protoporphyrin IX protect against hepatic IR injury but a much lower dose is required to protect steatotic livers, as they show higher HO-1 basal levels compared to non-steatotic livers[59].

Finally, it should be considered that the experimental model used to induce steatosis interferes with the mechanisms involved in IR injury. Thus, dietary and alcohol-induced steatosis induce the production of superoxide dismutase (SOD) and catalase insensitive ROS[60] and alcohol-induced steatotic livers are more vulnerable to IR injury due to the presence of neutrophils[2]. The mentioned observations indicate that IR therapies would be model-specific, which increases the difficulty to translate the experimental observations to the bedside.

1.2. LIVER MICROCIRCULATION AND THE HEPATIC SINUSOID

1.2.1 The vascular endothelium

1.2.1.1 Definition

The endothelium forms the inner cellular lining of blood vessels and plays an important role in many physiological functions by producing several molecules.

Histologically, the endothelium consists of a monolayer of endothelial cells (EC) aligned in the direction of blood flow with a cell thickness that varies from less than 0.1 um in capillaries to 1 um in the aorta[61]. EC show a marked heterogeneity in structure and function that can be observed between organs, areas of the same organ, and between micro- and macrovascular EC.

1.2.1.2 Structure

The endothelium may be continuous or discontinuous. Continuous endothelium is subdivided in fenestrated and non-fenestrated. Non-fenestrated continuous endothelium is found in arteries, veins and capillaries of the brain, skin, heart and lung. Fenestrated continuous endothelium presents transcellular pores of about 70 nm in diameter called fenestrae that possess a non-membranous diaphragm across their opening. This type of endothelium is found in locations where increased filtration or transendothelial transport is needed including endocrine glands, gastric and intestinal mucosa and glomeruli amongst others[62]. Discontinuous endothelium is found in certain sinusoidal vascular beds, mainly the liver, and possesses larger fenestrations (100-200 nm in diameter) that lack a diaphragm allowing free trafficking of blood and small molecules.

Two main types of intercellular junctions found in endothelium are tight junctions and adheren junctions, their function is to form a barrier to transport between EC and help in maintaining cell polarity[63]. EC possess clathrin-coated pits, clathrin-coated vesicles, multivesicular bodies and lysosomes, which represent the structural components of the endocytotic pathway[64]. In addition to endocytosis, EC are involved in transcytosis, a process by which molecules are transferred across the endothelium by specialized structures, including caveolae and vesiculo-vacuolar organelles.

1.2.1.3 Functions

Endothelial cells carry out many functions, most of which are performed by specific subsets of blood vessel types or vascular beds. Table 1 depicts the functional heterogeneity of the endothelium. The most closely related functions to the present doctoral thesis, highlighted in italics, will be discussed in the next section.

Function	Primary site
Permeability	Capillaries and postcapillary venules
Leukocyte transmigration	Postcapillary venules (skin, mesentery, muscle) and capillaries (lung and liver)
Homeostasis	Panvascular
Vascular tone	Arterioles
Thermoregulation	Bronchial and skin microcirculation
Sieve function	Liver sinusoids
Scavenging	Liver sinusoids
Immune tolerance	Liver sinusoids
Proliferation/Angiogenesis	Reproductive system

Table 1. Endothelial function heterogeneity. Adapted from Aird W et al. Circ Res 2007.

1.2.2 The hepatic sinusoid

1.2.2.1 Liver microcirculation

The liver microcirculation is unique among vascular beds due to its dual blood supply[65]. High pressure and oxygenated arterial blood flow mixes with low-pressure, de-oxygenated but nutrient-rich portal venous blood within the hepatic sinusoids, the specialized capillary network of the liver[66]. Due to this particular structure, hepatic cells tightly interact each other and is in these segments of the microcirculation where nutrient supply and removal of metabolic products takes place [67]. Blood flow passes through the sinusoids from the hepatic artery and the

portal vein of the periportal area to the central vein of each lobule, thus supplying the liver with oxygen and nutrients (Figure 3).

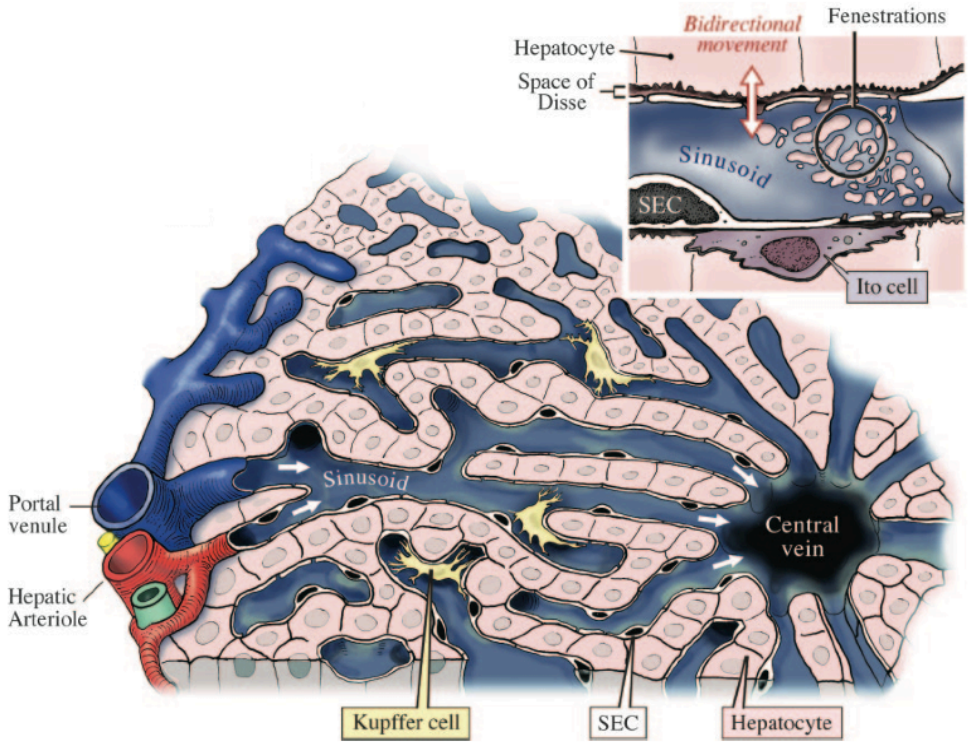

Figure 3. The sinusoidal architecture. Adapted from Aird W et al. Circ Res 2007.

1.2.2.2 Sinusoidal architecture

Liver sinusoids are formed by LSEC, which represent the façade of the sinusoids and constitute approximately 50% of non-parenchymal liver cells. LSEC are not in close contact with hepatocytes, there is a space between basal microvilli-rich surfaces of hepatocytes and LSEC known as the space of Disse, where molecules from the blood enter before making contact with hepatocyte microvilli.

Another unique cellular component of the hepatic sinusoids are the vitamin A storing perisinusoidal cells, known as hepatic stellate cells (HSC). HSC are located in the space of Disse in a quiescent state encircling the sinusoidal tube, resembling the tissue pericytes. Their functions in normal conditions are to regulate retinoid metabolism[68], modulate blood flow by contraction[69] and are implicated in metabolic

activities and cell growth of other cells by direct cell-cell interaction or by release of cytokines and growth factors[70].

The last cellular components of the hepatic sinusoids are Kupffer cells (KC), monocyte-derived resident macrophages found in the luminal side of the endothelium. KC constitute 15-20% of liver non-parenchymal cells and about 50% of all tissue macrophages in the body[71]. In contrast to HSC, which are distributed almost homogeneously throughout the different zones of the liver lobule, the majority of KC is found in periportal regions where they are larger and have greater phagocytic activity than those located in the pericentral region[72] (Figure 3).

1.2.2.3 LSEC specific features and functions

Liver sinusoidal endothelial cells were firstly identified as highly differentiated endothelial cells in the 70s by Prof. Wisse[73, 74]. These cells have typical functions of endothelial cells lying the vascular wall, they participate to all aspects of the vascular homeostasis and regulation of the vascular tone, but also to physiological or pathological processes like thrombosis, inflammation and vascular wall remodelling.

Although they share similar functions, LSEC are dissimilar from other endothelial cells because the lack of an organized basal membrane and the presence of open fenestrae of 100-200nm in diameter organized in clusters termed sieve plates, which comprise 20 to 50 aggregated pores and globally cover 2-20% of cell surface (Figure 4). Fenestrae are dynamic structures able to contract and dilate in response to alterations of blood flow and perfusion pressure changing in size but also in number[75, 76]. Hence, the liver endothelial wall is discontinuous and LSEC the most permeable of all endothelial cells[77].

Furthermore, fenestrae act as a selective sieving barrier for fluids, solutes, and particles, allowing the passage of small particles from blood to hepatocytes, and vice versa, contributing to the homeostatic control of the hepatic microcirculation. The reduced velocity of blood flow through the sinusoids compared with other vascular beds prolongs the interactions between blood and LSEC and thus promotes filtration[78].

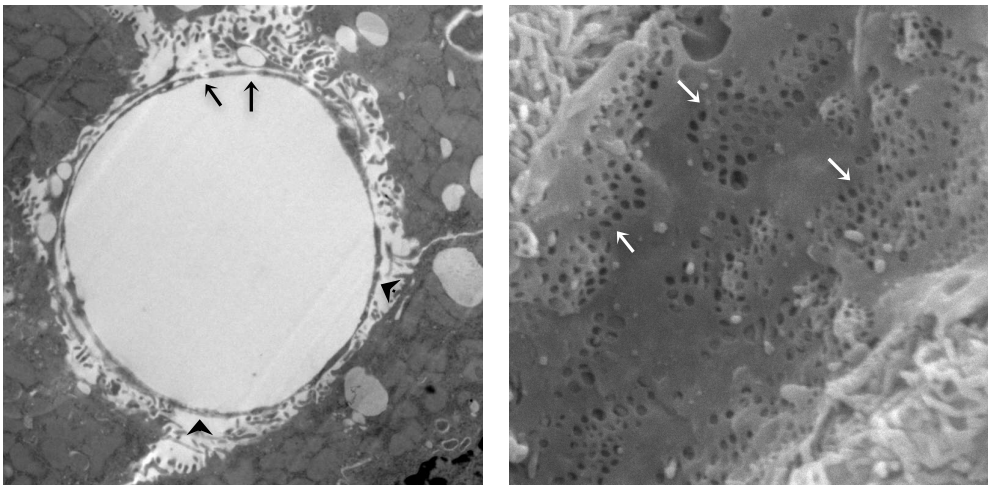

Figure 4. LSEC phenotype analysed by transmission and scanning electron microscopy. Arrow: fenestrae; arrowhead: lack of basal membrane.

In addition, LSEC exert scavenger functions by their high receptor-mediated endocytic capacity and clear the blood from many macromolecular waste products including hyaluronan, acetylated low-density lipoprotein (LDL), denatured albumin, advanced glycation end products and other modified macromolecules[79]. Another important function of LSEC relates to immunity. LSEC take up antigens via their scavenger receptors and present antigens via major histocompatibility complex. This, linked to the unique morphology of the LSEC which permits interactions between lymphocytes and hepatocytes through fenestrae, allow naive T-cells to interact with hepatocytes and thus to develop immune tolerance as lymphocytes pass through the liver[80].

1.2.3 Vascular tone regulation

1.2.3.1 Mechanisms

The balance between vasoconstrictors and vasodilators acting on the liver sinusoid determines the hepatic vascular tone and perfusion. Vasoactive molecules can be divided in vasoconstrictors such as angiotensin II (Ang II), endothelin-1, thromboxane A2 (TXA_2) and isoprostanes; and vasodilator factors including bradykinin, prostacyclin (PGI_2), nitric oxide (NO), adenosine, acetylcholine and

natriuretic peptide C (CNP)[76]. Vasoactive stimuli can be extrinsic to the liver or intrinsically produced by the cells forming the hepatic sinusoid.

Under physiological conditions, the influence of these vasoactive mediators on intrahepatic blood flow is rather small and their effects are balanced. However, any adverse stimulus may lead to an imbalance between constrictors and dilators affecting microvascular blood flow regulation. Thus the maintenance of an appropriate balance seems to be an attractive concept for an adequate regulation of hepatic blood flow, aiming at limiting microcirculatory dysfunction-associated liver injury[81].

Although there are many vasoactive mediators, particular emphasis is given on the enzymes producing NO.

1.2.3.2 Nitric oxide

Nitric oxide is a gaseous molecule with a half-life of 3-5 seconds and a size in the order of picometers. It regulates vascular tone and homeostasis and it is implicated in biological functions such as vasodilation, inhibition of platelet aggregation, angiogenesis, coagulation and leukocyte adhesion to the endothelium[82, 83]. The enzyme responsible for the formation of this signalling molecule is the nitric oxide synthase (NOS), which catalyses the production of NO from L-arginine. There are three main isoforms of the enzyme: the neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). Among the isoforms in the liver, iNOS is expressed in macrophages, hepatocytes, HSC and other cell types, and synthesizes elevated levels of NO after its induction by pro-inflammatory cytokines or LPS. eNOS is a calcium-calmodulin controlled cytosolic enzyme expressed constitutively in endothelial cells, where it produces small amounts of NO in response to biomechanical and humoral stimuli such as blood-derived shear stress, vascular endothelial grow factor (VEGF), acetylcholine and agonists of G-protein coupled receptors[84]. The formation and activity of this enzyme is a complex process that involves post-transcriptional and post-translational modifications. Among them, phosphorylation on serine, threonine and tyrosine residues, protein cofactors and intracellular localization of the enzyme play an important role[85]. NO derived from eNOS diffuses to the HSC modulating their contraction through the

activation of the soluble guanylate cyclase (sGC), an enzyme responsible for the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The main target of cGMP is a protein kinase called protein kinase G (PKG), which phosphorylates many substrates inducing reduction in intracellular Ca^{2+} levels and relaxation[86].

During liver injury, including IR injury, hepatic NO bioavailability decreases, mainly by a reduced activity of eNOS due to post-translational modifications including decreased bioavailability of the NOS cofactor tetrahydrobiopterin (BH4) or increased interaction with the inhibitory proteins caveolin-1, NOSIP and NOSTRIN[87-89] or decreased Akt-dependent phosphorylation[90, 91]. In addition, NO bioavailability within the liver is further reduced due to its scavenging by elevated levels of superoxide.

1.2.4 Endothelial dysfunction

The term endothelial dysfunction was coined to describe the change of the vascular endothelium from a modulatory phenotype to a phenotype unable to adapt, characterized by a generalized defect in the homeostatic mechanisms present in healthy endothelial cells. Endothelial dysfunction is considered one of the main mechanisms involved in pathological microcirculatory function in several vascular disorders including arterial hypertension[92], diabetes[93] and hypercholesterolemia[94]. In addition, our group has described that liver endothelial dysfunction is also responsible of microcirculatory derangements in models of cirrhosis[95], NAFLD[96] and cold preservation for transplantation[97].

As mentioned above, the term endothelial dysfunction was established to describe the phenotypical modifications observed in endothelial cells and conducing to derangements in liver microcirculation. However, recent advances in the field demonstrate the participation of all sinusoidal cells, not only LSEC, in the pathological vasoactive response. For example, in cirrhotic livers LSEC and KC acquire a vasoconstrictor phenotype and HSC become hyper-responsive to vasoconstrictors[98, 99]. Further studies demonstrate the existence of paracrine interactions between LSEC or KC and HSC, responsible for phenotypic deregulations

in both directions[100-103]. Thus the term 'liver microcirculatory dysfunction' appears much more appropriate to describe all the changes observed in the hepatic sinusoid[104].

The main functional manifestations of microcirculatory dysfunction include altered vasomotor responses, increased permeability, increased leukocyte adhesion and thrombotic complications[105]. At the cellular and molecular level, these manifestations of microcirculatory dysfunction reflect acute or chronic changes occurring in several endothelial functions including decreased production of vasodilators (mainly NO) and increased vasoconstrictor production, increased secretion of chemokines, expression of adhesion molecules, decrease in antioxidant pathways and reduced cell survival. In addition to this humoral mechanisms, changes in biomechanical forces created by blood flow can lead to endothelial dysfunction due to their influence in LSEC structure and the modulation of gene expression[106].

1.3. THERAPEUTIC APPROACHES IN LIVER ISCHEMIA-REPERFUSION

1.3.1 Antioxidant therapies

As previously described, oxidative stress due to increased reactive oxygen species formation and reduction of antioxidant cell capacity is one of the main disease mechanisms in ischemia-reperfusion injury. Many therapeutic strategies have been successfully used in experimental models of hepatic warm and cold ischemia and reperfusion with some of them beginning clinical trials. However, despite its universal role in the pathogenesis, nowadays no drug targeting ROS is in clinical use.

Antioxidant enzymes play a fundamental role in maintaining the delicate redox balance and are essential for preserving physiological function and neutralize oxidant species from endogenous or exogenous sources. Endogenous protective mechanisms would probably be more effective against ROS than pharmacological strategies based on antioxidant administration, which need to reach the site of action in adequate concentrations to be effective. Please see in table 2 a summary of proposed antioxidant strategies to ameliorate liver IR injury.

1.3.1.1 XOD inhibitors

Early investigations on the mechanisms of hepatic IR injury focused on intracellular sources of ROS, especially xanthine oxidase. This hypothesis was mainly based on the protective effect of the XOD inhibitor allopurinol[107, 108]. However, there are multiple reasons for the limited importance of XOD in clinical conditions. First, the conversion of XDH to XOD, the ROS-generating enzyme, requires lengthy ischemic times that are normally avoided in clinical practice[109]. In addition, ROS formation depends on the substrates xanthine and hypoxanthine that are relatively fast metabolized during reperfusion[110]. Allopurinol studies have shown its effectiveness protecting livers from IR injury only if animals were pre-treated multiple times with high doses[107, 111]. Because a single dose of allopurinol is completely effective in inhibiting XOD[112], the high doses used in IR injury have off-target effects, which may be responsible for the protection. Although the mechanism remains unclear, allopurinol has shown protective effects preventing mitochondrial injury and increasing NO bioavailability[111, 113, 114].

1.3.1.2 Superoxide dismutase and catalase

Superoxide dismutase, which catalyses the dismutation of O_2, is a major ROS scavenger. There are three isoforms of SOD, with unique subcellular locations. Cu/Zn-SOD is localized in the cytosol and nucleus of all cell types, and functions as the intracellular antioxidant system; manganese SOD (MnSOD) is exclusively localized in the mitochondria, and finally extracellular SOD (EC-SOD) offers antioxidant protection against ROS not only in the cytosol but also in the extracellular space. Catalase, a potent scavenger of H_2O_2, prevents the formation of HO^- when SOD is insufficient to remove O_2^- overload.

Some studies have reported that treatment with SOD and/or catalase reduces oxidative stress under hepatic IR[115, 116] showing only partial positive results in liver injury, probably due to the difficulty of getting these enzymes into the cells. To overcome this problem, gene delivery of SODs has been proposed. Positive results reducing IR liver injury were found using adenovirus encoding any of the three SOD isoforms[117, 118] or using transgenic mice overexpressing Cu/Zn-SOD[119]. However, there are a number of problems inherent to gene therapy, for example, vector toxicity, difficulties in increasing transfection efficiencies and protein expression at the appropriate time and site. Thus, potential antioxidant therapies using modified SODs or catalase are still needed for ameliorating liver IR injury.

1.3.1.3 GSH

Glutathione (GSH), a highly effective antioxidant present at high concentrations in hepatocytes[120], detoxifies H_2O_2 through glutathione peroxidase activity. Hydrogen peroxide and hypochlorous acid as well as peroxynitrite can also react spontaneously with GSH[121, 122], thus GSH can detoxify these ROS in the presence or absence of the peroxidase. Because GSH is continuously released from hepatocytes into the vascular space, it can detoxify ROS generated by KC[123]. Consequently, GSH intravenous administration has shown protection against vascular oxidative stress during reperfusion after warm or cold ischemia[122, 124, 125]. On the other hand, treatment with the GSH precursor N-acetylcysteine (NAC) maintained the intracellular GSH during IR and reduced ROS levels[126, 127]. Gene transfer is an especially effective option for GSH management, as GSH has a very short half-life.

Gene transfer of GSH synthesis components offered protection against IR injury by increasing intracellular GSH levels[128].

1.3.1.4 Preconditioning

Perhaps the most common investigated method to reduce IR injury is ischemic preconditioning (IPC), consisting in a brief period of IR before subsequent prolonged hepatic ischemia. The benefits of IPC in relation to pharmacological treatments rely in its capacity to induce endogenous cellular pathways. IPC has wide effects inducing many molecular cascades, which are complex and not completely understood.

In relation to oxidative stress, IPC has shown to induce antioxidants such as SOD, NO, and heat shock proteins, as well as to affect XDH/XOD[129-131]. IPC, through NO, reduced the accumulation of xanthine during ischemia and prevented the conversion of XDH to XOD, thus preventing ROS generation[108, 132]. Indirectly, the benefits of IPC with regard to mitochondrial dysfunction and neutrophil and KC activation in hepatic IR have been associated with reduced oxidative stress[133].

1.3.1.5 NADPH oxidase inhibition

More detailed investigations of ROS formation *in vivo* indicated that the main oxidant stress after moderate ischemia occurs in the extracellular space of the liver pointing to KC and neutrophils as the main source of ROS via its NADPH oxidase (NOX2) system[36]. In fact, NOX2 inhibitors have been shown to protect against IR injury[134]. In addition, numerous strategies that indirectly reduce the inflammatory oxidant stress, for example, blocking adhesion molecules, depletion of Kupffer cells or neutrophils are highly effective against IR injury[36].

1.3.1.6 Vitamins and herbal antioxidants

Vitamin E or α-tocopherol can provide a first line of defence against DNA oxidative damage. Its activity is basically that of a chain-breaking antioxidant in relation to lipid peroxidation of unsaturated fatty acids in the cell membrane. Pre-treatment with this compound has been shown to attenuate liver IR injury[135, 136].

In addition, natural antioxidants have been reported to be effective in reducing oxidative stress associated to hepatic IR injury by maintaining the expression and activity of the enzymes described in this section. Some of the evaluated antioxidants

include resveratrol[137], green tea catechins[138], tetrandine[139] and quercetin[140].

Drug	Species	Model	Effects
Allopurinol (XOD inhibitor)	Mouse	Warm IR	↑GSH ↓XOD, MDA and O_2^-
Allopurinol	Rat	Cold and warm IR	↓ROS and hepatic injury
SOD	Rat	Warm IR	↓ROS and hepatic injury
Cu/Zn-SOD (Adenovirus)	Rat	CS+LT	↓Hepatic injury
Cu/Zn-SOD (Adenovirus)	Mouse	Warm IR	↑SOD ↓ROS and hepatic injury
EC-SOD	Mouse	Warm IR	↑SOD and GSH
SOD (Polyethylene glycol)	Rat	Warm IR	↓MDA
SOD (transgenic)	Mouse	Warm IR	↑SOD ↓Hepatic injury
Catalase	Rat	Warm IR	↓H_2O_2 and hepatic injury
GSH	Rat	Cold and warm IR	↓ROS and hepatic injury
NAC (GSH precursor)	Rat	Warm IR	↓MDA and hepatic injury
IPC	Rat	Cold and warm IR	↑GSH, SOD and NO ↓XOD, ROS, MPO, MDA and hepatic injury
Apocynin (NADPH oxidase inhibitor)	Mouse	Warm IR	↑GSH ↓NADPH oxidase, MDA, O_2^-
α-tocopherol	Rat	Cold and warm IR	↑Catalase, GSH ↓Lipid peroxidation
Trans-resveratrol	Rat	Warm IR	↑Catalase, GSH and SOD
Green tea extract	Rat	Warm IR	↑SOD ↓MPO, ROS and hepatic injury
Quercin	Rat	Warm IR	↑GSH, SOD ↓MDA, ROS
Tetrandine	Mouse	Warm IR	↑SOD ↓MDA and neutrophil accumulation

Table 2. Antioxidant strategies on liver IR injury. Adapted from Elias-Miró M et al. Free Radic Res 2013. CS+LT: cold storage + liver transplantation; MDA: malondialdehyde; MPO: myeloperoxidase.

1.3.2 Vasoprotective strategies

Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemia–reperfusion[19]. Several strategies focused to protect liver microcirculation against IR injury have been developed in animal models and the efficacy of some of them has been subsequently confirmed in clinical studies. As a result, surgical procedures such as ischemic preconditioning (which indeed protects through a variety of mechanisms) have found their way into clinical practice, whereas clinical evaluation of pharmacological approaches remains limited.

1.3.2.1 Vasoactive mediators

Imbalance of vasoactive molecules plays a role in hepatic IR injury due to its implication in vascular tone and perfusion modulation. This has made many laboratories to be interested in testing some of these vascular mediators in experimental models of liver IR injury. Thus, ET receptor blockade[141], angiotensin II inhibition[142], PGE_1 analogues[143, 144], and angiotensin converting enzyme inhibitors[142] have shown to effectively reduce the severity of IR injury by improving hepatic sinusoidal perfusion. Moreover, A2 receptor stimulation (adenosine, dipyridamole)[145], NO supplementation (L-arginine, spermine NONOate)[146, 147], carbon monoxide pre-treatment[148], induction of HO-1[149, 150] and administration of atrial natriuretic peptide[151, 152] reduced warm IR injury.

Although the preliminary evidence from experimental approaches suggested that vasodilators hold therapeutic promise, only three randomized clinical trials have been performed. The two first studies using TXA_2 synthase inhibitor or PGE_1 showed improvements in plasma aminotransferase levels but with no effects on microcirculation and postoperative complications[153, 154]. The last study demonstrated that the vasodilator amrinone, which inhibits the breakdown of cAMP and cGMP by the phosphodiesterase (PDE3) enzyme, enhanced endothelial function and lactate metabolism during hepatic resection in patients with cirrhosis[155].

1.3.2.2 Ischemic preconditioning

As previously stated, IPC is maybe the most studied strategy in preventing IR injury, including hepatic IR. Although the mechanisms are not fully unravelled, a wide literature point to an improvement in liver microcirculation.

Early reports by Peralta et al. showed that the release of adenosine plays a key role promoting liver protection by ischemic preconditioning and that was associated with a slight improvement in liver perfusion[145, 156]. Functional evidence indicates that, besides adenosine, NO plays a prominent role mediating hepatic preconditioning. Increased NO levels after IPC have been associated with reduced ET-1 expression[157] which can lead to improved liver vasodilatation. After these pioneer studies, a large amount of papers have demonstrated a link between increased NO bioavailability and improved hepatic perfusion conducing to an improvement in liver function[145, 158-160]. However, the exact mechanism by which NO is increased after IPC still remains unclear.

Because to its simplicity and ability to modulate endogenous pathways, surgeons have been more predisposed to use IPC in the clinical practice and, at least, ten randomized clinical trials have been performed. The global results demonstrate that this strategy can reduce parenchymal damage and extend ischemia time[161-165].

1.3.2.3 Anaesthetic preconditioning

The concept of pharmacological preconditioning is based on up-regulating the protective mechanisms induced by IPC without the need of a surgical procedure. In particular, anaesthetic preconditioning with volatile anaesthetics such as sevoflurane and isoflurane has shown promising results including increased expression of HO-1 in response to isoflurane[166, 167]or increased hepatic NO levels after sevoflurane administration[168]; two vasoprotective signalling pathways that are also activated after IPC.

1.3.2.4 Statins

Statins are a group of drugs that inhibit the action of the hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase limiting cholesterol synthesis. Statins were firstly

described to lower lipid levels, but different studies reported many pleiotropic effects independent of cholesterol lowering[169, 170].

Several studies have investigated the role of statins in ischemia-reperfusion injury, some of them in hepatic IR, demonstrating its effectiveness in preserving microvascular function. Among the mechanisms involved, over-expression of HO-1 and eNOS, and reduction of ET-1 and iNOS[150, 171, 172] have been described. In line with these results, our laboratory described the benefits of simvastatin as a therapy to prevent microcirculatory injury in cold ischemia and reperfusion[48, 97]. Simvastatin effects were partly mediated by the activation of the transcription factor Kruppel-like factor 2 (KLF2).

KLFs are a subclass of zinc finger transcription factors that regulate cell growth and tissue development[173]. Although the zinc finger domains are very similar, the non-DNA-binding domains are highly divergent and mediate the transcriptional regulation by KLFs. KLF2 is known to be primarily expressed in the endothelium and is necessary for proper vessel development and homeostasis[174-176]. Indeed, it has been reported that KLF2 confers anti-inflammatory and anti-thrombotic properties to the endothelium by decreasing adhesion molecules such as E-selectin and VCAM-1[177] and induces the expression of eNOS[177] and thrombomodulin (TM)[178], a cell surface factor essential for coagulation inhibition. A summary of KLF2 regulation and functions is represented in Figure 5.

The expression of KLF2 is flow dependent[179] with high levels found in vascular regions exposed to laminar shear stress (defined as atheroprotective flow) and low levels in regions exposed to turbulent shear stress (atheroprone flow)[180]. Consequences of flow-dependent induction of KLF2 expression in endothelial cells include activated expression of eNOS and repressed expression of angiotensin converting enzyme, ET-1 and adrenomodullin, all of which are involved in vascular tone control in response to flow[181]. In fact, experiments performed in endothelial cells cultured under shear stress conditions, demonstrate the decay in KLF2 expression and its target genes in a time-dependent manner when flow is stopped [182] and how statins maintain the KLF2 pathway in static cold storage conditions.

The mechanisms involved in statin induction of KLF2 include the inhibition of small GTPases comprising RhoA, Rac1 and Ras[183],what increases the expression of KLF2 and its target genes[184-186]. In line with this evidence, some studies administering simvastatin in cardiac ischemia-reperfusion have shown prevention of microcirculatory injury by inhibition of RhoA/ROCK[171], however KLF2 levels were not evaluated.

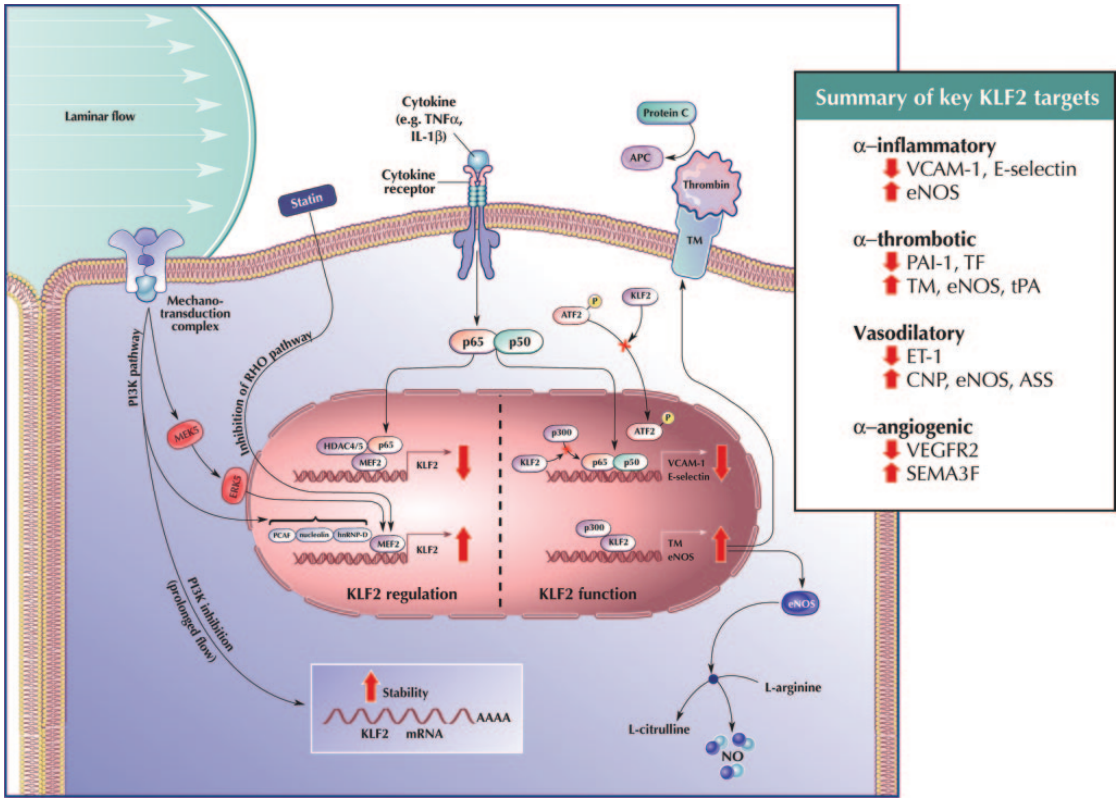

Figure 5. Regulation and function of KLF2 in endothelial cells. From Atkins GB Circ Res 2007

HYPOTHESIS & AIMS

2. HYPOTHESIS AND AIMS

Liver ischemia-reperfusion injury is a frequent pathological condition observed in surgical procedures needing vascular occlusion or during bleeding and resuscitation. This pathology, associated with microvascular dysfunction and the "no reflow phenomenon" causes a wide range of derangements in the liver, which may finally lead to hepatic failure in case of severe injury. Sinusoidal dysfunction is, in part, due to the reduction in vasodilator molecules, such as nitric oxide. The decrease in NO bioavailability is due to both, a reduction in its production by eNOS in endothelial cells and an increase of its scavenging by ROS like O_2^- [187]. Thus, therapies focused on increasing eNOS expression or reducing ROS production may be useful in preventing microcirculatory derangements associated with IR injury.

Despite the importance of the liver sinusoid in health and disease, a vast bibliography about the effects of IR in the parenchyma is available but scarce reports investigated the pathophysiological consequences of sinusoidal cell deregulation on hepatic IR injury, especially in warm ischemia. This has conduced to the erroneous idea that hepatocytes should be the main target for protection and therefore most of the therapies tested to prevent IR injury have focused on this cell type, without placing great importance to the liver microcirculation, in particular to the sinusoidal endothelium.

Recent studies oriented to understand sinusoidal microcirculation in the setting of IR have demonstrated that the lack of hemodynamic biomechanical stimuli during ischemia, in particular during cold preservation, is crucial for endothelial phenotype deregulation[182]. Furthermore, studies in experimental models of cold preservation for transplantation performed by our group have demonstrated the existence of endothelial dysfunction associated to this pathology[97]. The mechanisms involved include KLF2 downregulation due to the stop in blood flow, thus causing a reduced expression of its target genes involved in vasodilation, thrombosis prevention and other vasoprotective properties.

Despite the impact of IR injury in the clinical practice and the efforts of many groups in finding therapeutic strategies to reduce injury, nowadays no

pharmacological treatment is available, possibly due to the lack of microcirculatory protection of those drugs. Thus, we hypothesize that therapies focussed in preserving the sinusoidal endothelial phenotype during IR will conduce not only to a better microcirculation but also to a global improvement of the whole liver.

With this background, the global aim of the present PhD thesis was to characterize the liver microcirculation in the setting of ischemia-reperfusion injury and evaluate possible drugs that, through an improvement in the hepatic sinusoid, could maintain a correct hepatic phenotype during IR.

Study 1. A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischemia and reperfusion injury

Endothelial dysfunction occurring during cold storage for transplantation represents the first event in the development of liver injury by IR[97, 188]. Different mechanisms responsible of endothelial dysfunction have been described including ROS production during reperfusion. Despite the efforts in developing antioxidant treatments[118, 119], there is no available therapy in the clinical practice to prevent liver IR injury. Our hypothesis was that acute pre-treatment of the liver donor with an advanced antioxidant formulation just before organ procurement will inhibit ROS formation during reperfusion and will prevent its deleterious effects.

Therefore, the aim of this study was to evaluate the protective effects of a new recombinant form of the human manganese superoxide dismutase (rMnSOD) on the endothelial function and viability post-transplantation using rat healthy and steatotic livers, and also test its antioxidant efficacy in human tissues.

Study 2. Effects of warm ischemia and reperfusion on the liver microcirculatory phenotype of rats: underlying mechanisms and pharmacological therapy

Hepatic warm ischemia associated with liver resection or haemorrhagic shock is poorly tolerated by the liver and, although the ischemic times are shorter than in cold ischemia, the damage can conduce to primary non-function. As our group has demonstrated, cold ischemia causes microcirculatory dysfunction associated with

a reduction in KLF2 expression during ischemia[97, 182]. However, the vascular pathophysiology of livers undergoing warm ischemia is much less investigated.

The first aim of this study was to characterize liver microcirculation using an experimental model of hepatic warm ischemia reperfusion. The second aim was to test if pre-treatment with endothelial protective molecules such as statins, which have shown several vasoprotective benefits independent from their lipid-lowering effects, would significantly improve function and viability of the organ and finally characterize the pathways involved in this hepatoprotection.

COPY OF ORIGINAL ARTICLES

STUDY 1. A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischemia and reperfusion injury

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online data

Original Paper

A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischaemia and reperfusion injury

Diana HIDE*, Martí ORTEGA-RIBERA*, Anabel FERNÁNDEZ-IGLESIAS†, Constantino FONDEVILA‡, M. Josepa SALVADÓ†, Lluís AROLA†, Juan Carlos GARCÍA-PAGÁN*, Aldo MANCINI§, Jaime BOSCH* and Jordi GRACIA-SANCHO*

*Barcelona Hepatic Hemodynamic Laboratory, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Universitat de Barcelona, Barcelona, Spain
†Nutrigenomics Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain
‡Liver Surgery and Transplantation Unit, IDIBAPS, Hospital Clínic de Barcelona, CIBERehd, Universitat de Barcelona, Spain
§Molecular Biology and Viral Oncogenesis, National Cancer Institute, Naples, Italy

Abstract

Hepatic microcirculatory dysfunction due to cold storage and warm reperfusion (CS + WR) injury during liver transplantation is partly mediated by oxidative stress and may lead to graft dysfunction. This is especially relevant when steatotic donors are considered. Using primary cultured liver sinusoidal endothelial cells (LSECs), liver grafts from healthy and steatotic rats, and human liver samples, we aimed to characterize the effects of a new recombinant form of human manganese superoxide dismutase (rMnSOD) on hepatic CS + WR injury. After CS + WR, the liver endothelium exhibited accumulation of superoxide anion (O_2^-) and diminished levels of nitric oxide (NO); these detrimental effects were prevented by rMnSOD. CS + WR control and steatotic rat livers exhibited markedly deteriorated microcirculation and acute endothelial dysfunction, together with liver damage, inflammation, oxidative stress, and low NO. rMnSOD markedly blunted oxidative stress, which was associated with a global improvement in liver damage and microcirculatory derangements. The addition of rMnSOD to CS solution maintained its antioxidant capability, protecting rat and human liver tissues. In conclusion, rMnSOD represents a new and highly effective therapy to significantly upgrade liver procurement for transplantation.

Key words: cold storage, endothelium, liver sinusoidal endothelial cell (LSEC), oxidative stress, transplantation

INTRODUCTION

Liver transplantation is the only curative treatment for end-stage liver diseases. Although remarkable improvement in graft survival has been achieved during previous years, early organ damage continues to be an important problem and remains a major focus of therapeutic attention [1]. In addition, liver transplantation rates are limited by the shortage of adequate organs for clinical use, which have led to the use of steatotic liver grafts. Unfortunately, steatotic livers are more susceptible to ischaemia/reperfusion (I/R) injury, exhibit poorer outcome, and are associated with increased risk of primary graft dysfunction [2,3].

I/R injury is the phenomenon of deprivation and afterwards restoration of oxygen and blood-derived shear stress stimulation during the transplantation setting. In most liver transplant procedures, I/R injury derives from hypothermic preservation followed by warm reperfusion periods [cold storage and warm reperfusion (CS + WR)]. CS + WR is poorly tolerated by liver grafts, and liver sinusoidal endothelial cells (LSECs) represent one of the most affected cell types, which rapidly develop severe alterations including cell activation and apoptosis [4,5]. These

Abbreviations: Ach, acetylcholine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CS + WR, cold storage and warm reperfusion; DAF, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, haematoxylin and eosin; HFD, high-fat diet; ICAM-1, intracellular adhesion molecule-1; I/R, ischaemia/reperfusion; KLF2, Kruppel-like factor 2; LDH, lactate dehydrogenase; LSEC, liver sinusoidal endothelial cell; NOx, nitrites/nitrates; O_2^-, superoxide anion; rMnSOD, recombinant form of human manganese superoxide dismutase; ROS, reactive oxygen species; SOD, superoxide dismutase; vWF, von Willebrand factor.

Correspondence: Dr Jordi Gracia-Sancho (email jgracia@clinic.cat).

de-regulations cause acute endothelial dysfunction development, which correlates with poorer liver transplantation outcome [6,7].

Different mechanisms for endothelial damage during CS + WR have been described and include inflammation, vasoconstriction cascades and oxidative stress [5,8–11]. Indeed, during CS, lack of oxygen causes accumulation of respiratory chain intermediates which, during WR, are rapidly converted into reactive oxygen species (ROS) [12]. These ROS can cause important damages in DNA and protein structure and function. Furthermore, an excess of ROS acts as a scavenger of nitric oxide (NO), forming peroxynitrite [13] and further negatively affecting cell viability. Previous studies have investigated the role of reducing oxidative stress in liver grafts preserved for transplantation, showing partially positive results [14,15]; however, none of them has specifically evaluated the role of CS + WR-derived oxidative stress on the hepatic microcirculation.

Recently, a novel recombinant form of human manganese superoxide dismutase (rMnSOD) has been developed [16]. This new formulation of a key superoxide anoin (O_2^-)-degrading protein remains stable in solution, has a good biodistribution in all organs, effectively scavenges intra- and extra-cellular O_2^-, freely enters the cells and is constitutively active in the cytoplasm, nucleus and mitochondria [17,18]. The aim of the present study was to investigate whether rMnSOD could be a new therapeutic strategy to reduce hepatic and microcirculatory status of liver grafts preserved for transplantation.

MATERIALS AND METHODS

Animals and treatment

Male Wistar and Sprague–Dawley rats from the Charles River Laboratories weighing 300–325 g were used. Liver steatosis was induced by feeding animals with a safflower oil-based high-fat diet (HFD; 28% carbohydrates, 58% fat, 14% protein; #5ALX, TestDiet) for 7 days, as previously described by our group [11,19]. Livers from HFD-fed animals exhibited over 75% of hepatocytes with macro-vesicular fat, an 11.2-fold increase in lipid content determined by Oil Red staining, and significantly elevated levels of non-esterfied ('free') fatty acids (4.3 ± 0.5 compared with 6.0 ± 0.3 μmol/g) and triacylglycerol (1.0 ± 0.1 compared with 4.0 ± 0.4 mg/g). Animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

LSEC isolation and cold preservation

LSECs from control rats were isolated as described previously [20]. Highly pure and viable cells were used. After 1 h of isolation, LSECs were washed with PBS and incubated for 16 h at 37 °C (no CS group) or at 4 °C in Celsior solution (Genzyme) supplemented with rMnSOD (0.15 μM) or its vehicle (PBS). After this period, cells were incubated for 1 h at 37 °C in culture medium to partially mimic the WR period, and *in situ* determination of O_2^- and NO intracellular levels was performed as described in the following sections.

Liver vascular studies

Hepatic I/R injury was induced using the *ex vivo* model of liver CS + WR as previously described [5,21,22]. Although this experimental approach does not allow polymorphonuclear neutrophil infiltration, it indeed reproduces hepato-endothelial cell injury, inflammation, and microcirculatory dysfunction observed in transplantation models.

Under anaesthesia with intraperitoneal ketamine (100 mg/kg, Merial Laboratories) and midazolam (5 mg/kg, Baxter), rats were treated via the femoral vein with rMnSOD (50 μg/kg for controls and 150 μg/kg for steatotic rats), or its vehicle, 30 min before liver isolation (doses based on preliminary results in the present study, results not shown).

Liver vascular responses were assessed in the isolated, *in situ* liver perfusion system, as described previously [5,11,23]. Baseline pressures at a constant portal flow of 30 ml/min were recorded after 20 min of stabilization; afterwards, livers were flushed with cold Celsior solution and then cold-stored for 16 h in Celsior solution.

After CS, livers were exposed at room temperature (22 °C) for 20 min to mimic the normothermic ischaemia period and reperfused through the portal vein with Krebs buffer (37 °C). The perfused livers were continuously monitored for 1 h. Afterwards, liver endothelial function was evaluated analysing endothelium-dependent vasorelaxation in response to incremental doses of acetylcholine (Ach: 10^{-7} to 10^{-5} M) after pre-constriction with methoxamine (10^{-4} M) [24].

Control livers (no CS) were perfused, harvested, and immediately reperfused *ex vivo*. Aliquots of the perfusate were sampled for the measurement of transaminases and lactate dehydrogenase (LDH) using standard methods at the Hospital Clinic of Barcelona's CORE laboratory.

Histological analysis

Liver samples were fixed in 10% formalin, embedded in paraffin, and sectioned, and slides were stained with haematoxylin and eosin (H&E) to analyse the hepatic parenchyma [25]. Hepatic histology was analysed and scored by a third researcher under blinded conditions. To detect neutral lipids, snap-frozen livers were fixed in a freezing medium (Jung, Leica Microsystems) and stained with Oil Red for 2 h at room temperature [19].

The samples were photographed and analysed using a microscope equipped with a digital camera and the assistance of AxioVision software (Zeiss). Five fields of each sample were randomly selected, photographed at a magnification of 40× with an inverted optical microscope equipped with a digital camera (Zeiss Axiovert) and then quantified using AxioVision software. The red-stained area per total area was determined using a morphometric method. The results were expressed as a steatosis ratio (%), calculated as the ratio of the Oil Red-positive area to the total area.

O_2^- and NO detection

In situ O_2^- and NO levels in LSECs and hepatic tissue were assessed with the oxidative fluorescent dye dihydroethidium (DHE; 10 μM; Molecular Probes) or with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA; 10 μM; Molecular Probes) as described previously [13,26,27]. Specificity of the assays was ensured using superoxide dismutase (SOD; 200 units/ml) or N^G-nitro-L-arginine methyl ester (L-NAME) (1.5 mM) as negative controls [23,28]. Fluorescence images were obtained with a fluorescence microscope (Olympus BX51), and quantitative analysis of at least 20 images per condition was performed with ImageJ 1.44 m software (National Institutes of Health).

In addition, levels of cGMP, a marker of NO bioavailability, were analysed in liver homogenates using an enzyme immunoassay (Cayman Chemical) as previously described [5].

Hepatic nitrites/nitrates (NOx) production was assessed in aliquots of perfusate using specific microelectrodes (Lazar Laboratories).

SOD activity

Total SOD activity was determined using a commercially available kit (Superoxide activity assay kit, Cayman Chemical). Briefly, livers were homogenized in buffer containing 20 mM Hepes, 1 mM EDTA, 210 mM mannitol and 70 mM sucrose. After centrifugation at 1500 ***g*** for 5 min at 4 °C, the supernatant was collected and the protein concentration was quantified. SOD activity assay was performed according to the manufacturer's instructions.

Nitrotyrosine and von Willebrand factor immunohistochemistry

After antigen-retrieval procedure and endogenous peroxidase activity inhibition, sections were incubated with anti-nitrotyrosine (1:200 dilution; Millipore) or anti-von Willebrand factor (vWF; 1:400 dilution; Dako) for 1 h at room temperature. Horseradish peroxidase (HRP)-conjugated rabbit/mouse (Dako) secondary antibody was added. Colour development was induced by incubation with a diaminobenzidine (DAB) kit (Dako), and the sections were counterstained with haematoxylin. Sections were dehydrated and mounted. The specific staining was visualized, and images were acquired using a microscope equipped with a digital camera and the assistance of AxioVision software. vWF and nitrotyrosine relative volume was determined by point-counting morphometry on immunoperoxidase-stained sections, using a point grid to obtain the number of intercepts over vWF/nitrotyrosine-positive cells over the tissue. Six fields were counted in each liver. All measurements were performed by two independent blinded observers. The relative volume was calculated by dividing the number of points over that particular cell type by the total number of points over liver tissue.

Nitrotyrosine fluorohistochemistry

Quantitative tyrosine nitration detection was performed as previously described [29]. Briefly, slides were deparaffinized, hydrated, incubated with aqueous sodium dithionite solution (10 mM) for 10 min, washed with distilled water and then incubated overnight at 4 °C with an equimolar solution of aluminium chloride and salicylaldehyde (200 μM). Afterwards, the aqueous solution was removed, and sections were mounted in Fluoromount G medium (Southern Biotech). Negative and positive internal controls were included. Fluorescence images were obtained with a fluorescence microscope, and quantitative analysis of at least six images per sample was performed with ImageJ 1.44 m software.

Western blot analysis

Liver samples were processed and Western blot analysis was performed as described previously [23]. Primary antibodies against endothelial nitric oxide synthase (eNOS) (BD Transduction Laboratories) and intracellular adhesion molecule 1 (ICAM-1) (R&D Systems), both at 1:1000 dilution, were used. Blots were revealed by chemiluminescence, and protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film). Blots were also assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) content as standardization of sample loading.

Glutathione levels and catalase activity

Total hepatic glutathione was determined as previously described [30]. Briefly, in the presence of glutathione reductase (50 units/ml), total GSH reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Sigma) to generate 2-nitro-5-thiobenzoic acid, a yellow compound absorbing at 412 nm.

To measure catalase activity, liver homogenates containing same amount of protein were mixed with 30 mM hydrogen peroxide (H_2O_2) (Panreac) and 50 mM of phosphate buffer, and the absorbance was measured for 60 s. The enzymatic activity was calculated using the H_2O_2 molar absorbance coefficient ($\varepsilon = 0.0436\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$) [31].

rMnSOD as a CS solution supplement for rat and human livers

Rats were anaesthetized, the abdomen was opened and liver was washed with saline solution. Liver biopsies were taken and preserved 16 h at 4 °C in Celsior solution supplemented with rMnSOD (0.15 μM), or its vehicle. Afterwards, an *in vitro* WR period was mimicked incubating liver biopsies in complete culture medium for 1 h at 37 °C. At the end of the study, tissue was snap-frozen for O_2^- detection using DHE staining.

Furthermore, O_2^- levels were evaluated in human liver samples obtained from healthy donors accepted for liver transplantation. A biopsy from each donor was divided into the following two parts: (i) cold-stored for 16 h in Celsior solution and (ii) cold-stored for 16 h in Celsior solution with 0.15 μM rMnSOD. After this time, liver tissues were incubated for 1 h at 37 °C in culture medium, and O_2^- levels were determined. The present study was approved by the Ethical Committee of the Hospital Clinic de Barcelona.

Analysis of hepatic triacylglycerol and non-esterified fatty acids

Frozen livers samples were homogenized (1:3, w/v) in buffer composed of 50 mM Tris, 150 mM NaCl, and 5 mM EDTA, and

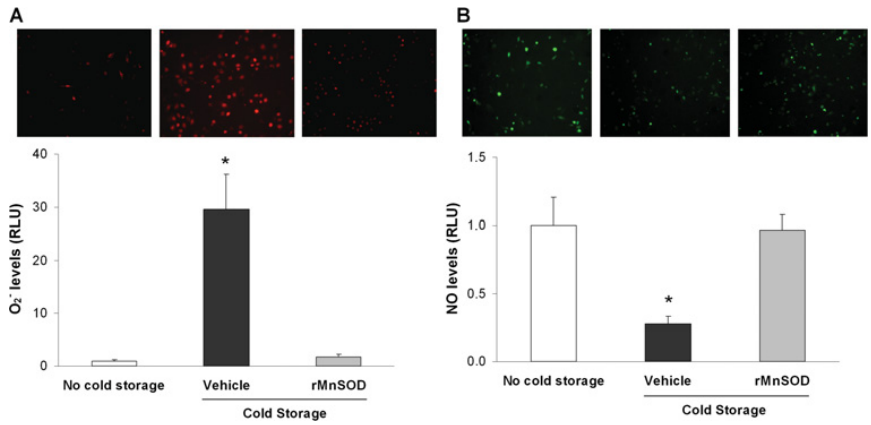

Figure 1 **rMnSOD prevents O_2^- accumulation and maintains NO levels in LSECs**

Freshly isolated LSECs were incubated for 16 h at 37 °C (control group) or at 4 °C in Celsior solution supplemented with rMnSOD, or its vehicle. (**A**) Endothelial oxidative stress was assessed as O_2^- levels. (**B**) LSEC NO levels were determined by DAF staining. Fluorescent intensity was divided by the total number of cultured cells. (Images ×20; $n = 5$ per group; $^*P < 0.01$ compared with no CS and rMnSOD).

triacylglycerols and non-esterified fatty acids were analysed with standard methods at the Hospital Clinic de Barcelona CORE lab.

Statistical analysis

Statistical analyses were performed with the IBM SPSS Statistics 19 for Windows statistical package. All results are expressed as means ± S.E.M. Comparisons between groups were performed with ANOVA followed by least-squares difference (LSD) test, or with Student's *t* test or Mann–Whitney *U* test when adequate. Differences were considered significant at $P < 0.05$.

RESULTS

rMnSOD prevents O_2^- accumulation and maintains NO levels in LSECs

Cold-stored and warm-reperfused LSECs exhibited significantly higher levels of O_2^- (Figure 1A) and reduced NO (Figure 1B) compared with no cold-stored cells. These detrimental effects of CS + WR were prevented in LSECs preserved with rMnSOD.

rMnSOD pre-treatment prevents O_2^- accumulation and improves viability of cold-stored and warm-reperfused control livers

As shown in Figure 2(A), CS + WR markedly increased O_2^- levels in hepatic tissue without significant changes in SOD activity (Figure 2B). Rats pre-treated with a single dose of rMnSOD exhibited significantly increased hepatic SOD activity, which led to reduced levels of O_2^-, demonstrating that intravenously administered rMnSOD reaches the liver where it is functionally active.

Furthermore, cold-stored and warm-reperfused livers exhibited hepatocellular lesions, mainly in centrilobular areas, defined by loss of cohesion of cell plates, hepatocyte necrosis, the presence of Councilman bodies and anoxia-derived small fat vacuoles (Figure 2A). Hepatocellular damage was accompanied by increased levels of ICAM-1 and a significantly greater release of transaminases and LDH in comparison with no cold-stored grafts (Figures 2C and 2D). Pre-treatment with rMnSOD significantly reduced, or even prevented, these parameters of liver injury (Figures 2A, 2C and 2D).

rMnSOD improves microcirculation and endothelial function in cold-stored and warm-reperfused control livers

Livers cold-stored for 16 h exhibited significantly deteriorated microcirculation upon reperfusion, as demonstrated by the markedly increased portal perfusion pressure compared with no cold-stored livers. Hepatic microcirculation de-regulation was prevented in liver grafts from rats pre-treated with rMnSOD (Figure 3A).

In addition, cold-stored and warm-reperfused livers exhibited endothelial dysfunction defined as a significant reduction in the endothelium-derived vasodilatation in response to Ach in comparison with no cold-stored livers. Liver vasorelaxation was significantly improved in rats pre-treated with rMnSOD (Figure 3A).

Interestingly, development of acute endothelial dysfunction caused by CS + WR was accompanied by a decrease in eNOS protein expression (Supplementary Figure S1 at http://www.clinsci.org/cs/127/cs1270527add.htm) and diminished NO production and bioavailability, measured by the release of NOx and cGMP respectively (Figure 3B), together with increased intrahepatic accumulation of nitrotyrosinated proteins (Figure 3C and Supplementary Figure S1). rMnSOD pre-treatment was effective in improving NO bioavailability, most probably through a reduction in its scavenging as demonstrated by diminished levels of nitrotyrosinated proteins (Figure 3C and Supplementary Figure S1).

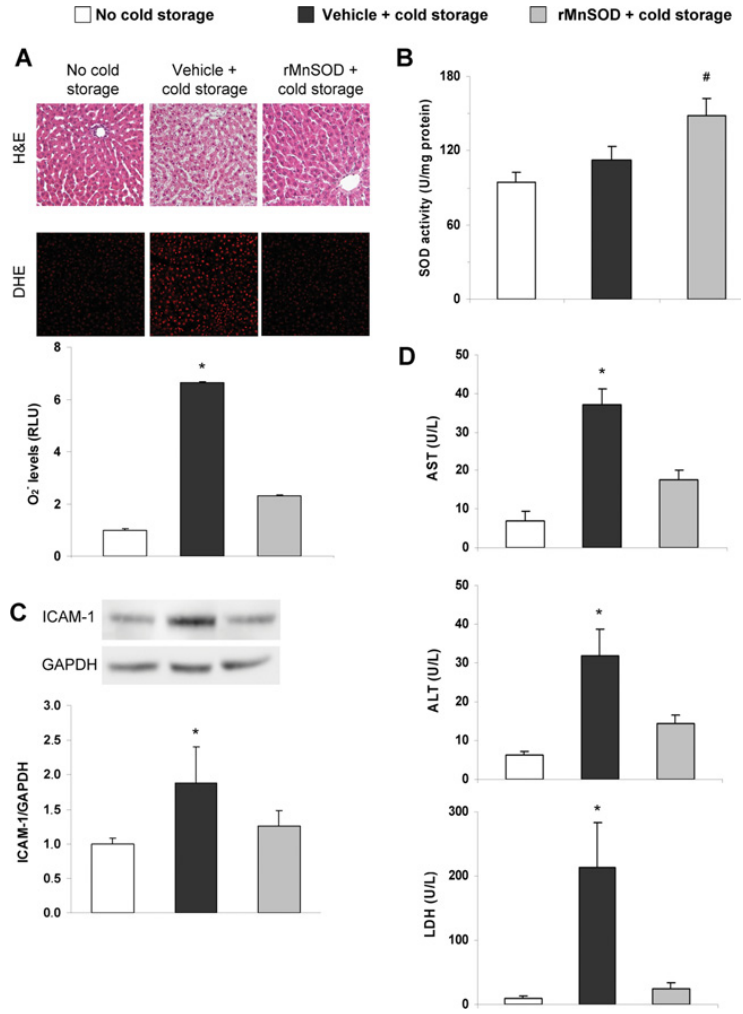

Figure 2 **rMnSOD improves hepatic I/R injury in control grafts**
Hepatic damage was evaluated at the end of WR in grafts not cold-stored (control group) and in livers from rats receiving rMnSOD, or its vehicle, after 16 h of CS. (**A**) Upper: hepatic morphological changes were assessed by H&E staining; lower: representative images of oxidative stress detection using DHE staining and quantitative analysis (all images $\times 20$). (**B**) SOD activity determined in liver tissue. (**C**) Representative hepatic ICAM-1 immunoblot and densitometric analysis normalized to GAPDH. (**D**) Hepatic injury measured as release of transaminases (AST and ALT) and LDH. ($n = 8$ per group; $^{*}P < 0.05$ compared with no CS and rMnSOD; $^{\#}P < 0.05$ compared with no CS and vehicle). RLU, relative light units.

CS + WR induced a significant increase in the hepatic expression of the LSEC capillarization marker vWF, which was prevented by administering rMnSOD (Figure 3C).

rMnSOD prevents hepatic O_2^- accumulation and improves liver microcirculation and endothelial function in rats with steatosis

As shown in Figure 4(A), analysis of hepatic histology showed that HFD-fed rat livers exhibited massive micro- and macrovesicular fat deposition in all cases, characterized by the presence of multiple small vacuoles surrounding the nuclei of hepatocytes and large fat vacuoles distorting the nuclei respectively (Figure 4A).

Although rMnSOD was effective in maintaining SOD activity during CS + WR and, therefore, preventing hepatic O_2^- accumulation when administered to dietary-induced steatotic rats (Figures 4B and 2C), it was not associated with a reduction in hepatocellular damage biochemical markers (AST: 122.8 ± 28.2 units/l in vehicle compared with 110.1 ± 15.5 units/l in rMnSOD; ALT: 65.3 ± 18.2 units/l compared with 42.6 ± 8.5 units/l; LDH: 2688 ± 662 units/l compared with 2678 ± 614 units/l]. The lack of a reduction in liver injury after rMnSOD treatment may be explained by intrahepatic accumulation

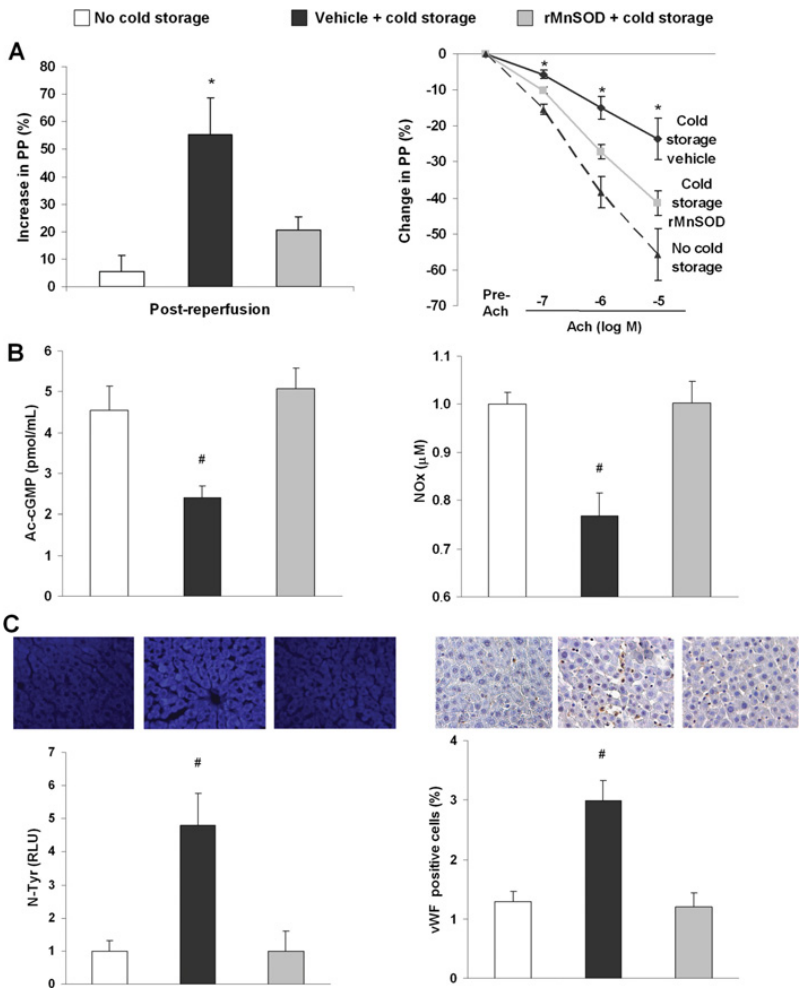

Figure 3 rMnSOD improves hepatic microcirculation and endothelial function in control livers

(**A**) Left: portal pressure increment observed during WR in livers not cold-stored or livers pre-treated with a single dose of rMnSOD, or its vehicle, and afterwards cold-stored for 16 h. Right: hepatic endothelial function evaluation by relaxation to incremental doses of Ach. (**B**) cGMP levels (left) and NOx release (right) in livers described in (**A**). (**C**) Representative images and quantitative analysis of nitrotyrosinated proteins fluorohistochemistry (left) and vWF immunohistochemistry (right) determined in livers described in (**A**) (×40 magnification). ($n = 8$ per group; $^{*}P < 0.05$ compared with no CS and rMnSOD; $^{\#}P < 0.01$ compared with no CS and rMnSOD).

of the SOD final product H_2O_2. Indeed, H_2O_2-scavenger systems glutathione and catalase were significantly diminished in steatotic livers undergoing CS + WR (Supplementary Figure S2 at http://www.clinsci.org/cs/127/cs1270527add.htm).

Cold-stored steatotic livers exhibited increased portal pressure upon reperfusion, together with acute endothelial dysfunction development compared with no cold-stored grafts (Figure 5A). These negative microcirculatory effects of CS + WR were significantly improved when steatotic rats were pre-treated with a single dose of rMnSOD.

In addition, an increase in hepatic nitrotyrosinated proteins and vWF-positive cells due to CS + WR was prevented in rMnSOD pre-treated rats (Figure 5B).

rMnSOD addition to cold-storage solution prevents oxidative stress accumulation in rat and human livers

The potential beneficial effects of rMnSOD as a supplement of a commercially available CS solution were evaluated in liver tissue from rats and humans. Figures 6(A) and 6(B) show significantly lower levels of O_2^- in control and steatotic rat hepatic tissues

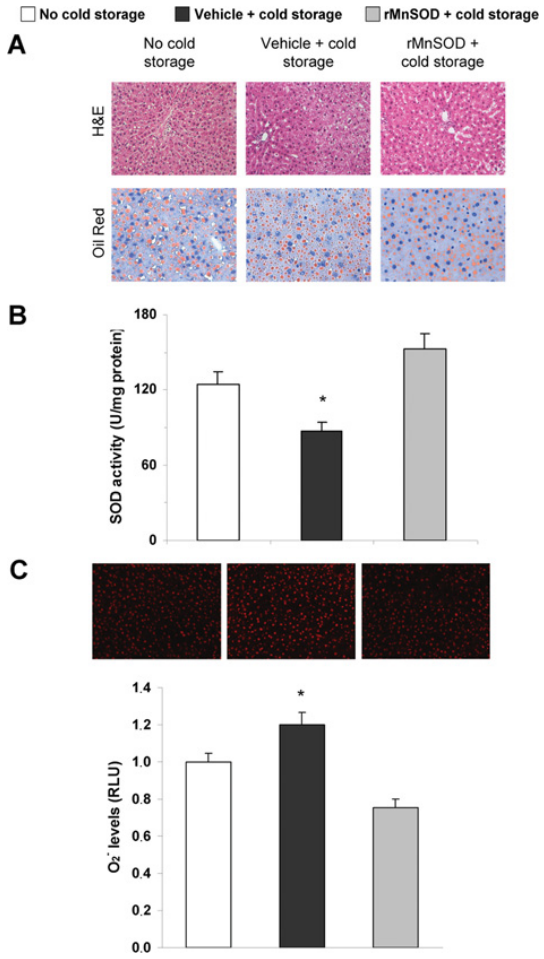

Figure 4 rMnSOD maintains SOD activity preventing O_2^- accumulation in severe steatotic liver grafts

Rats with severe steatosis due to 7-day HFD received a single intravenous dose of rMnSOD, or its vehicle, 30 min before graft explant, 16 h of CS and 1 h of WR. A control group of steatotic animals without the CS period was included. (**A**) Hepatic architecture status was assessed by H&E staining (upper panel; ×20) and advanced liver steatosis was confirmed by Oil Red staining (lower panel; ×40). (**B**) SOD activity evaluated in liver tissue. (**C**) Representative images of hepatic oxidative stress (O_2^-) detection using DHE (×20) and its corresponding quantification. ($n = 7$ per group; $^*P < 0.05$ compared with no CS and rMnSOD). RLU, relative light units.

cold-stored in Celsior solution supplemented with rMnSOD and afterwards warm reperfused, compared with livers cold stored in non-supplemented Celsior solution.

In addition, human liver biopsies preserved in rMnSOD-supplemented CS solution exhibited significantly lower intrahepatic oxidative stress levels than those preserved in standard Celsior solution, demonstrating the effectiveness of rMnSOD in human liver tissue (Figure 6C).

DISCUSSION

Endothelial phenotype de-regulation due to CS + WR injuries during transplantation is probably the first event in the development of graft dysfunction post-transplantation, which is followed by neutrophil recruitment and parenchymal damage [32,33]. Several features inherent to CS + WR negatively affect the endothelial phenotype. These include loss of haemodynamic stimulation and oxygen supply during cold ischaemia [8,34], as well as the production and accumulation of ROS upon reperfusion [35,36]. Previous studies from our group have focused on improving liver graft viability by maintenance of the Kruppel-like Factor 2 (KLF2) pathway [5,25]. Nevertheless, this novel therapeutic strategy had a limited effect in improving hepatic ROS accumulation. Previous studies aimed at decreasing oxidative stress in experimental models of liver ischaemia and reperfusion, including some using native SOD or derivatives, showed promising results; however, none of them reached clinical application probably because of poor stability of the antioxidant molecules and/or controversy in the use of adenoviral vectors in humans [15,37–40]. A new rMnSOD, which is constitutively active, has a good biodistribution, is stable in solution and freely enters cells, has been proposed as novel therapeutic agent for humans [17,18,41]. Therefore, the present study aimed at evaluating the effects of rMnSOD-lowering O_2^- accumulation as a new strategy to improve graft circulation, endothelial function and viability in experimental models of CS + WR.

We first characterized the impact of CS + WR, and the possible benefits of rMnSOD, on oxidative stress levels and NO bioavailability in primary cultured LSECs. In the present study, we demonstrate for the first time that CS + WR induces a marked increase in O_2^- levels in LSECs that is accompanied by a reduction in NO bioavailability. Importantly, rMnSOD blunts the O_2^- burst, which results in the maintenance of NO levels. These *in vitro* data suggest that rMnSOD may effectively improve hepatic vascular function and thereby viability of livers undergoing I/R injury.

This hypothesis was tested by administering a single dose of rMnSOD 30 min before graft procurement for transplantation in control and steatotic rats and determining the microcirculatory status and graft injury/viability after WR.

In control animals, and confirming previously reported findings [5,11,22], CS + WR induced liver damage, as shown by histological findings, increased aminotransferases and LDH release, which were accompanied by increased oxidative stress and inflammation, together with microcirculatory de-regulation and endothelium dysfunction manifested by loss of KLF2 and eNOS expression and abnormal endothelium-dependent vasorelaxation in response to incremental doses of Ach. Notably, our data show that rMnSOD pre-treatment almost entirely prevented endothelial dysfunction, microcirculatory damage and liver injury after CS + WR, probably due to the inhibition of ROS-mediated cell injury and ICAM-1 activation.

As stated above, endothelial protection during CS + WR is a key step in maintaining graft viability after transplantation; thus, we investigated the molecular mechanisms responsible for liver microcirculation protection by rMnSOD. The vasodilator

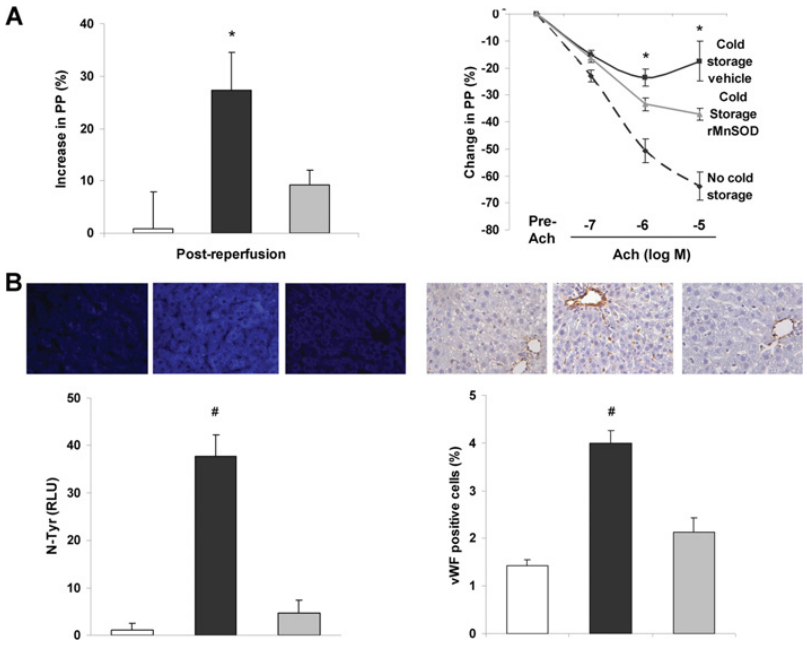

Figure 5 **rMnSOD ameliorates microcirculatory and endothelial dysfunction in severe steatotic liver grafts**
(**A**) Microcirculatory dysfunction was estimated as portal pressure increment during WR (left) and endothelial function as relaxation to incremental doses of Ach (right). (**B**) Detection of hepatic nitrotyrosinated proteins by fluorohistochemistry (left) and vWF expression by immunohistochemistry (right), with their corresponding quantifications ($n = 7$ per group; $^{*}P < 0.05$ compared with no CS and rMnSOD; $^{\#}P < 0.01$ compared with no CS and rMnSOD; images $\times 40$).

NO plays a critical role in modulating liver microcirculation, and its decreased availability is a marker of endothelial dysfunction [24,42]. It has been demonstrated that, when large amounts of O_2^- are found in the same environment as NO, this is rapidly scavenged to form peroxynitrite, which reduces NO bioavailability and increases vascular tone [13,43]. Therefore, we characterized the NO pathway in liver grafts included in the present study. These experiments showed that rMnSOD administration led to an increase in hepatic NO bioavailability as measured by two different final products, cGMP and NOx, without changes in eNOS expression, or in the expression of the vasoprotective transcription factor KLF2 that orchestrates eNOS expression (results not shown). In fact, the increase in NO was associated with a reduction in hepatic nitrotyrosinated proteins, a marker of peroxynitrite formation, thus confirming lower O_2^--mediated NO scavenging in livers from rMnSOD-treated rats. Together with low NO, elevated levels of the glycoprotein vWF have been associated with liver sinusoidal endothelial phenotype de-regulation [44,45]. Indeed, vWF is not expressed in healthy LSECs but, after acute or chronic injury, LSECs change their phenotypic pattern and express vWF. Thus, an increase in this protein is associated with endothelial dysfunction. Interestingly, in the present study, we demonstrate that vWF levels within the liver are rapidly increased after CS + WR, reinforcing the relatively novel concept of acute endothelial dysfunction development due to organ procurement for transplantation, and more importantly that rMnSOD pre-treatment prevents vWF up-regulation. Taken together, our findings suggest that rMnSOD efficiently maintains liver endothelial function after CS + WR, which ultimately will positively contribute to preserve global liver function and viability.

It is well known that liver steatosis is increasingly found in liver grafts donated for transplantation. Although moderate steatosis (30–60%) does not prevent transplantation, these grafts may exhibit worse post-operative viability and function [46]. Therefore, new therapeutic options to improve steatotic graft procurement are desirable, especially due to the rising trend in obesity affecting most developed countries. Accordingly, in the present study, we also investigated the possible beneficial effects of rMnSOD in the procurement of liver grafts with severe steatosis [11]. Our experiments confirm previous reports demonstrating that steatotic livers undergoing CS + WR exhibit enhanced parenchymal damage as shown by a more severe degree of histological changes, elevated transaminases and LDH release, and increased oxidative stress [47]. Furthermore, these steatotic grafts show impaired hepatic microcirculation after CS + WR [11], together with exacerbations in O_2^--mediated NO scavenging, and endothelial phenotype de-regulation. Pre-treatment with rMnSOD significantly reduced hepatic oxidative stress levels, which were associated with a reduction in peroxynitrites and vWF levels and improvement in hepatic microcirculation and endothelial function. Nevertheless, in spite of the beneficial effects of rMnSOD, no reduction in hepatic parenchymal damage was observed. These partially positive effects of rMnSOD

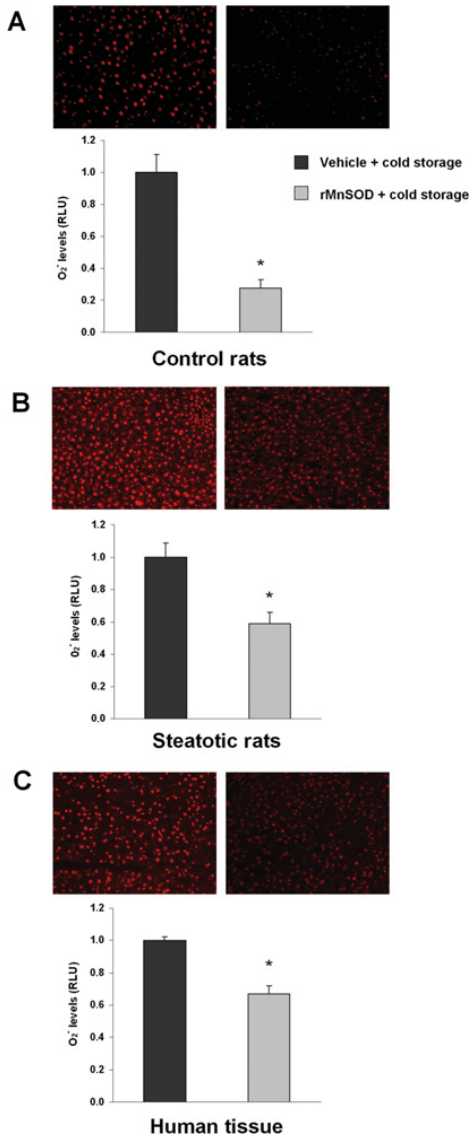

Figure 6 rMnSOD addition to cold storage solution prevents oxidative stress accumulation in rat and human livers

In situ hepatic O_2^- levels were determined in liver biopsies from control rats (**A**), advanced steatotic rats (**B**) and healthy humans (**C**) preserved for 16 h in Celsior solution supplemented with rMnSOD, or its vehicle, and afterwards warm-incubated for 1 h. (×20 magnification; $n = 5$ per condition; $^*P < 0.01$ compared with vehicle). RLU, relative light units.

in liver grafts with steatosis can be explained by an undesirable accumulation of H_2O_2 within the hepatocytes, partly due to insufficient activity/levels of catalase and glutathione, which may ultimately damage the parenchyma [48]. Future experiments evaluating the effects of combined antioxidant therapies are desirable.

Owing to the possible controversies caused by pharmacological pre-treatment of the donor, we evaluated the option to supplement a commercially available CS solution with rMnSOD to prevent ROS accumulation in liver tissues from healthy and steatotic rats and, importantly, also from healthy humans. In all cases, we found that addition of rMnSOD to the preservation solution prevents O_2^- accumulation derived from CS + WR. These results, comparable with those observed with the pre-treatment strategy, demonstrate the effectiveness of this new recombinant protein preventing oxidative stress accumulation in human liver, thus suggesting a global improvement in hepatic CS + WR injury.

Conclusions

The results of the present study demonstrate that donor pre-treatment with rMnSOD shortly before graft procurement protects the liver parenchyma in healthy rat donors and maintains a correct microcirculatory status in both control and steatotic rats. Although additional experimental transplantation studies, where polymorphonuclear neutrophils and other relevant inflammatory cells will contribute to CS + WR injury, are required, we propose rMnSOD as a new and highly effective supplement of the preservation solution for the procurement of liver grafts for transplantation.

CLINICAL PERSPECTIVES

- Liver transplantation is the unique solution for several end-stage liver diseases. Non-steatotic, but especially steatotic, grafts are highly susceptible to I/R injury, exhibiting poor viability and function upon transplantation. One of the main underlying mechanisms of I/R injury is oxidative stress accumulation.
- A single intravenous administration of rMnSOD to the donor shortly before organ procurement significantly improves graft function and prevents microcirculatory derangements, in both non-steatotic and steatotic rats. Addition of rMnSOD to a commercially available CS solution retains its antioxidant properties, inhibiting oxidative stress accumulation in rat and human livers procured for transplantation.
- Pre-treatment of healthy and extended-criteria organ donors with a single dose of rMnSOD could improve the clinical results of liver transplantation and, more importantly, it would increase organ donor pool for transplantation.

AUTHOR CONTRIBUTION

Diana Hide designed the research, conceived the study, performed the experiments, analysed the data and wrote the manuscript. Martí Ortega-Ribera and Anabel Fernández-Iglesias performed the experiments and analysed the data. Constantino Fondevila provided essential materials and critically revised the manuscript. Josepa Salvadó analysed the data and critically revised the manuscript. Lluís Arola and Juan García-Pagán critically revised the manuscript. Aldo Mancini provided essential reagents and critically revised the manuscript. Jaime Bosch conceived the study, critically revised the manuscript and obtained funding. Jordi Gracia-Sancho designed the research, conceived the study, wrote the manuscript, obtained funding and directed the study. All authors edited and reviewed the final manuscript.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischaemia and reperfusion injury

Diana HIDE*, Martí ORTEGA-RIBERA*, Anabel FERNÁNDEZ-IGLESIAS†, Constantino FONDEVILA‡, M. Josepa SALVADÓ†, Lluís AROLA†, Juan Carlos GARCÍA-PAGÁN*, Aldo MANCINI§, Jaime BOSCH* and Jordi GRACIA-SANCHO*

*Barcelona Hepatic Hemodynamic Laboratory, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Universitat de Barcelona, Barcelona, Spain
†Nutrigenomics Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain
‡Liver Surgery and Transplantation Unit, IDIBAPS, Hospital Clínic de Barcelona, CIBERehd, Universitat de Barcelona, Spain
§Molecular Biology and Viral Oncogenesis, National Cancer Institute, Naples, Italy

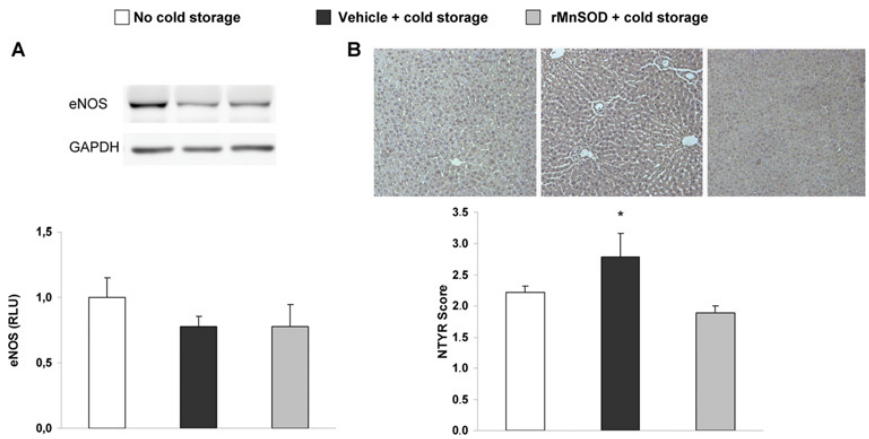

Figure S1 **Effects of CS + WR and rMnSOD on eNOS and nitrotyrosination in control rat livers**
(**A**) Representative images of hepatic eNOS immunoblot and densitometric analysis normalized to GAPDH. (**B**) Detection of hepatic nitrotyrosinated (NTYR) proteins by immunohistochemistry with the corresponding quantification (×20). ($n = 8$ per group; $^*P < 0.05$ compared with no CS and rMnSOD).

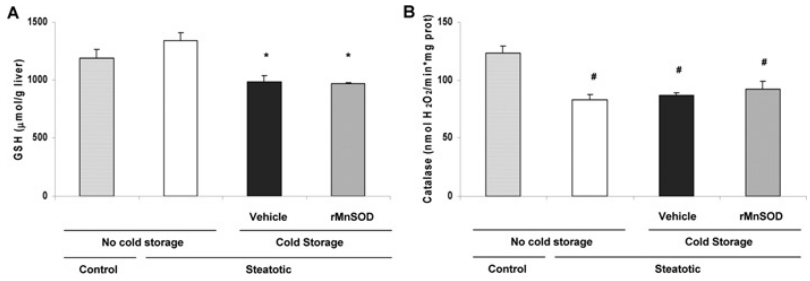

Figure S2 **Effects of CS + WR on H_2O_2-scavenger systems in steatotic rat livers**
Total glutathione (GSH) levels (**A**) and catalase activity (**B**) were evaluated in liver homogenates ($n = 7$ per group; $^{*}P < 0.05$ compared with no CS; $^{\#}P < 0.05$ compared with control).

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STUDY 2. Effects of warm ischemia and reperfusion on the liver microcirculatory phenotype of rats: underlying mechanisms and pharmacological therapy

Diana HIDE, Martí ORTEGA-RIBERA, Juan-Carlos GARCIA-PAGAN, Carmen PERALTA, Jaime BOSCH, Jordi GRACIA-SANCHO

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Effects of warm ischemia and reperfusion on the liver microcirculatory phenotype of rats: underlying mechanisms and pharmacological therapy

Diana Hide[1], Martí Ortega-Ribera[1], Juan-Carlos Garcia-Pagan[1], Carmen Peralta[2], Jaime Bosch[1] & Jordi Gracia-Sancho[1]

Warm ischemia and reperfusion (WIR) causes hepatic damage and may lead to liver failure, however the mechanisms involved are largely unknown. Here we have characterized the microcirculatory status and endothelial phenotype of livers undergoing WIR, and evaluated the use of simvastatin in WIR injury prevention. Male Wistar rats received simvastatin, or vehicle, 30 min before undergoing 60 min of partial warm ischemia (70%) followed by 2 h or 24 h of reperfusion. Hepatic and systemic hemodynamics, liver injury (AST, ALT, LDH), endothelial function (vasodilatation in response to acetylcholine), KLF2 and nitric oxide pathways, oxidative stress, inflammation (neutrophil and macrophage infiltration) and cell death were evaluated. Profound microcirculatory dysfunction occurred rapidly following WIR. This was evidenced by down-regulation of the KLF2 vasoprotective pathway, impaired vasodilatory capability and endothelial activation, altogether leading to increased hepatic vascular resistance and liver inflammation, with significant leukocyte infiltration, oxidative stress and cell death. Simvastatin preserved the hepatic endothelial phenotype, and blunted the detrimental effects of WIR on liver hemodynamics and organ integrity. In conclusion, WIR-induced injury to liver sinusoidal endothelial cells is mitigated by pre-treatment with Simvastatin probably through a KLF2-dependent mechanism.

Ischemia/reperfusion injury is the phenomenon of interruption of blood supply followed by restoration of blood flow and the accompanying oxygen, nutrient supply and shear stress. Clinically, warm ischemia/reperfusion (WIR) injury is almost unavoidable in liver resection surgery, liver transplantation, and in blood transfusion for hemorrhagic shock, and may lead to delayed graft function and liver failure. Two different phases can be distinguished during reperfusion: an early phase (within 2 h after restoring reperfusion), which is characterized by the release of reactive oxygen species (ROS) and production of inflammatory mediators (TNFα, chemokines)[1,2], and a late phase (6–48 h after reperfusion), in which inflammatory responses caused by neutrophil and macrophage infiltration exacerbate the liver damage[3]. As demonstrated recently by our group, the effects of ischemia reperfusion are already significant under cold ischemia when further deterioration of hepatic microcirculation and endothelial dysfunction is seen[4].

Kruppel-like factor 2 (KLF2) is a transcription factor predominantly expressed by the endothelial cell[5] that induces expression of vasodilator, anti-thrombotic and anti-inflammatory genes (e.g. endothelial nitric oxide synthase (eNOS) and thrombomodulin)[6,7] and inhibits the expression of adhesion molecules (vascular cell adhesion molecule 1 (VCAM-1) and E-selectin)[6,8] maintaining a vasoprotective endothelial phenotype. Experimental studies using endothelial cells have demonstrated that KLF2 expression is induced by physiological blood flow-derived shear stress[9,10].

Statins are HMG-CoA inhibitors originally designed to lower cholesterol levels, however they have shown other therapeutic effects independent on lipid lowering[11]. Administration of simvastatin in experimental models

[1]Barcelona Hepatic Hemodynamic Lab. IDIBAPS Biomedical Research Institute – Hospital Clinic de Barcelona – CIBEREHD. Barcelona, Spain. [2]IDIBAPS & CIBEREHD. Correspondence and requests for materials should be addressed to J.G.-S. (email: jgracia@clinic.cat)

of chronic and acute liver injury has demonstrated its effectiveness in protecting the liver sinusoidal endothelium[12,13,4], nevertheless its potential applicability in situations of hepatic WIR is unknown. These beneficial effects of statins are due in part to the activation of KLF2 pathway that contributes in maintaining/restoring a healthy endothelial phenotype[14,15].

In the present study we have characterized for the first time the hepatic microcirculatory status and sinusoidal endothelial phenotype of livers undergoing warm ischemia and reperfusion injuries, and evaluated the applicability of simvastatin to improve or prevent warm ischemia/reperfusion injury.

Results

WIR causes microcirculatory derangements and endothelial dysfunction. Livers undergoing warm ischemia and reperfusion (WIR) exhibited significantly impaired microcirculation evidenced by a marked increase in intrahepatic vascular resistance at 2 h and 24 h of reperfusion, associated with increased portal pressure and a reduction of portal blood inflow (Fig. 1a). Furthermore, WIR promoted the development of acute endothelial dysfunction defined as a reduced response to the endothelial-dependent vasodilator acetylcholine (Fig. 1b) and an increase in the sinusoidal expression of the liver sinusoidal endothelial cells (LSEC) capillarization marker von Willebrand Factor (vWF, Fig. 1b). The detrimental effects of WIR on liver endothelial function observed at 2 h of reperfusion were exacerbated after 24 h (Fig. 1b).

In addition, WIR caused a significant increase in the release of transaminases and LDH compared to sham operated animals especially at the early phase of reperfusion (Fig. 1c). At 24 h of reperfusion these parameters were decreased but still were significantly higher than in sham animals.

WIR reduces KLF2 expression and NO bioavailability. WIR caused a reduction in KLF2 liver protein expression at the early and late phases of reperfusion (Fig. 2a left), which was accompanied by a decrease in phosphorylated eNOS (Fig. 2a right) and a diminution in NO bioavailability (Fig. 2b). O_2^- production and nitrotyrosine formation (caused by the scavenging of NO by O_2^-) were significantly increased at both time points of reperfusion (Fig. 2c).

WIR promotes hepatic inflammation and cell death. WIR injury induced a rapid activation of the hepatic endothelium, as demonstrated by the increases in P-Selectin (5.8 fold and 2.3 fold increments at 2 h and 24 h, respectively, $p < 0.05$) and VCAM-1 (Fig. 3), which was associated with subsequent infiltration and accumulation of macrophages and, especially, neutrophils within the liver parenchyma (Fig. 3). In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed that there was a significant 15-fold increase in cell death after 2 h of reperfusion, which was partly resolved at 24 h but remaining significantly higher than in sham operated animals (Fig. 3).

Simvastatin improves WIR-mediated liver microcirculatory dysfunction. Simvastatin administration 30 minutes before ischemia prevented the WIR-derived increment in intrahepatic vascular resistance, thus improving liver perfusion without changes in portal pressure (Fig. 4a). In addition, simvastatin significantly attenuated the development of acute endothelial dysfunction both at 2 and 24 h of reperfusion as evidenced by improved vasodilation in response to Ach (Fig. 4b top) and reduced sinusoidal vWF expression (Fig. 4b bottom). The improvement in hepatic hemodynamics was associated with a much less marked increase in transaminases and lactate dehidrogenase (LDH) release (Fig. 4c).

Simvastatin prevents KLF2 down-regulation and maintains NO bioavailability. Administration of simvastatin prior to ischemia maintained liver KLF2 protein expression and eNOS phosphorylation levels, which was especially evident at 2 h of reperfusion (Fig. 5a). This was accompanied by higher NO bioavailability (Fig. 5b), and by decreased O_2^- production and O_2^--mediated NO scavenging (Fig. 5c).

Simvastatin administration inhibits endothelial activation, inflammation and cell death. Liver endothelial phenotype maintenance due to simvastatin administration prevented an increased expression in adhesion molecules (P-Selectin 55% lower at 2 h of reperfusion, and 98% at 24 h vs. vehicle-treated rats, $p < 0.05$; VCAM-1 Fig. 6), and neutrophil and macrophage infiltration (Fig. 6), both at the early and late phases of reperfusion. Moreover, simvastatin-treated animals showed a significant reduction in hepatic cells death (Fig. 6).

Simvastatin increases KLF2 and improves the phenotype of hepatic cells *in vitro*. Simvastatin increased KLF2 mRNA expression not only in LSEC but also in primary hepatocytes and Kupffer cells (Supplementary Fig. S1a). This correlated with a slight improvement in the hepatocyte phenotype observed by a trend to an increase in the transcription factor HNF4α and a reduction in the Mrp3 transporter expression (Supplementary Fig. S1b). Regarding Kupffer cells, simvastatin treatment induced a polarization towards an anti-inflammatory M2 phenotype, evidenced by significant increases in the expression of Arg-1 and IL-10, without significant changes in the markers of M1 phenotype TNF-α and iNOS (Supplementary Fig. S1c).

LSEC treated with simvastatin showed a statistically significant 8-fold and 2.6-fold increase in KLF2 and eNOS mRNA expression respectively after reperfusion. This was associated with a decrease in the mRNA expression of the pro-inflammatory & capillarization markers iNOS (1.00 ± 0.16 vs. 0.76 ± 0.19) and VCAM-1 1.00 ± 0.06 vs. 0.85 ± 0.14). Furthermore, LSEC pre-treated with simvastatin exhibited lower levels of oxidative stress, measured as O_2^-, compared with vehicle-treated cells (1.00 ± 0.18 vs. 0.72 ± 0.18; $p = 0.1$).

Discussion

Liver sinusoidal endothelial cells (LSEC) damage following ischemia reperfusion (IR) injury is the first event in the development of graft failure. Although this was already suggested in the late 1980 s[16], most research to date

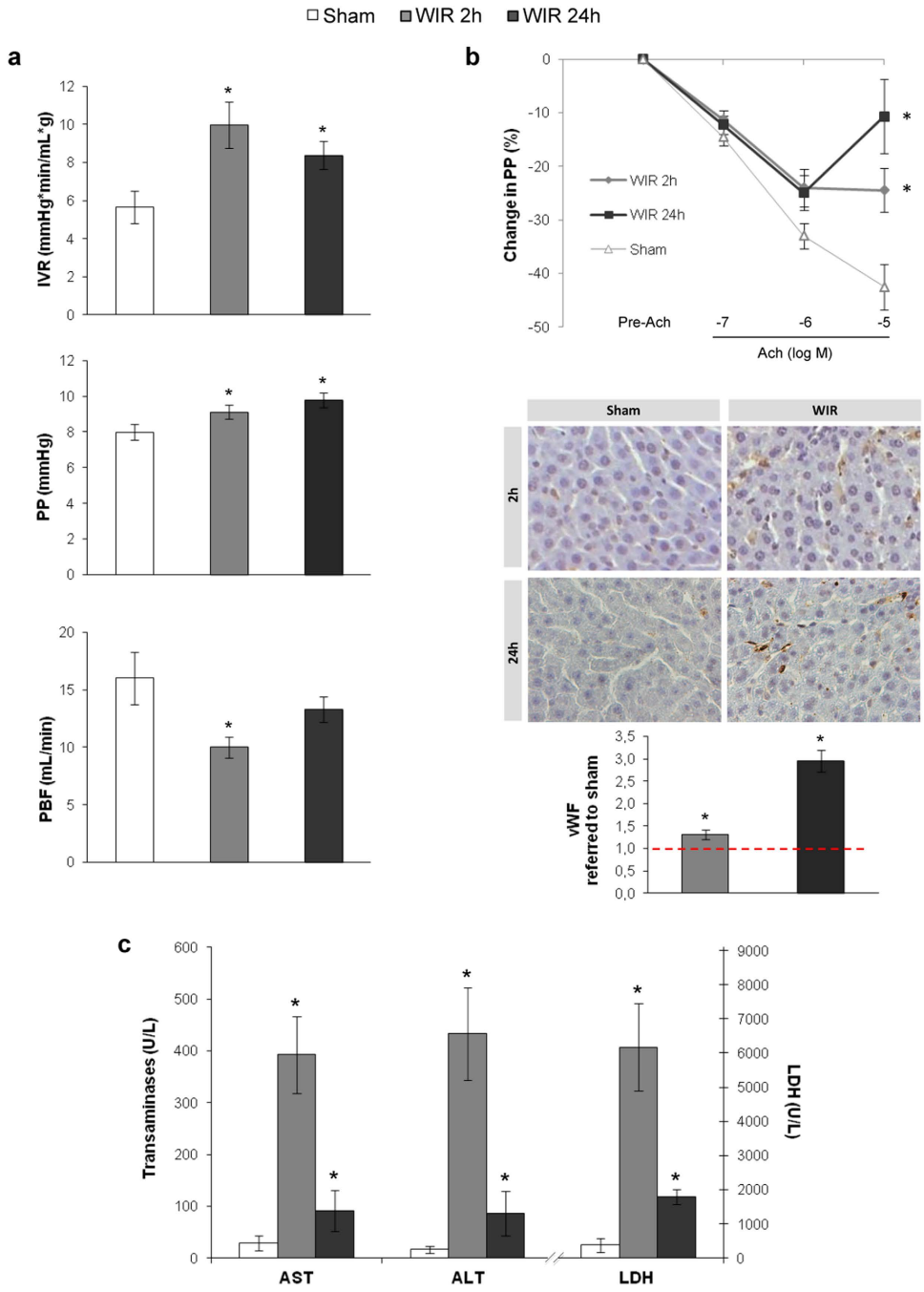

Figure 1. Liver warm ischemia and reperfusion leads to acute hepatic microvascular dysfunction. (**a**) Intrahepatic vascular resistance (IVR), portal pressure (PP) and portal blood flow (PBF) determined in rats that underwent 70% hepatic warm ischemia followed by 2 h or 24 h of reperfusion (WIR). **(b)** Hepatic endothelial function evaluation analyzing relaxation response to incremental doses of Ach (top) and vWF protein expression (down) in rats described in A. Representative images (40 × magnification). **(c)** Hepatic injury evaluated as release of transaminases (ALT, AST) and LDH in liver perfusate from rats described in A. ($n = 8$ per group; $^*p < 0.05$ vs. sham).

has focused on other liver cell types. It is accepted that after IR there is an increase in LSEC ROS, together with an imbalance between decreased NO bioavailability (due to reduced production by eNOS and increased scavenging

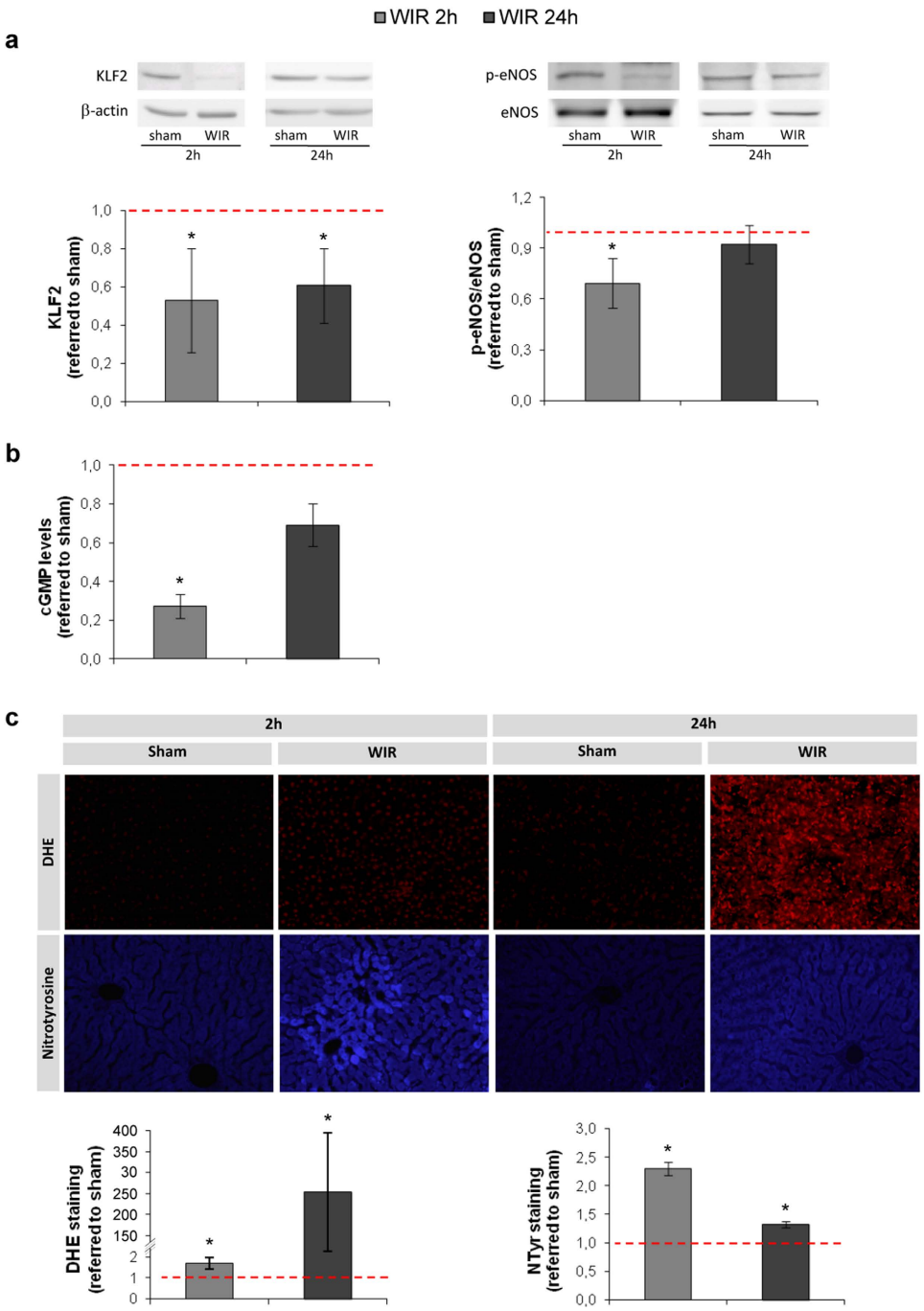

Figure 2. Hepatic warm ischemia and reperfusion induces a rapid down-regulation in KLF2 and its derived vasoprotective programs. **(a)** Representative images of hepatic KLF2, phosphorylated eNOS (p-eNOS) and total eNOS immunoblots and densitometric analysis normalized to β-actin from rats undergoing 1 h of partial ischemia followed by 2 h or 24 h of reperfusion (WIR), compared to the sham group. **(b)** cGMP levels in livers described in A. **(c)** Representative images and quantitative analysis of DHE staining and nitrotyrosinated proteins fluorohistochemistry (20 × magnification) (n = 8 per group; *p < 0.05 vs. sham). The red dotted line represents the sham group.

by ROS) and increased endothelin and thromboxane A2 levels, altogether promoting the expression of adhesion molecules, neutrophil adhesion and platelet aggregation[17–19]. However elucidation of the underlying mechanisms

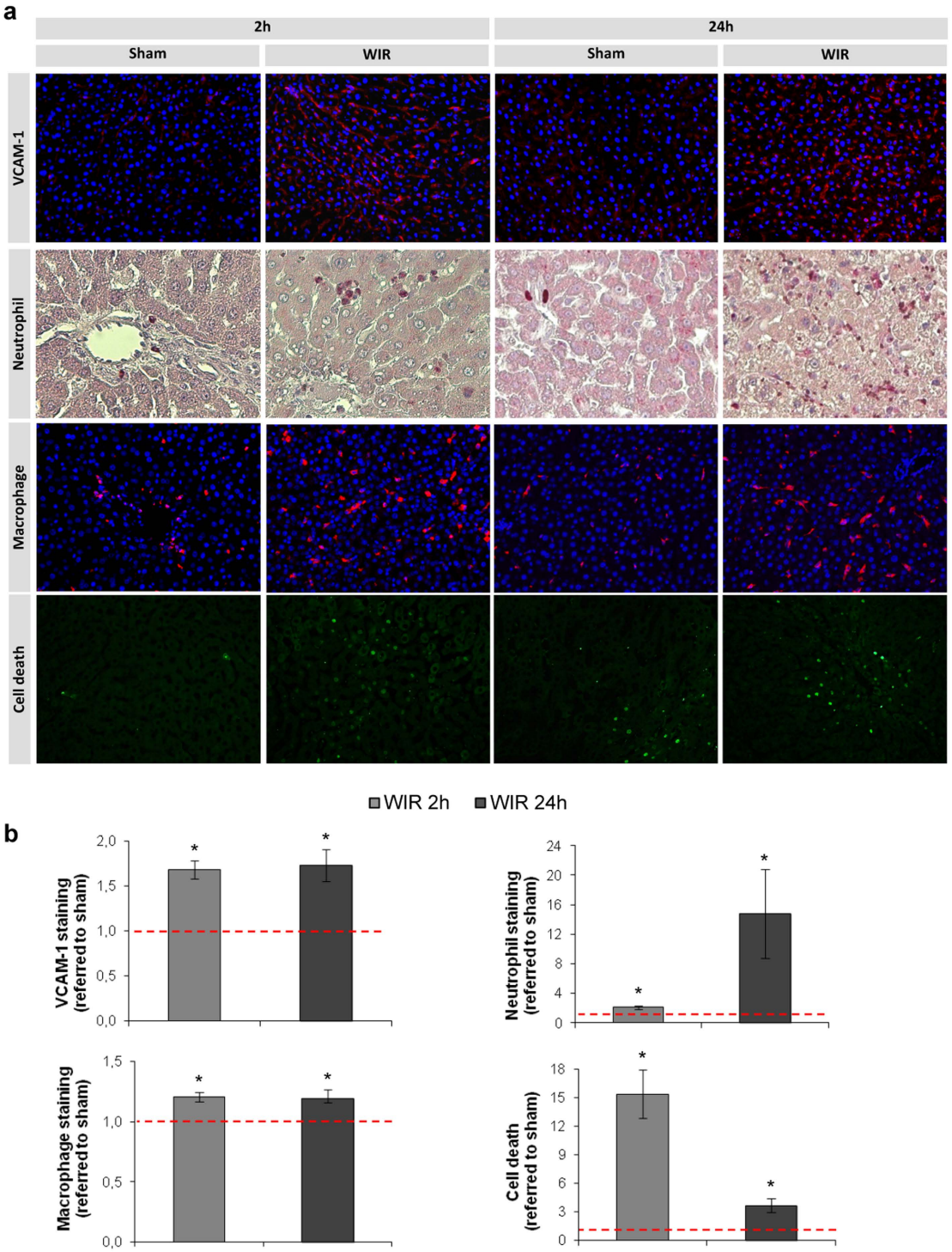

Figure 3. Liver warm ischemia and reperfusion promotes endothelial cell activation, leukocyte infiltration and cell death. (a) Representative images of VCAM-1 and CD68 immunohistochemistry, neutrophil infiltration and TUNEL staining determined in livers from rats that underwent 1 h of partial hepatic ischemia followed by 2 h or 24 h of reperfusion (WIR) (neutrophil magnification 40×, other 20×); **(b)** quantitative analysis of at least 8 fields/sample referred to its sham group (red dotted line). (n = 8 per group, *$p < 0.05$ vs. sham).

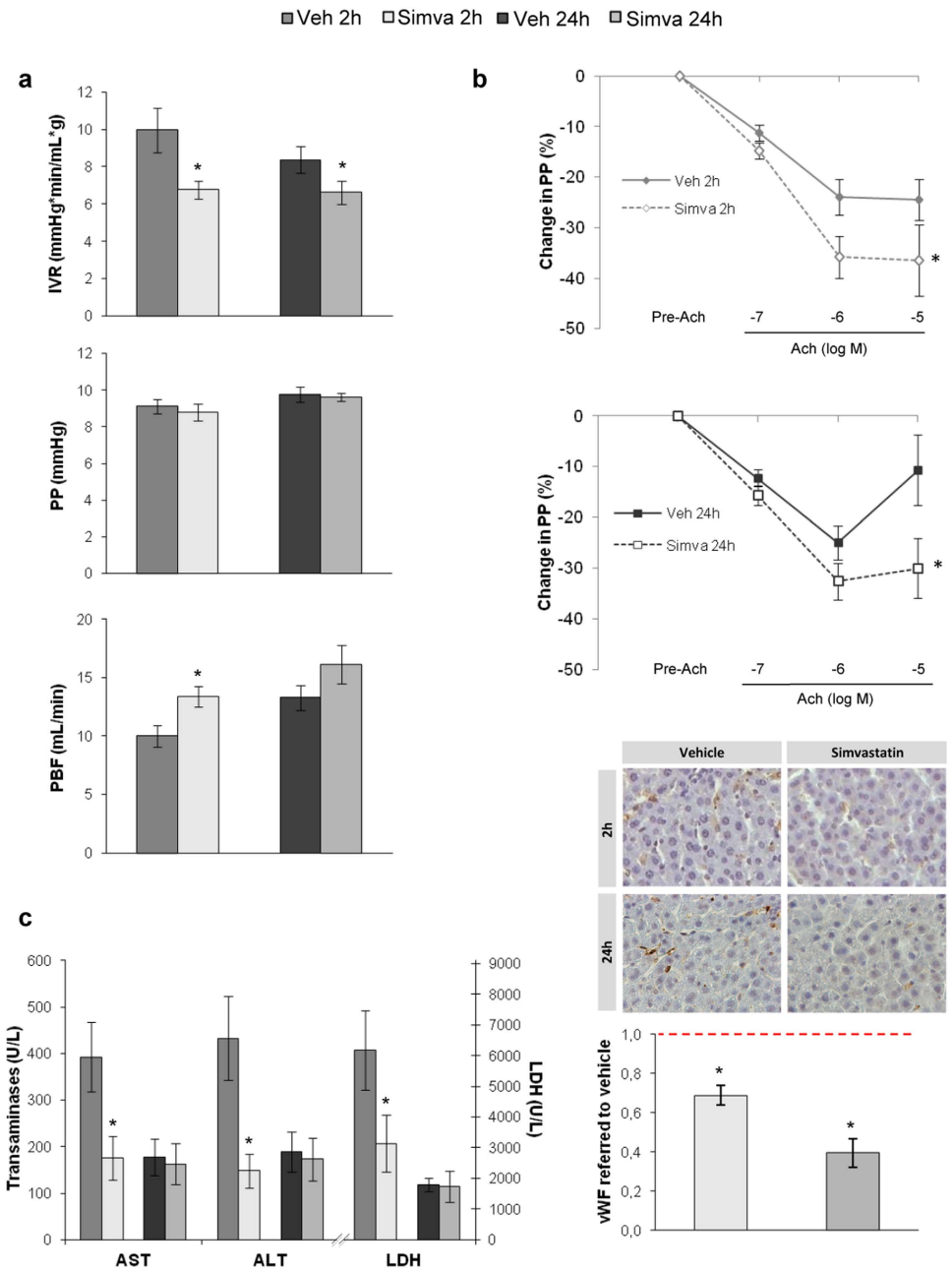

Figure 4. Simvastatin prevents liver vascular dysfunction produced by warm ischemia and reperfusion injury. (**a**) Intrahepatic vascular resistance (IVR), portal pressure (PP) and portal blood flow (PBF) in rats pre-treated with vehicle or simvastatin and afterwards undergoing liver warm ischemia and reperfusion. **(b)** Endothelial function evaluation (top) and vWF immunohistochemistry (down) in animals described in A (40 × magnification), normalized to its corresponding vehicle-treated group (red dotted line). **(c)** Hepatic injury evaluation. (n = 8 per group; *$p < 0.05$ vs. vehicle).

of LSEC damage during hepatic IR injury, and its impact on the global reduction in organ viability and function after liver resection or transplantation is scarce. In this work we have characterized the hepatic microcirculation in a well-established model of warm ischemia and reperfusion of the liver, focusing predominantly on changes in LSEC phenotype, its mechanisms, consequences and prevention.

In the present study, we demonstrate for the first time that a short period of warm ischemia is enough to cause striking deleterious effects on the hepatic microcirculation markedly increasing the intrahepatic vascular

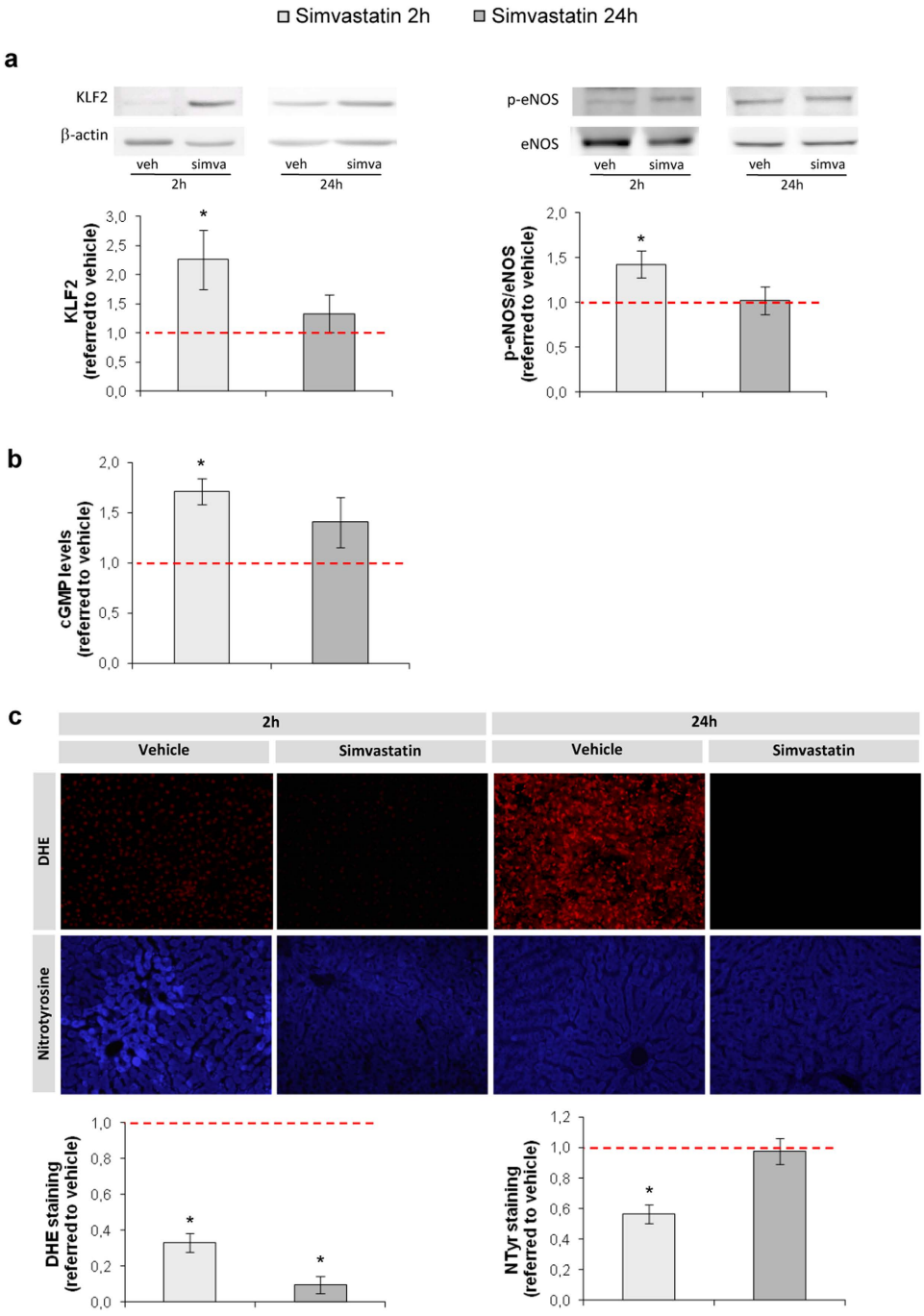

Figure 5. Simvastatin pre-treatment maintains the KLF2-derived vasoprotection during warm ischemia and reperfusion injury. **(a)** Representative images of hepatic KLF2, p-eNOS and eNOS immunoblots and densitometric analysis normalized to β-actin. **(b)** Hepatic cGMP levels. **(c)** Representative images and quantitative analysis of DHE staining and nitrotyrosinated proteins fluorohistochemistry (20×). All quantifications normalized to its corresponding vehicle (red dotted line). (n = 8 per group; *$p < 0.05$ vs. vehicle).

resistance (IVR), both at the early and late phases of reperfusion, leading to increased portal pressure and to a significant reduction in hepatic perfusion. Furthermore, animals suffering WIR develop acute endothelial dysfunction evidenced by a decreased response to the endothelial dependent vasodilator acetylcholine[20] and by increased

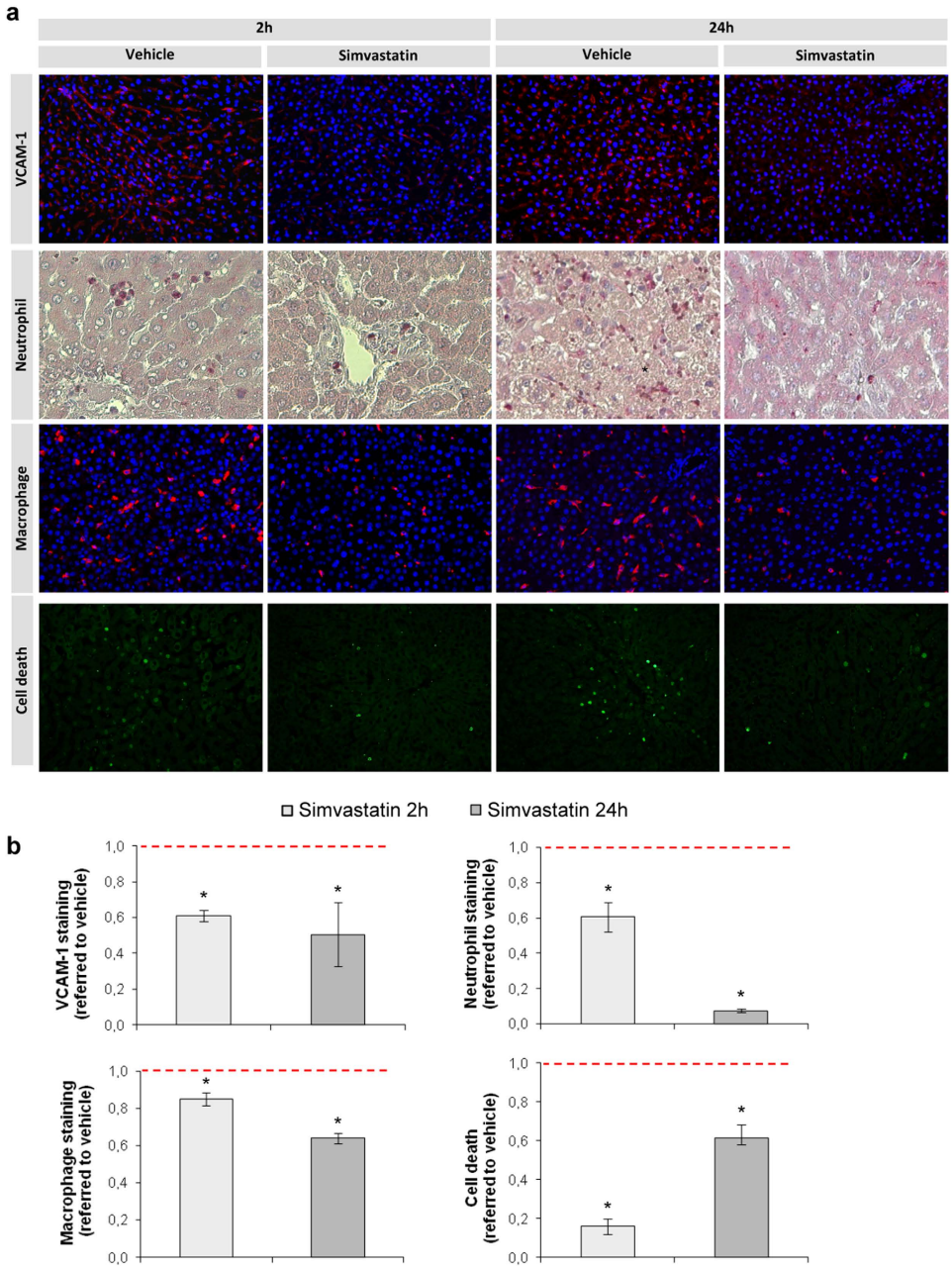

Figure 6. Simvastatin reduces hepatic endothelial activation, leukocyte infiltration and cell death derived from warm ischemia and reperfusion injury. Representative images of VCAM-1, neutrophil and macrophage infiltration, and cell death detection in livers from rats pre-treated with simvastatin, or its vehicle, before undergoing 1 h of warm ischemia followed by 2 h or 24 h of reperfusion (**a**) and its corresponding quantifications (**b**). Neutrophil 40×, other 20×, values normalized to its vehicle group (red dotted line). (n = 8 per group, *$p < 0.05$ vs. vehicle).

expression of vWF[21,22]. In addition WIR caused liver damage, measured as increments in AST, ALT and LDH, as described previously[23,24], especially at 2 h of reperfusion. At the late phase of reperfusion, levels of transaminases and LDH seem to improve most likely due to an initial resolution of liver injury; nevertheless they remain significantly higher than in sham-operated animals.

It has been described that blood flow-derived shear stress maintains a vasoprotective endothelial phenotype owing to the activation of the transcription factor KLF2, which mediates the transcription of several protective

genes[8,9,25]. Indeed, recent studies have demonstrated that lack of the biomechanical stimulus determined by shear stress deteriorates the endothelial phenotype by down-regulating the expression of KLF2[4,10]. Thus, we hypothesized that during WIR blood inflow blockade may also decrease liver endothelial KLF2 expression, and this reduction will cause a loss of liver vasoprotection during reperfusion.

To evaluate our hypothesis we measured KLF2 protein expression in liver tissue observing a significant reduction after WIR, both at the early and the late phases of reperfusion. Interestingly, this decrease was accompanied by a reduction in eNOS activation, one of KLF2 target genes. We further evaluated the bioavailability of the vasodilator NO by measuring the levels of cyclic guanosine monophosphate (cGMP) as a second messenger of NO and found a significant reduction in cGMP levels at the early phase of reperfusion. The reduction in NO bioavailability is explained by a combination of two factors: less synthesis due to reduced eNOS activity and; increased NO-scavenging by the high amount of O_2^- produced during reperfusion. This scavenging causes the formation of the free radical peroxynitrite that may rapidly react with cell components such as proteins, lipids, and DNA further damaging the cell[26].

We further characterized the consequences of liver endothelial de-regulation during WIR by analyzing liver inflammation at two reperfusion time-points: early (2 h) and late (24 h). We evaluated hepatic VCAM-1 and P-selectin protein expression due to their implication in neutrophil adhesion and extravasation[27–29] and found an up-regulation at early and late reperfusion periods. Indeed, neutrophil infiltration was slightly increased after 2 h of reperfusion and greatly increased at 24 h. Furthermore macrophage activation and infiltration was also significantly increased at both reperfusion times. As it has been described, leukocytes produce pro-inflammatory cytokines and ROS further contributing to tissue damage[30,31], this can also be observed in our results, with a vast O_2^- production at the late reperfusion phase. Microvascular dysfunction and parenchymal damage associated with WIR induced hepatic cell death.

Statins or HMG-CoA inhibitors were primarily designed to decrease cholesterol levels but they have also shown to have beneficial cholesterol-independent effects such as up-regulating KLF2-derived transcriptional programs that improve endothelial function[14,32]. Considering the detrimental effects of WIR on liver microcirculation, KLF2 vasoprotective cascades, and recent data from our group demonstrating the beneficial effects of simvastatin in the cirrhotic liver[33], we have applied an acute pharmacological pre-treatment with simvastatin to preserve liver endothelial function during WIR. Some previous experimental studies have applied statins to WIR and have shown benefits in terms of organ injury and inflammation[34–38], however they have not evaluated the effects of statins on liver microvascular function and phenotype, especially when administered shortly before ischemia, thus discarding possible concomitant effects of lipid-lowering.

Our results demonstrate that simvastatin administration 30 min before ischemia prevents the increase in hepatic vascular resistance during reperfusion, and that is associated with better liver perfusion at early and late reperfusion periods. Simvastatin pre-treatment also improved liver endothelial function and prevented the peak in transaminases and LDH release observed at 2 h of reperfusion.

Evaluation of the KLF2 pathway revealed that simvastatin is able to prevent liver KLF2 protein expression decay observed at the early reperfusion period, thus maintaining the activation of its target gene eNOS, enhancing NO bioavailability. Moreover, simvastatin-treated animals showed reduced levels of ROS. This might be explained by the antioxidant properties of KLF2 through activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and up-regulation of heme oxygenase 1 (HO-1)[39,33]. Finally, we found that simvastatin prevented the expression of adhesion molecules such as VCAM-1 and P-selectin on the endothelial surface reducing leukocyte infiltration, which may also be attributed to the anti-inflammatory properties of KLF2[6,40].

The results obtained *in vivo* were validated *in vitro*. LSEC cultured in an anoxia/reoxygenation system showed that simvastatin was able to partly abrogate the burst in oxidative stress and inflammation due to I/R, most probably due to the induction of the KLF2 protective pathway[10]. Furthermore, we herein demonstrate for the first time that simvastatin also activates the KLF2 pathway in other hepatic cells (ie. Hepatocytes and Kupffer cells), in which it also confers a protective phenotype. These observations complement previous data demonstrating the beneficial effects of statins in another non-parenchymal cell type, the hepatic stellate cells[33], altogether reinforcing the concept of global hepatic protection in response to statins.

Despite the positive results achieved in this study using simvastatin in the setting of WIR injury, we have to acknowledge some limitations. First, the model of partial ischemia without hepatic resection, although representative of the WIR that occurs *in vivo* after episodes of shock and/or hypovolemia, does not allow extrapolating what happens in liver resection surgery. On the other hand, the role of KLF2 in protection against liver WIR could be further delineated using genetic models where KLF2 expression would be knocked-down specifically.

In summary, the results herein reported shed light on the pathophysiology of liver ischemia reperfusion injury demonstrating that a warm ischemia period, even of short duration, profoundly affects the liver endothelial phenotype, which becomes dysfunctional, leading to an immediate increase in the hepatic vascular tone, inflammation, polymorphonuclear cells infiltration, and hepatic cell death. WIR-derived damage to the hepatic endothelium may be, at least in part, due to KLF2 down-regulation. Indeed, simvastatin pre-treatment maintains KLF2 vasoprotective pathways and efficiently protects the hepatic microcirculation from WIR and prevents subsequent liver injury. Our data strongly suggest that simvastatin administration may represent an effective and simple approach to prevent or reduce liver damage in clinical situations associated with warm ischemia-reperfusion liver injury.

Methods

Animals and treatment. Male Wistar rats from Charles River Laboratories (Barcelona, Spain) weighting 300–350 g were used. Rats were treated with simvastatin (1 mg/kg i.v., Calbiochem, San Diego, CA) or its vehicle (DMSO 0.1%) 30 minutes before ischemia. Animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS). All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance

ith European Community guidelines for the protection of animals used for experimental or other scientific urposes (EEC Directive 86/609).

iver Vascular Studies. Under anesthesia with intraperitoneal ketamine (100 mg/Kg, Merial Laboratories, arcelona, Spain) and midazolam (5 mg/Kg, Normon, Tres Cantos, Madrid, Spain) partial warm ischemia affect-ıg 70% of liver volume was induced by clamping the portal triad irrigating the medial and left lateral lobes with ı atraumatic clamp for 1 h[41], which was followed by 2 h (short-term) or 24 h (long-term) of reperfusion. Sham perated animals were included. After the WIR period, hemodynamic studies were performed as previously escribed[42]. Briefly, a tracheotomy was performed and a polyethylene PE-240 tubing was inserted into the trachea ɔ ensure a patent airway. PE-50 catheters were introduced into the femoral artery, for arterial pressure recording nm Hg), and into the portal vein through the ileocolic vein, to measure portal pressure (mmHg). Then, the ortal vein was carefully dissected free from connective tissue, and a non-constrictive perivascular transit-time ltrasonic flow probe (Transonic Systems, Ithaca, New York, USA) was placed around the vessel. The flow probe as connected to a flow-meter, to measure the portal vein blood flow (mL/min). Intrahepatic vascular resist-ıce (IVR; mmHg/mL·g·min^{-1}) was calculated as: portal pressure/(portal vein blood flow/liver weight). Blood ressures and flows were registered on a multichannel computer-based recorder (PowerLab; ADInstruments, olorado Springs, Colorado, USA). The external zero reference point was placed at the midportion of the animal. lemodynamic data were collected after a 20-min stabilization period.

Immediately after the *in vivo* hemodynamic study, liver vascular responses were assessed in the isolated, *ı situ* liver perfusion system, as described[42]. Livers were perfused at a constant portal flow of 35 mL/min and after ɔ minutes of stabilization, liver endothelial function was evaluated analyzing endothelium-dependent vasorelaxa-on to incremental doses of acetylcholine (Ach; 10^{-7} to 10^{-5} M) after pre-constriction with methoxamine (10^{-4} M).

Aliquots of the perfusate were sampled for the measurement of transaminases and lactate dehydrogenase LDH) using standard methods at the Hospital Clinic of Barcelona's CORE laboratory. At the end of the study, ver samples from lobules that suffered WIR were stored for molecular analysis as described below.

iver cells isolation and treatments. Hepatocytes, Kupffer cells and liver sinusoidal endothelial cells LSEC) were isolated from control rat livers by collagenase digestion followed by percoll gradient[43].

Hepatocytes and Kupffer cells were treated with simvastatin (5 μM) or its vehicle for 24 h. LSEC were treated ith simvastatin (1 μM) or its vehicle 1 h before undergoing 1 h anoxia using a BD Gaspak™ System followed by 4 h reoxygenation.

'on Willebrand Factor and P-Selectin immunohistochemistry. Liver samples were fixed in 10% for-ıalin, embedded in paraffin and sectioned. After antigen retrieval procedure and endogenous peroxidase activ-y inhibition, sections were incubated with anti-von Willebrand Factor (1:400; Dako, Glostrup, Denmark) or ıti-P-selectin (1:400; Biovision, Milpitas, CA) 1 h at room temperature. HRP-Rabbit/Mouse (Dako) secondary ıtibody was added. Colour development was induced by incubation with a DAB kit (Dako) and the sections were ɔunterstained with hematoxylin. Sections were dehydrated and mounted. The specific staining was visualized and nages were acquired using a microscope equipped with a digital camera and the assistance of Axiovision software. WF relative volume was determined by point-counting morphometry on immunoperoxidase-stained sections, sing a point grid to obtain the number of intercepts over vWF positive cells over the tissue. Six fields were counted ı each liver. All measurements were performed by two independent blinded observers. The relative volume was cal-ılated by dividing the number of points positive in sinusoidal areas by the total number of points over liver tissue.

Vestern Blotting. Liver samples were processed and western blot performed as described[42]. Used pri-ıary antibodies: KLF2 (Santa Cruz Biotech, Santa Cruz, CA), phosphorylated eNOS at Ser1177 (Cell Signaling, anvers, MA), and total eNOS (BD Transduction Laboratories, Lexington, KY), all 1:1000. Blots were revealed y chemiluminescence and protein expression was determined by densitometric analysis using the Science Lab 001, Image Gauge (Fuji Photo Film, Düsseldorf, Germany). Blots were also assayed for β-actin (Sigma-Aldrich) ɔntent as standardization of sample loading.

uperoxide and nitric oxide bioavailability. *In situ* O_2^- levels were assessed in LSEC and liver tis-ıe with the oxidative fluorescent dye dihydroethidium (DHE 10 μM; Molecular Probes Inc., Eugene, OR) as escribed[44,33]. Fluorescence images were obtained with a fluorescence microscope (Olympus BX51, Tokyo, ıpan), and quantitative analysis of at least eight images per condition was performed with Image J 1.44 m soft-are (National Institutes of Health, Bethesda, MD).

Levels of cGMP, a marker of NO bioavailability, were analyzed in liver homogenates using an enzyme immu-oassay (Cayman Chemical Co., Ann Arbor, MI) as previously described[4].

itrotyrosine fluorohistochemistry. Quantitative tyrosine nitration detection was performed as pre-iously described[45,22]. Briefly, slides were deparaffinized, hydrated, incubated with aqueous sodium dithionite ɔlution (10 mM) for 10 min, washed with distilled water and then incubated overnight at 4 °C with an equimolar ɔlution of $AlCl_3$ and salicylaldehyde (200 mM). Afterwards, the aqueous solution was removed and sections were ıounted in Fluoromount G medium (Southern Biotech, Birmingham, AL). Negative and positive internal con-ɔls were included. Fluorescence images were obtained with a fluorescence microscope and quantitative analysis f at least six images per sample was performed with Image J 1.44 m software.

Neutrophil infiltration. For the evaluation of neutrophil infiltration, paraffin embedded slides were stained for specific esterase activity using a commercial available kit (Naphthol AS-D Chloroacetate Kit, Sigma-Aldrich) following manufacturer's instructions. Six images per preparation were obtained and positive cells per field were counted.

VCAM-1 and CD68 immunofluorochemistry. Liver cryosections of 6 μm thickness were fixed with acetone at −20 °C for 10 min, incubated with anti-VCAM-1 (1:150; BD Biosciences) 2 h at room temperature and incubated with secondary antibody Alexa Fluor 555 (1:300; Life Technologies, Alcobendas, Madrid, Spain) and 4′,6-diamino-2-fenilindol (1;3000; DAPI, Sigma-Aldrich) and mounted in Fluoromount G medium.

For CD68 detection paraffin embedded samples were used. After antigen retrieval procedure, sections were incubated with anti-CD68 (1:100; BioRad, El Prat de Llobregat, Barcelona, Spain) 1 h at room temperature and incubated with secondary antibody and mounted as described before.

Ten images per sample were obtained with a fluorescence microscope and percentage of positive area (VCAM-1) or positive cells per field (CD68) were quantified.

Cell death. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed in deparaffined liver sections using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Sant Cugat del Valles, Barcelona, Spain) according to the manufacturer's instructions.

Statistical Analysis. Statistical analyses were performed with the IBM SPSS Statistics 19 for Windows statistical package. All results are expressed as mean ± standard error of the mean. Comparisons between groups were performed with analysis of variance followed by LSD post-hoc test, or with Student's *t* test when adequate. Differences were considered significant at $p < 0.05$.

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Author Contributions

D.H. conceived the study, designed the research, performed the experiments, analysed the data and wrote the manuscript. M.O.-R. performed the experiments and analysed the data. J.C.G.-P. and C.P. critically revised the manuscript. J.B. critically revised the manuscript and obtained funding. J.G.-S. conceived the study, designed the research, wrote the manuscript, obtained funding and directed the study. All authors edited and reviewed the final manuscript.

Additional Information

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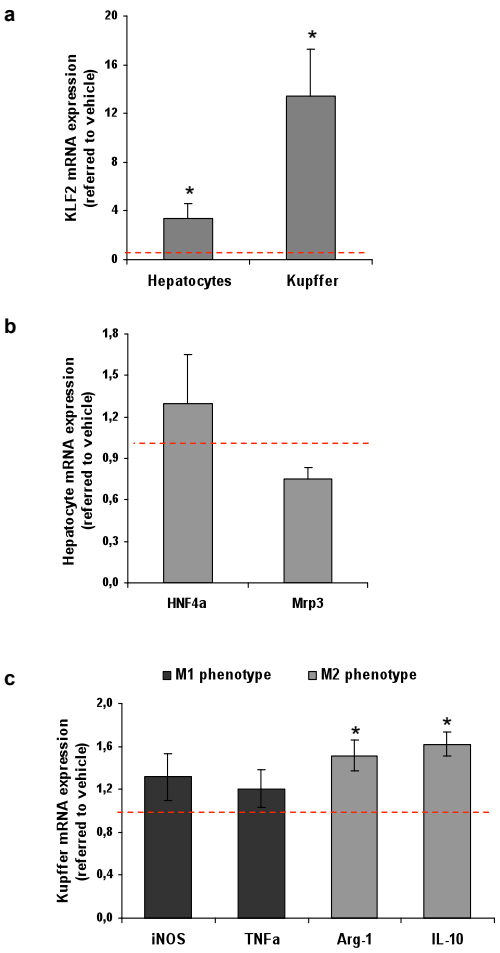
Supplementary information:



Supplementary Figure S1. Simvastatin up-regulates KLF2 expression and improves the phenotype of hepatic cells *in vitro*. (a) KLF2 mRNA expression in primary rat hepatocytes and Kupffer cells after 24h of simvastatin treatment. **(b)** Expression of hepatocyte phenotype markers in cells described in a. **(c)** Expression of Kupffer cells phenotype markers in cells described in a. Values normalized to its vehicle group (red dotted line). (n=6 per group, *p<0.05 vs. vehicle).

SUMMARY OF RESULTS

4. SUMMARY OF RESULTS

Study 1: A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischemia and reperfusion injury

- LSEC undergoing CS+WR show increased O_2^- and reduced NO levels. rMnSOD treatment prevents these effects on LSEC.
- CS+WR increases O_2^- levels in hepatic tissue. *In vivo* rMnSOD administration maintains ROS at lower concentrations.
- CS+WR causes hepatocellular lesions associated with increased expression of adhesion molecules and liver damage, evaluated by transaminases and LDH release, compared to control livers. rMnSOD pre-treatment significantly reduces the parameters of hepatic damage evaluated.
- Cold stored livers present a significant deterioration in liver microcirculation upon reperfusion, as shown by the increase in portal perfusion pressure and the development of endothelial dysfunction, evaluated by the response to the vasodilator acetylcholine and the sinusoidal protein expression of the capillarization marker vWF. Hepatic microcirculatory deregulation is prevented by rMnSOD pre-treatment.
- Endothelial dysfunction after CS+WR is associated with reduced hepatic eNOS expression and NO bioavailability, together with an accumulation of nitrotyrosinated proteins. rMnSOD improves NO bioavailability by reducing O_2^- levels as evidenced by the reduction in nitrotyrosine formation.
- Steatotic livers show elevated O_2^- levels and liver damage after IR. rMnSOD treatment prevents O_2^- formation due to the increase in SOD expression but this is not enough to prevent hepatic injury.
- In steatotic livers undergoing CS+WR portal perfusion pressure is significantly increased and acute endothelial dysfunction is developed. These negative effects on liver microcirculation are partly prevented by rMnSOD.
- Supplementation of a preservation solution for transplantation with rMnSOD significantly reduces O_2^- levels produced in hepatic tissue from healthy and

steatotic rats and, importantly, also in healthy human tissue undergoing CS+WR.

Study 2: Effects of warm ischemia and reperfusion on the liver microcirculatory phenotype of rats: underlying mechanisms and pharmacological therapy

- Livers undergoing WIR show hepatic microcirculatory derangements as evidenced by the increased intrahepatic vascular resistance at 2h and 24h of reperfusion, associated with increased portal pressure and reduced portal blood flow.
- WIR promotes endothelial dysfunction development, evaluated by the vWF sinusoidal staining and acetylcholine response. Furthermore, WIR causes an increase in transaminases and LDH in comparison to control animals, especially at 2h of reperfusion.
- WIR reduces the hepatic KLF2 expression at both time points of reperfusion and is accompanied by a reduction in eNOS phosphorylation and NO bioavailability. O_2^- production and protein nitrotyrosination are significantly increased at both reperfusion times.
- In livers undergoing WIR, a rapid activation of hepatic endothelium is observed, determined by increased expression of adhesion molecules. This increase is associated with a rise in macrophage and neutrophil infiltration into the hepatic parenchyma. Finally, WIR causes a significant increase in cell death, evaluated by TUNEL staining.
- Simvastatin treatment prevents the increase in intrahepatic vascular resistance thus, improving hepatic perfusion. The drug also attenuates endothelial dysfunction and reduces transminases and LDH levels.
- Pre-treatment with simvastatin maintains hepatic KLF2 expression and eNOS activation, especially at 2h of reperfusion. This is accompanied by increased NO bioavalilability.

- Simvastatin inhibits endothelial activation, preventing adhesion molecules expression and reducing neutrophil and macrophage infiltration. Globally contributing to reduce cell death.
- Administration of simvastatin to LSEC undergoing anoxia/reoxygenation increases KLF2 and eNOS expression and reduces capillarization markers. Additionally, simvastatin reduces O_2^- levels in this cell type.
- Simvastatin increases KLF2 expression not only in LSEC by also in hepatocytes and KC. This correlates with a slight improvement in the phenotype of these cells.

DISCUSSION

4. DISCUSSION

Ischemia-reperfusion injury is a clinical pathological condition characterized by an initial deprivation and afterwards restoration of oxygen and blood-derived shear stress stimulation. The known involved mechanisms of injury include energy depletion during ischemia and generation of ROS, activation of Kupffer cells and leukocytes, expression of adhesion molecules in endothelial cells and neutrophils, and infiltration of inflammatory cells in the liver parenchyma during reperfusion. In general, two different types of ischemia can be defined depending on the ischemic temperature: cold ischemia, mainly occurring during preservation for transplantation, and warm ischemia, common in numerous clinical conditions including hepatic resection, haemorrhagic shock as well as in liver transplantation during organ harvesting and revascularization.

The studies included in the present doctoral thesis firstly aimed to advance in the knowledge of the pathophysiology of IR injury, both cold and warm, specially focusing on the liver microcirculation and using proper pre-clinical models, to secondly apply therapeutic approaches designed to target the cellular and molecular de-regulations discovered.

In the first study we assessed the effects of cold preservation for transplantation and warm reperfusion (CS + WR) on the phenotype of primary LSEC. Our results demonstrated, for the first time, that CS+WR induced a significant increase in O_2^- levels in LSEC, which lead to markedly reduced NO levels.

After this initial ascertainment, we aimed to study the effectiveness of rMnSOD reducing oxidative stress during IR *in vivo*. For this purpose, we used a well established model of hepatic cold storage and warm reperfusion *ex vivo*[48, 97]. Confirming previous results, CS+WR induced liver damage, increased aminotransferases release, oxidative stress and inflammation[97]. Furthermore, it also caused deregulation of microcirculation and endothelial dysfunction, manifested by reduced KLF2 and eNOS hepatic expression and impaired vasorelaxation in response to the endothelial-dependent vasodilator

acetylcholine[189]. NO bioavailability, evaluated measuring hepatic cGMP levels and nitrites and nitrates (NOx), was reduced due to the mentioned decrease in eNOS expression and due to the rapid scavenging by the large amount of O_2^- produced during reperfusion[190]. Overall, the results of this study reinforced the novel concept of acute endothelial dysfunction development due to organ procurement for transplantation.

In the second study we become interested in the effects of warm ischemia-reperfusion injury on liver microcirculation because only a few reports centred its attention to LSEC in this pathology. Accordingly, we fully characterized the hepatic microcirculation in a model of partial warm-ischemia and reperfusion, focusing predominantly on changes in LSEC phenotype, its mechanisms, consequences and prevention.

The first evidence from this study was the observation that a short period of warm ischemia was enough to cause important disturbances on the hepatic microcirculation by markedly increasing the intrahepatic vascular resistance (IHVR) at the early (2h) and late (24h) phases of reperfusion, leading to increased portal pressure and significant reduction in liver perfusion. Furthermore, these animals developed acute endothelial dysfunction evidenced by a decreased response to the vasodilator acetylcholine and increased sinusoidal expression of vWF, which is only expressed in dysfunctional sinusoidal endothelial cells[191]. In addition, WIR caused liver damage, measured by transaminases and LDH release, which was especially relevant at early reperfusion times.

As it has been mentioned, cold preservation causes KLF2 down-regulation due to the lack of biomechanical stimulus determined by shear stress[97, 182]. Thus, we hypothesized that during WIR, and although the ischemic time is much shorter than in CS, the same effect would be also occurring and this could be the cause of the observed loss in liver vasoprotection during reperfusion. We therefore measured KLF2 protein expression in liver tissue observing a marked decrease after WIR, both at early and late reperfusion times. Finally, this was accompanied by a reduction in eNOS activation. Evaluating NO bioavailability by measuring

cyclic guanosine monophosphate (cGMP), as a second messenger of NO, we found a significant reduction in hepatic cGMP at the early phase of reperfusion. The reduction in NO levels was found to be due to a diminished synthesis by reduced eNOS activity, but also increased NO-scavenging by the high amount of O_2^- produced during reperfusion, indirectly measured by peroxynitrite protein modification[192].

Analysis of the consequences of microcirculatory dysfunction revealed an increase in adhesion molecules such as VCAM-1 and P-selectin in the cell surface of liver endothelial cells, and consequent increased macrophage and neutrophil infiltration. These inflammatory cells caused a massive production of O_2^- in the liver parenchyma at the late reperfusion phase, and all this damage finally conduced to cell death.

In summary, the results here reported shed light on the pathophysiology of liver ischemia reperfusion injury demonstrating that a warm ischemia period, even of short duration, profoundly affects the liver endothelial phenotype, which becomes dysfunctional, leading to an immediate increase in the hepatic vascular tone, inflammation, polymorphonuclear cells infiltration, and hepatic cell death. WIR-derived damage to the hepatic endothelium may be, at least in part, due to KLF2 down-regulation.

Once the microcirculation was properly characterized, our first approach to reduce microcirculatory injury due to ischemia-reperfusion was the administration of a single dose of the antioxidant rMnSOD shortly before ischemia in a model of CS+WR. This new formulation of the human manganese superoxide dismutase has many advantages in comparison to other SOD formulations or derivatives: it is constitutively active, has a good biodistribution, is stable in solution and freely crosses the cell membrane, being able to reduce intracellular and extracellular ROS. Furthermore its antioxidant capacity has been proven in experimental models of cancer and cirrhosis[193, 194]. Thus, the application of this molecule can easily overcome the problems of other approaches using unstable SOD derivatives or

adenoviral vectors, which are ineffective or provoke many safety concerns[118, 195, 196].

The first results of this study using primary cultured LSEC demonstrated the effectiveness of rMnSOD blunting O_2^- levels specifically in this cell type. This was associated with the maintenance of NO levels, probably due to the prevention of its scavenging by O_2^-.

As it has been mentioned, previous studies from our group have focused on testing vasoprotective drugs in the setting of cold preservation showing positive results in terms of microvascular protection. However their effects improving ROS accumulation were limited. In this sense, the *in vivo* studies using the antioxidant rMnSOD demonstrated that a single dose of this drug administered only 30 minutes before organ procurement and cold storage almost entirely prevented endothelial dysfunction, microcirculatory damage and liver injury after CS+WR, probably owing to the inhibition of ROS-mediated cell injury and the reduction in the expression of adhesion molecules.

Considering that endothelial protection is a key step in maintaining graft viability after CS+WR, we investigated the molecular mechanisms responsible for rMnSOD protection of liver microcirculation. Due to the critical role of NO in modulating the vascular tone and our findings showing reduced levels of NO in LSEC after CS+WR, we characterized the NO pathway in the liver grafts included in this study. The results showed that rMnSOD administration led to an increase in hepatic NO bioavailability measured by two different final products, cGMP and NOx, without changes in eNOS expression or activity. The increase in NO was associated with a reduction in hepatic nitrotyrosinated proteins, a marker of peroxynitrite formation, altogether confirming lower O_2^--mediated NO scavenging.

Owing to possible controversies due to pharmacological treatment of liver donors, we additionally evaluated the supplementation of a commercially available preservation solution with rMnSOD to prevent ROS accumulation as a proof of concept. We tested the supplemented solution in liver rat tissue and, importantly, in biopsies from human donors. In all cases we found that addition of rMnSOD to

the preservation solution reduced hepatic O_2^- levels derived from CS+WR. Although further evaluation in *in vivo* models is needed, these results demonstrated the effectiveness of this protein in preventing oxidative stress accumulation even in human tissues, suggesting a possible global improvement in hepatic CS+WR injury.

In the second study, we centred our attention in protecting the liver microcirculation during warm ischemia-reperfusion using vasoprotective drugs able to maintain KLF2-derived transcription programs. Simvastatin was our first choice due to its beneficial effects on endothelial function in experimental models of cirrhosis and cold preservation[97, 102, 197] and for being the most effective statin increasing KLF2 levels in LSEC[103]. We applied an acute pharmacological pre-treatment with simvastatin 1h before warm ischemia to discard possible concomitant effects of lipid lowering when administering it for prolonged times.

Some previous studies in experimental models of WIR have applied statins showing benefits in terms of liver injury and inflammation[150, 198, 199], however their effects on hepatic microvascular function were not evaluated. In this sense, our study is pioneer demonstrating the effectiveness of simvastatin pre-treatment in preventing intrahepatic vascular resistance increase during reperfusion *in vivo* and this associates with better liver perfusion at early and late reperfusion periods. The treatment also improved hepatic endothelial function and prevented the peak in transaminases and LDH release observed at 2h of reperfusion.

When evaluating the KLF2 pathway, simvastatin pre-treatment was able to prevent the decrease in liver KLF2 protein expression observed during early reperfusion, thus maintaining the activation of its target gene eNOS and enhancing NO bioavailability. Furthermore, simvastatin-treated animals showed reduced levels of ROS, what might be explained by the antioxidant properties of KLF2 through the activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and up-regulation of hemeoxygenase 1 (HO-1) as previously described[102, 200]. Finally, simvastatin maintenance of a correct sinusoidal phenotype prevented the expression of adhesion molecules in the endothelial cell surface (VCAM-1 and P-selectin)

reducing leukocyte rolling and infiltration, which is in agreement with previous studies demonstrating the anti-inflammatory properties of KLF2[177, 186].

To confirm the results observed *in vivo*, we performed some *in vitro* experiments using primary LSEC cultured under anoxia/reoxygenation conditions in order to mimic WIR. Simvastatin pre-treatment in these cells was able to partly abrogate the increase in oxidative stress and inflammation caused by anoxia/reoxygenation, most probably due to the activation of KLF2 pathway[182]. Interestingly, we also evaluated the effect of simvastatin on KLF2 protein expression in other liver cell types demonstrating, for the first time, the activation of KLF2 pathway in hepatocytes and Kupffer cells treated with statins. The increase in KLF2 protein expression was associated with an improvement in hepatocyte phenotype and a polarization towards an anti-inflammatory phenotype in Kupffer cells. This observation complements previous data demonstrating the beneficial effects of statins in another non-parenchymal cell type, the hepatic stellate cells[102] and reinforces the concept of global hepatic protection in response to statins.

In the occidental countries a rise in obesity and fatty liver has been observed during the past years and this trend is expected to dramatically increase in the near future. Although moderate steatosis does not prevent transplantation, steatotic livers exhibit worse post-operative viability and function[201]. Therefore, the characterization of those marginal organs to improve their outcome is of great relevance. Accordingly, we also investigated the effects of CS+WR in livers with diet-induced severe steatosis[48]. We confirm that steatotic grafts exhibit enhanced parenchymal damage, elevated transaminases release and increased oxidative stress[48, 202]. Furthermore, steatotic livers undergoing CS+WR showed impaired hepatic microcirculation, reduction in antioxidant capacity as evidenced by reduced activity of SOD, exacerbation in NO scavenging by O_2^-, and endothelial phenotype deregulation.

In fatty livers, pre-treatment with rMnSOD significantly reduced hepatic oxidative stress levels, which was associated with a reduction in peroxynitrite and improvement of hepatic microcirculation and endothelial function. Nevertheless, in

spite of rMnSOD beneficial effects, no reduction in parenchymal damage was observed. It is known that the antioxidant capacity of steatotic livers is impaired[203] and this was confirmed in our steatotic livers, which showed insufficient hepatic levels of catalase and glutathione, suggesting H_2O_2 accumulation as the reason for reduced improvement in hepatic injury in comparison to control livers. Future studies evaluating combined antioxidant therapies to totally re-establish ROS detoxification pathways in steatotic livers would be of great interest.

Taken together, the results derived from the two studies of the present PhD thesis strongly suggest that protection of liver microcirculation represents a key point in the prevention of hepatic damage in any setting were ischemia-reperfusion injury occurs. This is also applicable to haemorrhagic shock situations, for example in cirrhosis, where recent results from a randomized controlled trial demonstrate that cirrhotic patients under statin treatment show a significant increase in overall survival[204].

In conclusion, we provide evidences that, by preserving sinusoidal function, livers will benefit of a reduction in inflammation and cell death, which ultimately will result in the maintenance of a correct global function and viability.

CONCLUSIONS

6. CONCLUSIONS

Study 1: A novel form of the human manganese superoxide dismutase protects rat and human livers undergoing ischemia and reperfusion injury

- Cold storage + warm reperfusion produce a worst impairment of hepatic microcirculation in steatotic livers compared with healthy livers.
- rMnSOD pre-treatment shortly before organ procurement maintains a correct microcirculatory status both in control and steatotic rats, resulting in the protection of the liver parenchyma.
- Supplementation of a preservation solution with rMnSOD efficiently reduces O_2^- accumulation in rat control and steatotic liver tissue and, importantly, in human biopsies.

These data suggest that rMnSOD would be an effective pre-treatment or supplement of the preservation solution in the procurement of liver grafts for transplantation.

Study 2: Effects of warm ischemia and reperfusion on the liver microcirculatory phenotype of rats: underlying mechanisms and pharmacological therapy

- A short warm ischemia period deeply affects the liver endothelial phenotype that becomes dysfunctional leading to an increase in vascular tone.
- WIR-derived damage to the endothelium is due, in part, to KLF2 down-regulation.
- The reduction in KLF2 vasoprotection and microcirculatory impairment is associated with increased inflammation and cell death, especially at the late reperfusion phase.
- Simvastatin pre-treatment protects hepatic microcirculation from WIR preventing subsequent liver injury.

- KLF2 vasoprotective pathways mediate, in part, the beneficial effects of simvastatin.

Simvastatin administration is an effective and simple strategy to reduce liver damage in clinical situations associated with hepatic warm ischemia-reperfusion injury

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SUMMARY

8. SUMMARY

8.1 Introducció

8.1.1 Isquèmia-Reperfusió

La isquèmia-reperfusió (IR) és una condició patològica bifàsica caracteritzada per una interrupció inicial del flux sanguini, el seu estímul biomecàmic i l'aportació d'oxigen (O_2) seguit del subseqüent restabliment de la perfusió, comportant un restabliment d'O_2, nutrients i estrès per fregament.

Existeixen dos tipus principals d'IR, la isquèmia en calent (WIR; de les inicials en anglès) que es desenvolupa *in situ* durant la resecció hepàtica, transplantament o shock hemorràgic, i pot conduir a una funció hepàtica tardana; i la isquèmia freda, que succeeix durant la preservació d'empelts per transplantament *ex vivo* i normalment està unida a dues fases d'isquèmia calenta abans i després de la preservació. Alguns dels mecanismes de dany comuns als dos tipus d'isquèmia inclouen generació d'espècies reactives d'oxigen (ROS), activació de les cèl·lules de Kupffer (KC) i els neutròfils, augment en l'expressió de molècules d'adhesió i infiltració de limfòcits i monòcits circulants.

Durant la isquèmia, la falta de nutrients produeix un descens en els nivells d'O_2 i d'ATP amb subseqüent deprivació energètica. En aquestes condicions, els processos cel·lulars s'aturen i els metabòlits intermedis s'acumulen. Durant la reperfusió, el metabolisme és restablert produint una gran quantitat de productes de rebuig. La reperfusió es pot dividir en dues fases, una primerenca (fins a les 6h de reperfusió) i una fase tardana (de 6 a 48h).

Durant la fase primerenca, l'alliberació massiva de ROS supera la capacitat antioxidant de la cèl·lula produint la modificació de lípids, proteïnes i àcids nucleics; a més promou la secreció de compostos pro-inflamatoris i l'expressió de molècules d'adhesió. En aquesta fase es produeixen també canvis en la microcirculació hepàtica, com són la descompensació entre vasodilatadors i vasoconstrictors a favor dels segons, edema de les KC i cèl·lules endotelials sinusoïdals (LSEC) i augment de l'adhesió plaquetària. Tots aquests canvis condueixen a un estretament del sinusoide hepàtic i un increment en la resistència

al pas del flux, comportant un empitjorament de la perfusió hepàtica. En la fase tardana de reperfusió, la resposta inflamatòria causada per l'activació i infiltració dels neutròfils incrementa el dany hepàtic i, finalment, condueix a la mort cel·lular.

8.1.2 L'endoteli vascular

L'endoteli consisteix en una monocapa de cèl·lules endotelials que recobreix els vasos sanguinis i juga un paper important en el manteniment de l'homeòstasi. Les cèl·lules endotelials tenen estructures i funcions molt heterogènies entre òrgans però també en diferents àrees del mateix òrgan.

En particular, la circulació hepàtica és única degut a la seva aportació sanguínia dual, on la sang oxigenada arterial es barreja amb la sang venosa portal rica en nutrients i pobre en oxigen. Tot això succeeix a la xarxa de capil·lars especialitzats del fetge, coneguts com a sinusoides hepàtics.

Els sinusoides estan formats per LSEC que composen la cara interna dels vasos. D'altra banda, els hepatòcits no estan en contacte directe amb les LSEC si no que existeix un espai conegut con a espai de Disse on les molècules sanguínies entren abans de contactar amb ells. En aquest espai es troben les cèl·lules estrellades hepàtiques (HSC), especialitzades en acumular vitamina A en estat quiescent, regular el to vascular i activitats metabòliques i de creixement cel·lular. L'últim tipus cel·lular son les KC, macròfags residents que es troben al lumen dels sinusoides, principalment a les zones periportals on hi ha una major activitat fagocítica.

L'endoteli hepàtic sinusoïdal presenta una estructura i funció característica. Aquestes cèl·lules participen en totes les funcions típiques endotelials mantenint l'homeòstasi vascular i regulant el to vascular, i també són clau en processos fisiològics o patològics com són la trombosi, inflamació i remodelació de la paret vascular. Les LSEC són diferents d'altres cèl·lules endotelials perquè els hi manca la membrana basal i presenten porus oberts de 100-200 nm coneguts com a fenestres. Aquest porus s'organitzen en grups de 20-50 anomenats 'plaques de tamís'. Es tracta d'estructures dinàmiques que poden contraure o dilatar en resposta a alteracions del flux sanguini i de la pressió de perfusió. A més, les fenestres són un filtre selectiu que permet el pas de petites partícules de la sang

cap als hepatòcits i viceversa. Les LSEC també presenten una gran capacitat endocítica mitjançant els receptors presents a la seva superfície, permetent l'eliminació de certs productes de rebuig. Un altra funció important de les LSEC és la relacionada amb la immunitat ja que presenten antígens a través dels seus complexos majors d'histocompatibilitat.

El to vascular és determinat pel balanç de vasoconstrictors i vasodilatadors que actuen sobre el sinusoide hepàtic. En condicions fisiològiques ambdós tipus de compostos estan compensats però, qualssevol estímul advers pot conduir al desajust d'aquest balanç i afectar al flux sanguini causant disfunció microcirculatòria i dany hepàtic. Tot i que existeixen molts mediadors vasoactius, un dels més estudiats en la circulació és l'òxid nítric (NO). Aquesta molècula gasosa amb capacitat vasodilatadora és produïda per l'òxid nítric sintasa (NOS). Existeixen tres isoformes d'aquest enzim: la neuronal (nNOS), l'induïble (iNOS) i l'endotelial (eNOS). Al fetge, iNOS és present a diversos tipus cel·lulars i produeix elevats nivells de NO en resposta a compostos pro-inflamatoris. eNOS s'expressa de forma constitutiva a les LSEC i produeix baixos nivells de NO en resposta a estímuls humorals i biomecànics. Durant el dany hepàtic, incloent la IR, la biodisponibilitat de NO disminueix principalment degut a una disminució en la seva síntesi per canvis post-traduccionals en l'eNOS. A més, els nivells de NO al fetge també es veuen reduïts pel seu segrest per part de l'anió superòxid (O_2^-).

El terme disfunció endotelial descriu els canvis produïts a l'endoteli que passa d'un fenotip modulable a un fenotip incapaç d'adaptar-se. La disfunció endotelial es considera el mecanisme clau involucrat en la funció microcirculatòria patològica en desordres vasculars com la hipertensió arterial, diabetis, hipercolesterolèmia, cirrosi y preservació en fred per transplantament. Tot i la descripció inicial d'aquest terme, investigacions recents demostren que els canvis produïts en la microcirculació es deuen a canvis en tots els tipus cel·lulars sinusoïdals, incloent les HSC i les KC. Per tant, el terme "disfunció microcirculatòria hepàtica" sembla molt més correcte per anomenar aquest fenomen.

Les principals manifestacions de la disfunció microcirculatoria inclouen alteracions en la resposta vasomotora, permeabilitat incrementada, augment en

l'adhesió de neutròfils i trombosi. A nivell molecular, la disminució en vasodilatadors i augment de vasoconstrictors, secreció de quimiocines, l'expressió de molècules d'adhesió i la disminució d'antioxidants són les principals vies implicades. A més a més, canvis en les forces biomecàniques creades pel flux sanguini poden conduir també a disfunció microcirculatòria degut a la seva influència sobre l'expressió gènica en les LSEC.

8.1.3 Aproximacions terapèutiques en isquèmia-reperfusió

Com s'ha descrit anteriorment, l'estrès oxidatiu és un dels mecanismes principals en el dany per IR. Els enzims antioxidants juguen un paper important mantenint l'equilibri redox i són essencials per preservar les funcions fisiològiques. A data d'avui, moltes estratègies antioxidants tant endògenes com exògenes s'han provat amb èxit en models experimentals d'IR i alguns d'aquests fàrmacs han començat assajos clínics. Tot i així, actualment cap fàrmac orientat a reduir ROS es troba en ús a nivell clínic.

Entre les diferents estratègies antioxidants avaluades destaquen aquelles dirigides a augmentar els nivells d'antioxidants com la superòxid dismutasa (SOD), catalasa i glutatió (GSH), trobant resultats positius en termes de producció de ROS i dany hepàtic. Un altra estratègia ha sigut la de reduir els nivells d'enzims encarregats de produir ROS emprant inhibidors específics; aquest és el cas de l'al·lopurinol, inhibidor de la xantina oxidasa (XOD) o els inhibidors de la NADPH oxidasa. L'administració de diverses vitamines i antioxidants naturals també ha resultat efectiva reduint l'estrès oxidatiu associat a la IR. Finalment, una altra aproximació terapèutica amb propietats antioxidants és el precondicionament isquèmic (IPC), procediment quirúrgic consistent en un breu període d'IR precedent el període principal d'isquèmia. Aquesta estratègia ha demostrat la seva eficàcia augmentant els nivells d'antioxidants com la SOD, NO i les proteïnes de xoc tèrmic.

Durant la isquèmia-reperfusió els problemes en la perfusió hepàtica són determinants per el desenvolupament de disfunció microcirculatòria. Així, s'han desenvolupat diferents estratègies enfocades a protegir la microcirculació durant la IR. Entre les més destacades es troba la regulació dels nivells de molècules vasoactives, reduint els nivells de vasoconstrictors com l'endotelina-1 (ET-1) o

angiotensina II o augmentant la disponibilitat de vasodilatadors mitjançant estimulació del receptor A2, suplementació de NO i monòxid de carboni o la inducció de HO-1. En quant a estratègies quirúrgiques, el IPC ha sigut una de les més estudiades mostrant una millora de la microcirculació hepàtica que s'ha associat a un increment en els nivells de NO i una reducció de ET-1. Finalment, les estatines, originalment dissenyades per reduir els nivells de colesterol, també han demostrat efectes beneficiosos en condicions d'IR. Els mecanismes implicats inclouen la sobreexpressió de HO-1, eNOS i la reducció de ET-1 i iNOS. Aquest efectes estan relacionats amb l'increment en l'expressió del factor de transcripció Krupel-like factor 2 (KLF2) i dels seus gens diana amb efectes antiinflamatoris, antitrombòtics i vasoprotectors.

8.2 Hipòtesi i objectius

El dany per isquèmia-reperfusió és observat en procediments quirúrgics que necessiten oclusió vascular o durant el sagnat i ressuscitació. Durant aquest dany es produeix un empitjorament de la microcirculació hepàtica que pot conduir a fallida hepàtica. Per tant, teràpies enfocades a augmentar la producció de substancies vasodilatadores o a reduir l'estrès oxidatiu per tal de millorar la microcirculació poden ser beneficioses en aquest context.

Tot i el paper principal de l'endoteli en l'homeòstasi i les malalties hepàtiques, fins ara la major part de la bibliografia s'ha centrat en conèixer el dany produït per la IR sobre el parènquima i s'ha investigat molt poc els efectes deleteris sobre el sinusoide hepàtic. Recentment, alguns estudis orientats a avaluar la microcirculació sinusoïdal han demostrat que la falta d'estímul biomecànic durant la isquèmia produeix un descens de vies vasoprotectores regulades pel factor de transcripció KLF2 conduint a una desregulació del fenotip endotelial.

L'objectiu global de la present tesi doctoral fou caracteritzar la microcirculació hepàtica en el context del dany per isquèmia-reperfusió i avaluar possibles fàrmacs que, mitjançant la millora del sinusoide hepàtic, puguin mantenir un correcte fenotip hepàtic durant la IR.

Estudi 1: *Una nova forma de la manganès superòxid dismutasa humana protegeix els fetges de rates i humans sotmesos a dany per isquèmia-reperfusió*

La disfunció endotelial que s'observa durant la preservació en fred per transplantament representa el primer esdeveniment en el desenvolupament de dany per IR. Entre els mecanismes implicats es troba la producció de ROS. Tot i que s'han provat diversos tractaments antioxidants, cap ha arribat a la pràctica clínica. La nostra hipòtesi és que el tractament agut del donant hepàtic abans de la cirurgia amb una nova formulació antioxidant pot inhibir la formació de ROS i prevenir els efectes deleteris de la IR.

L'objectiu d'aquest estudi és doncs el d'avaluar els efectes protectors d'una nova forma recombinant de la manganès superòxid dismutasa (rMnSOD) sobre la funció endotelial i la viabilitat post-transplantament en rates sanes i esteatòsiques per augmentar el nombre de donants disponible.

Estudi 2. *Els efectes de la isquèmia-reperfusió calenta en el fenotip microcirculatori hepàtic: mecanismes implicats i teràpies farmacològiques*

La isquèmia calenta associada a la resecció hepàtica o el xoc hemorràgic és poc tolerada pel fetge i condueix a disfunció primerenca. S'ha demostrat que la isquèmia en fred causa disfunció microcirculatòria associada a la reducció de KLF2 durant la isquèmia. Tot i així, la microcirculació hepàtica ha estat molt menys estudiada en isquèmia calenta.

El primer objectiu d'aquest estudi és caracteritzar la microcirculació del fetge en models experimentals d'IR en calent. El segon objectiu és avaluar si el pretractament amb molècules vasoprotectores com les estatines pot millorar significativament la funció i viabilitat de l'òrgan i finalment caracteritzar les vies implicades.

8.3 Resultats

Estudi 1: *Una nova forma de la manganès superòxid dismutasa humana protegeix els fetges de rates i humans sotmesos a dany per isquèmia-reperfusió*

- Les LSEC sotmeses a preservació en fred i reperfusió calenta (CS+WR) presenten major nivells d'O_2^- i una reducció de NO. La rMnSOD prevé aquests efectes en les LSEC.
- El CS+WR augmenta els nivells d'O_2^- en teixit hepàtic. L'administració *in vivo* de rMnSOD manté els nivells hepàtics d'estrès oxidatiu més baixos.
- CS+WR causa lesions hepatocel·lulars associades amb un increment en l'expressió de molècules d'adhesió i un increment en el dany hepàtic avaluat per l'alliberació de transaminases i LDH en comparació amb fetges control. El pretractament amb rMnSOD redueix significativament tots els paràmetres de dany hepàtic.
- La microcirculació dels fetges preservats en fred 16h es veu significativament deteriorada després de la reperfusió, com ho demostra l'increment en la pressió portal de perfusió, la disfunció endotelial avaluada per corbes dosi-resposta al vasodilatador acetilcolina i l'expressió proteica sinusoïdal de vWF. La desregulació de la microcirculació hepàtica es prevé mitjançant el pretractament amb rMnSOD.
- La disfunció endotelial observada després de CS+WR s'associa amb una reducció en l'expressió de eNOS i una menor producció i biodisponibilitat de NO, juntament amb una acumulació de proteïnes nitrotirosinades. La rMnSOD és efectiva millorant la biodisponibilitat de NO probablement degut a la reducció de O_2^- com evidencia la reducció de proteïnes nitrotirosinades.
- Les rates esteatòsiques presenten elevats nivells de O_2^- i dany hepatocel·lular després de la IR. Tot i que el pretractament amb rMnSOD prevé la formació de O_2^- gràcies al manteniment en l'expressió de SOD, aquesta no és capaç de prevenir totalment el dany hepàtic.
- Els fetges esteatòsics sotmesos a CS+WR mostren un increment en la pressió portal de perfusió i disfunció endotelial aguda. Els efectes negatius sobre la microcirculació son previnguts significativament pel tractament amb rMnSOD.

- La suplementació d'una solució de preservació per transplantament amb rMnSOD redueix els nivells de radicals lliures produïts en teixit hepàtic de rates sanes i esteatòsiques i, de forma important, en biòpsies humanes de teixit sà sotmeses a CS+WR.

Estudi 2: *Els efectes de la isquèmia-reperfusió calenta en el fenotip microcirculatori hepàtic: mecanismes implicats i teràpies farmacològiques*

- Els fetges sotmesos a isquèmia i reperfusió en calent (WIR) mostren un empitjorament en la microcirculació hepàtica evidenciat per l'increment en la resistència vascular intrahepàtica a 2h i 24h de reperfusió, associat amb un increment en la pressió portal i una reducció en la perfusió hepàtica.
- La WIR promou el desenvolupament de disfunció endotelial, avaluat pel marcador de capil·laritzación vWF i la resposta al vasodilatador acetilcolina. A més, WIR causa un increment de les transaminases i el LDH en comparació amb animals control, especialment a 2h de reperfusió.
- La WIR causa una reducció en l'expressió hepàtica de KLF2 en ambdós temps de reperfusió que s'acompanya d'un descens en la fosforilació d'eNOS i una disminució de la biodisponibilitat de NO. La producció de O_2^- i la formació de nitrotirosines es veu significativament incrementada als dos temps de reperfusió.
- La WIR indueix una ràpida activació de l'endoteli hepàtic, demostrat per l'increment en l'expressió de molècules d'adhesió. Aquest increment s'associa amb una major infiltració de neutròfils i macròfags al parènquima hepàtic. Finalment, WIR causa un augment significatiu en la mort cel·lular avaluada per tinció de TUNEL.
- El tractament amb simvastatina prevé l'increment en la resistència vascular intrahepàtica, millorant així la perfusió hepàtica. El fàrmac també és capaç d'atenuar la disfunció endotelial i reduir l'alliberació de transaminases i LDH.
- L'administració de simvastatina manté l'expressió hepàtica de KLF2 i l'activació de eNOS, especialment a 2h de reperfusió. Això s'acompanya d'una major biodisponibilitat de NO.

- La simvastatina inhibeix l'activació endotelial, prevenint l'expressió de molècules d'adhesió, redueix la infiltració de neutròfils i macròfags i, globalment, redueix la mort cel·lular.
- L'administració de simvastatina a LSEC sotmeses a anòxia/reoxigenació augmenta l'expressió de KLF2 i eNOS i redueix els marcadors de capil·larització. A demés, la simvastatina redueix els nivells de superòxid en LSEC sota IR.
- La simvastatina augmenta l'expressió de KLF2 no només en LSEC sinó també en hepatòcits i KC. Això es correlaciona amb una lleugera millora del fenotip en aquests tipus cel·lulars.

8.4 Discussió

Els estudis de la present tesi doctoral tenen com a primer objectiu avançar en el coneixement actual sobre fisiopatologia de la IR freda i calenta emprant models experimentals per a continuació aplicar teràpies dissenyades per corregir les desregulacions cel·lulars i moleculars descobertes.

Els resultats del primer estudi confirmen resultats previs demostrat com CS+WR causa dany hepàtic, estrès oxidatiu i inflamació. A més, aquests fetges presenten una desregulació de la microcirculació hepàtica i disfunció endotelial, acompanyat d'un descens en la biodisponibilitat d'òxid nítric degut a una disminució de la seva síntesi i un increment del seu segrest per part del O_2^-. En resum, els resultats de l'estudi reforcen el nou concepte de desenvolupament de disfunció microcirculatòria durant la preservació d'òrgans per transplantament.

El segon estudi es centra en els efectes de la isquèmia calenta sobre la microcirculació, fent servir un model d'isquèmia parcial i centrat principalment en els canvis produïts en les LSEC. Els resultats de l'estudi demostren, per primera vegada, que la WIR causa un increment de la resistència vascular intrahepàtica a fases primerenques (2h) i tardanes (24h) de reperfusió, associat amb un increment de la pressió i reducció del flux portal. Aquest fetges també presenten disfunció endotelial aguda i dany hepàtic.

La hipòtesi d'aquest segon estudi fou que, el descens de KLF2 durant la parada del flux en el període d'isquèmia era la causa de la manca de vasoprotecció durant la reperfusió. Aquesta hipòtesi va ser avaluada amb èxit demostrant-se que els nivells hepàtics de KLF2 estan reduïts a 2h i 24h de reperfusió i això correlaciona amb una reducció en l'activació d'eNOS i uns nivells inferiors de NO. Les conseqüències de la desregulació microcirculatòria són l'augment en l'expressió de molècules d'adhesió com VCAM-1 i P-selectina i l'increment en l'infiltrat de neutròfils i macròfags al parènquima hepàtic; amb el corresponent increment en la mort cel·lular.

Una vegada que la microcirculació va ser caracteritzada correctament en ambdós models d'isquèmia, la nostra primera aproximació per reduir el dany associat a CS+WR va ser l'administració d'una única dosi de rMnSOD just abans de la isquèmia. Aquesta nova formulació de la manganès superòxid dismutasa humana presenta molts avantatges en comparació amb altres formulacions sent activa constitutivament, estable en solució i capaç de travessar la membrana plasmàtica, el que li permet reduir els ROS intracel·lulars i extracel·lulars.

Els resultats de l'estudi demostren que la rMnSOD redueix els ROS de forma més efectiva que altres fàrmacs testats en aquest model i prevé la disfunció endotelial i el dany sobre la microcirculació hepàtica. Això produeix un increment en els nivells de NO que no es deuen a l'activació de eNOS si no a la prevenció del segrest de NO per part del O_2^-.

Degut a les possibles controvèrsies originades pel tractament del donant hepàtic amb un fàrmac vam suplementar una solució de preservació comercial amb rMnSOD, trobant resultats positius en termes de reducció dels nivells de superòxid en biòpsies hepàtiques de rates i, cal destacar, també de mostres procedents de donants humans. Tot i que es necessiten futurs estudis, aquests resultats són una primera evidència de l'eficiència de rMnSOD en teixit humà.

En el segon estudi, centrat en la isquèmia calenta, vam decidir utilitzar un fàrmac vasoprotector capaç de mantenir el programes derivats de KLF2. La nostra elecció va ser la simvastatina pels seus demostrats efectes beneficiosos sobre la funció endotelial en models de dany com són la cirrosi i la preservació en fred i per ser l'estatina més efectiva incrementant els nivells de KLF2 entre diverses testades.

Tot i que les estatines ja havien estat emprades en aquest context, el nostre estudi és pioner demostrant l'efectivitat d'un pre-tractament agut amb simvastatina prevenint l'increment de la resistència vascular intrahepàtica durant la reperfusió, el qual s'associa amb una millora de la perfusió hepàtica. La simvastatina també millora significativament la funció endotelial i redueix el dany hepàtic.

Entre els mecanismes implicats, s'observa un manteniment en l'expressió de KLF2 en fases inicials de reperfusió, mantenint així l'activació de eNOS i millorant la biodisponibilitat de NO. Els nivells de O_2^- també es veuen reduïts en el grup tractat. Tots aquests efectes beneficiosos sobre la microcirculació condueixen a una preservació del fenotip endotelial, que expressa menys molècules d'adhesió i prevé l'infiltrat de cèl·lules immunes.

Els resultats *in vitro* amb LSEC cultivades sota anòxia/reoxigenació demostren com la simvastatina redueix els nivells d'estrès oxidatiu i inflamació mitjançant l'activació de KLF2. De manera interessant, la simvastatina també té efectes beneficiosos sobre els hepatòcits i les cèl·lules de Kupffer causant una activació de la via de KLF2 i produint una lleugera millora del fenotip d'aquestes cèl·lules.

Per últim, degut a l'increment en la presència l'obesitat i fetge gras en els països occidentals, vam decidir caracteritzar aquests fetges marginals per tal de millorar la seva viabilitat mitjançant algun fàrmac i augmentar així el nombre d'òrgans disponibles per transplantament. Els resultats confirmen que els fetges grassos sotmesos a CS+WR presenten un major dany hepàtic i un dany microcirculatori exacerbat; acompanyat d'una reducció en la capacitat antioxidant amb el conseqüent increment de O_2^- i segrest de NO.

El pre-tractament dels fetges esteatòsics amb rMnSOD redueix l'estrès oxidatiu, prevenint la formació de peroxinitrit i millorant la funció microcirculatòria i endotelial. Tot i així, els efectes sobre el dany hepàtic van ser mínims, possiblement per la capacitat antioxidant disminuïda d'aquests fetges. El qual suggereix l'ús de teràpies antioxidants combinades per tal de millorar aquests efectes.

En conclusió, els resultats de la present tesi doctoral suggereixen que la protecció de la microcirculació hepàtica representa un punt clau en la prevenció del dany hepàtic en qualssevol patologia on la isquèmia-reperfusió es trobi implicada;

probablement també incloent els episodis de sagnat durant la cirrosi. Així doncs, la preservació de la microcirculació conduirà a una reducció en la inflamació i la mort cel·lular el qual, en última instància, millorarà la funció i la viabilitat global del fetge.

8.5 Conclusions

Estudi 1. *Una nova forma de la manganès superòxid dismutasa humana protegeix els fetges de rates i humans sotmesos a dany per isquèmia-reperfusió*

- La isquèmia freda + reperfusió calenta produeix l'empitjorament de la microcirculació hepàtica, sent aquest més greu en fetges esteatòsics que en fetges sans.
- El pretractament amb rMnSOD breument abans de la isquèmia protegeix la microcirculació hepàtica tant en fetges sans com esteatòsics, resultant en la protecció del parènquima hepàtica.
- El suplement de la solució de preservació amb rMnSOD redueix de forma eficient l'acumulació de O_2^- al teixit hepàtic de fetges murins sans i esteatòsics i, de forma important, també en biòpsies hepàtiques humanes.

Aquestes dades suggereixen que la rMnSOD pot ser efectiva com a pretractament o suplement de la solució de preservació en l'obtenció i preservació d'empelts hepàtics.

Estudi 2. *Els efectes de la isquèmia-reperfusió calenta en el fenotip microcirculatori hepàtic: mecanismes implicats i teràpies farmacològiques*

- Un període curt d'isquèmia calenta afecta significativament el fenotip endotelial hepàtic, que esdevé disfuncional conduint a un increment del to vascular.
- El dany endotelial derivat de la isquèmia i reperfusió calenta es deu, en part, a la disminució en l'expressió de KLF2 durant la fase primerenca de reperfusió.
- La reducció en la vasoprotecció derivada de KLF2 i el desajust microcirculatori està associat amb un augment en la inflamació i la mort cel·lular, especialment en la fase tardana de reperfusió.

- El pretractament amb simvastatina protegeix la microcirculació hepàtica de la isquèmia-reperfusió calenta prevenint el subseqüent dany hepàtic.
- Les vies vasoprotectores derivades de KLF2 mitjancen, en part, els efectes beneficiosos de la simvastatina.
- L'administració de Simvastatina és una estratègia simple i efectiva per reduir el dany hepàtic en situacions clíniques associades amb dany per isquèmia-reperfusió calenta.

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