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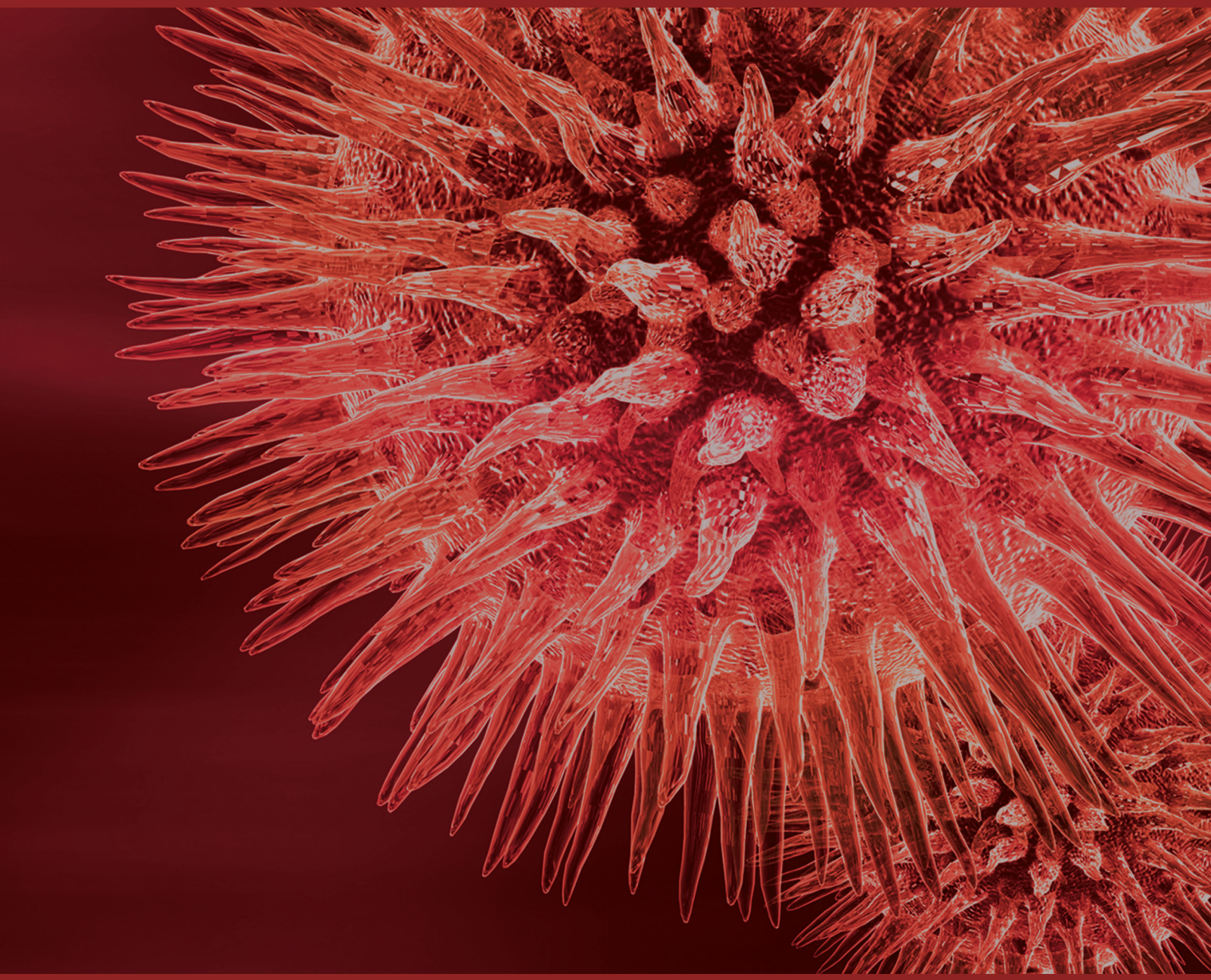
Guest Editors: Mariapia Vairetti, Hartmut Jaeschke, Diethard Monbaliu, Jae-Sung Kim, and Andrea Ferrigno



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Editorial

Innovative Pharmacological/Therapeutic Approaches against Hepatic Ischemia/Reperfusion Injury

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Prolonged oxygen deprivation corresponding to the ischemic period is observed during liver resection, transplantation, and trauma and subsequent oxygen restoration always leads to reperfusion injury. In particular, the ischemic period represents an inevitable event during conventional organ preservation before transplantation and the magnitude and severity of reperfusion injury depend on the time of liver preservation. Faced with the increasing shortage of donor organs for transplantation, there is renewed interest in marginal livers, such as those obtained from donation after circulatory death or fatty livers, which are particularly susceptible to ischemia/reperfusion (I/R) injury.

The decreased tolerance towards I/R observed in fatty livers has not been fully elucidated yet, and underlying mechanisms appear different in lean and steatotic livers. The paper of A. Matsuda et al. proposed the use of activated protein C (APC), an anticoagulant, found to induce an initial attenuation of tissue damage by inhibiting inflammatory cell infiltration and sinusoidal endothelial injury in normal liver. Of interest, in steatotic mice livers, APC did not affect initial liver damage but attenuated late damage, suggesting the existence of an additional pathway to the anti-inflammatory cytoprotective effect of APC which may occur via activation of adenosine monophosphate-activated protein kinase (AMPK) phosphorylation.

A further attempt to reduce the fatty liver susceptibility was reported by E. Pantazi et al. that used a peroxisome proliferator-activated receptor α (PPARα) agonist WY-14643. PPARα is a nuclear receptor highly expressed in liver and it functions as a lipid sensor. Treatment with WY-14643 reduced liver injury in fatty rat livers, enhanced the deacetylase enzyme sirtuin 1 (SIRT1), recently found to be a target for preventing I/R, and prevented endoplasmic reticulum stress.

M. Bejaoui et al. investigated the use of polyethylene glycols (PEGs) that, besides their usefulness as oncotic agents in preservation solution, have been shown to protect against cold injury and ischemic damage. The intravenous PEG 35 administration by a unique dose protected steatotic rat liver grafts against the deleterious effects of cold storage and the subsequent reperfusion. The rationale for the intravenous administration of PEG 35 was to induce a pharmacological preconditioning effect; its protective mechanisms are related to preservation of mitochondria and the induction of protective cell signaling pathways (eNOS, Akt, and AMPK).

An additional model of liver preconditioning by pharmacological induction of adenosine A2a receptor (A2aR) was reported by E. Alchera et al. The analysis of the molecular changes induced by A2aR stimulation revealed multiple mechanisms of liver cell protection that can be both immediate (early preconditioning) and delayed (late preconditioning). Note that the A2aR activation also protects against

lipotoxicity by preventing lipoapoptosis in primary rat hepatocytes.

Among the hepatic mechanisms involved in the development of I/R, mitochondrial dysfunction represents a crucial cellular event contributing to I/R injury. As reported by K. Go et al., the elimination of abnormal and dysfunction mitochondria by mitophagy appears to increase both the quality of the mitochondria and cell survival. Although the mechanisms underlying the onset and propagation of mitophagy remain elusive, this event represents an early adaptive response to facilitate cell survival during I/R.

Articles published in this issue not only help to identify new pharmacological approaches for improving liver function during ischemia/reperfusion damage but also stimulate the identification of molecular mediators of hepatic injury.

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Review Article

Mitochondrial Dysfunction and Autophagy in Hepatic Ischemia/Reperfusion Injury

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Ischemia/reperfusion (I/R) injury remains a major complication of liver resection, transplantation, and hemorrhagic shock. Although the mechanisms that contribute to hepatic I/R are complex and diverse involving the interaction of cell injury in hepatocytes, immune cells, and endothelium, mitochondrial dysfunction is a cardinal event culminating in hepatic reperfusion injury. Mitochondrial autophagy, so-called mitophagy, is a key cellular process that regulates mitochondrial homeostasis and eliminates damaged mitochondria in a timely manner. Growing evidence accumulates that I/R injury is attributed to defective mitophagy. This review aims to summarize the current understanding of autophagy and its role in hepatic I/R injury and highlight the various therapeutic approaches that have been studied to ameliorate injury.

1. Introduction

Ischemia/reperfusion (I/R) injury is the phenomenon by which cellular damage in an organ initiated during hypoxia or anoxia becomes exacerbated when oxygen delivery and tissue pH are restored [1]. I/R begins as a localized process leading to an initial parenchymal cell death and progresses to a profound inflammatory response that involves direct and indirect cytotoxic mechanisms [2]. Low-flow states, trauma, liver resection surgery for treatment of benign and malignant disease, and liver transplantation are among the scenarios that predispose the liver to I/R. Liver transplantation is the standard care for patients with the end-stage liver disease and those with irreversible tumors of hepatic origin [3]. However, organ shortage has led to extending the donor selection criteria, including older, steatotic, or non-heart-beating donors as well as organs that have been exposed to extended periods of ischemia [1]. Hepatic I/R remains a source of major complication in clinical practice affecting perioperative morbidity, mortality, and recovery. Despite its profound clinical importance, therapies to suppress I/R at the bedside remain limited largely due to the complex mechanisms that contribute to I/R. In this review, we will discuss the cellular and molecular mechanisms that trigger warm I/R injury and summarize current therapeutic approaches to ameliorate warm I/R injury.

2. Types of I/R Injury

The liver is the second largest organ in the body. Due to its highly aerobic nature, as inferred from its unique dual blood supply, liver cells are particularly susceptible to ischemic insult. Hepatic I/R can be categorized into warm and cold ischemia. Whereas warm I/R is observed in vascular occlusion during hepatic resection surgery or during exposure to low-flow incidences such as trauma, hemorrhagic shock, cardiac arrest, and hepatic sinusoidal obstruction syndrome, cold I/R is evident during hepatic transplantation, where the graft is subjected to hypothermic preservation prior to a warm reperfusion phase [4]. Although tissue death is the final outcome from either cold or warm ischemia, the injury mechanisms are quite distinct. For instance, while cold I/R induces injury primarily to sinusoidal endothelium and nonparenchymal cells [5], hepatocytes are a major target of warm I/R injury [6].

Hepatic endothelial and nonparenchymal damage initiates reperfusion injury after cold ischemia. Cellular injury to

endothelial and Kupffer cells adversely affects graft microcirculation by increasing platelet activation, vasoconstriction, upregulation of adhesion molecules, and generation of reactive oxygen species (ROS). These events further activate Kupffer cells and recruit neutrophils, ultimately potentiating hepatocyte death [7].

In biochemical aspects, warm ischemia causes three major changes in hepatocytes: (1) anoxia, (2) nutrition depletion, and (3) cytosolic acidosis. The loss of oxygen during ischemia depletes hepatocytes of cellular adenosine triphosphate (ATP), leading to disruption of energy-dependent metabolic and transport processes [6]. Sodium, chloride, and calcium homeostasis, which are tightly regulated by ATP-dependent channels and exchangers, are significantly compromised [6]. Cessation of blood flow likewise results in nutrient depletion and further potentiates ATP loss. Accumulation of lactate and hydrogen ion via anaerobic glycolysis and ATP hydrolysis, respectively, generates the acidic milieu in the cytoplasm, which, in turn, suppresses a myriad of enzymes that optimally operate in a neutral pH [8]. Though prolonged ischemia and severe tissue acidosis eventually cause liver cell death, the acidic environment confers protection to the liver parenchyma during the acute ischemic period [9]. Paradoxically, restoration of blood flow and return of normal pH independently aggravate ischemic damage.

Cold I/R is observed solely in the setting of orthotopic liver transplantation, whereas clinical settings leading to warm I/R are more numerous and occur more frequently. While the reader should be aware that two types of I/R exist and occur through two separate mechanisms, this review will focus on the mechanisms and subsequent therapeutic interventions involved in mitigating warm I/R due to its higher incidence.

3. The Mitochondrial Permeability Transition (MPT)

Individual hepatocytes possess hundreds of mitochondria in order to meet the high amount of energy required to execute multiple metabolic functions. While functional mitochondria are absolutely necessary for cell survival and anabolic events, these double-membrane organelles are also causatively involved in both apoptotic and necrotic cell death. When hepatocytes are exposed to oxidative stress, calcium overloading, or I/R, the high conductance permeability transition pores in the mitochondria open and subsequently initiate onset of the MPT [10]. Calcium, inorganic phosphate, alkaline pH, and ROS promote the MPT onset, whereas cyclosporin A (CsA), Mg^{2+}, acidic pH, and trifluoperazine prevent the opening of permeability transition pores [11]. Once the MPT initiates, the permeability barrier of the mitochondrial inner membranes collapses and solutes with a molecular mass of up to 1.5 kDa can diffuse freely across the mitochondrial inner membranes. Consequently, mitochondria depolarize, uncouple, and swell, leading to ATP depletion and necrotic cell death (Figure 1). Although necrosis is a predominant type of cell death after I/R, onset of the MPT can also induce apoptosis in ischemic liver and other organs [12]. Rupture of the mitochondrial outer membranes after onset of the MPT and subsequent mitochondrial swelling releases cytochrome *c* that is normally sequestered at the intermembrane space of the mitochondria. Since this 12 kDa protein is an integral element of the apoptosome, the release of cytochrome *c* into the extracellular medium triggers a caspase- and ATP-dependent apoptosis. However, when the cells are depleted of ATP, such as in the setting of ischemia, they do not undergo apoptosis. Instead, these ATP-deficient cells develop necrotic cell death even in the presence of the upstream proapoptotic signals. Thus, ATP availability is a key switch from necrosis to apoptosis [13]. The importance of the MPT in both apoptotic and necrotic hepatocyte death after I/R is further substantiated by the fact that CsA blocks reperfusion-induced apoptosis, whereas tacrolimus, an immunosuppressing agent that does not block the MPT, has no effects on I/R injury [14]. Thus, the MPT is a common pathway leading to both types of cell death after I/R.

Mitochondrial calcium and ROS likely trigger onset of the MPT after I/R. Imaging analysis with isolated rodent hepatocytes shows that increase of mitochondrial calcium and ROS precedes onset of the MPT after reperfusion [15]. Since the chelation of intramitochondrial calcium, but not cytosolic calcium, blocks the generation of ROS and cell death after reperfusion, calcium-mediated mitochondrial ROS formation appears to be the molecular event triggering the MPT after I/R. In contrast, cytosolic ROS play a minimal role in I/R injury, as demonstrated by lack of cytoprotection by apocynin or diphenyleneiodonium chloride, inhibitors of cytosolic NADPH oxidase.

MPT onset remains a critical component in cell death following I/R and therapies to block MPT have been studied. CsA inhibits onset of the MPT by binding to cyclophilin D in the mitochondrial matrix [16]. Although beneficial effects of CsA on reperfusion injury have been documented in animal models [10, 14, 17], its use in human livers remains limited for following reasons. First, CsA has a very narrow therapeutic efficacy of blocking the MPT. High concentrations of CsA cause nephrotoxicity [18]. Second, CsA inhibits calcineurin, provoking immunosuppression. Although nonimmunosuppressive derivatives of CsA such as N-methyl-val-CsA and NIM811 have been introduced [19], their benefits have not been recognized in the clinic. Third, cells can develop unregulated MPT when they are exposed to high concentrations of chemical inducers and excessive stresses [16]. Despite the structural similarity to regulated permeability pores, the unregulated pores do not require calcium for conductance and are not blocked by CsA. It is, however, noteworthy that endeavors are continuously pursued to develop a new class of MPT blockers. For instance, TRO40303 blocks the MPT by binding to the cholesterol site of the peripheral benzodiazepine receptor of the mitochondria [20]. This new agent is currently in clinical trial.

4. Autophagy

Macroautophagy (referred to as autophagy hereafter) is an intracellular self-digesting pathway to remove abnormal organelles, malformed proteins, and surplus or unnecessary

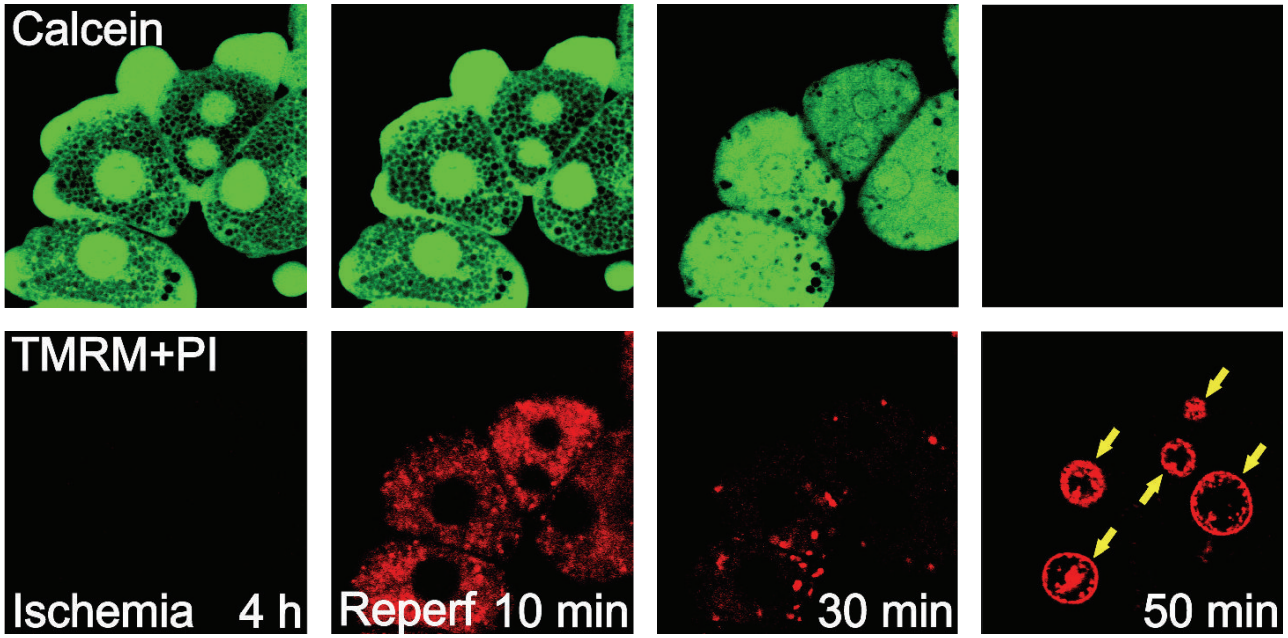

Figure 1: Onset of the MPT after I/R in primary rodent hepatocytes. After 4 hours of simulated ischemia, hepatocytes were reperfused and confocal images of calcein, tetramethylrhodamine methyl ester (TMRM), and propidium iodide (PI, arrows) were simultaneously collected. Polarized mitochondria take up red fluorescing TMRM while simultaneously excluding green fluorescing calcein due to the closed conformation of permeability transition pores. After 4 hours of ischemia, anoxia depolarized the mitochondria and TMRM fluorescence was undetectable. At the same time, the mitochondria in the green channel appeared as dark and round voids where each void represents a single, polarized mitochondrion, indicative of the absence of MPT onset during ischemia. After reperfusion, the mitochondria transiently repolarized within 10 minutes, but the MPT initiated thereafter, as shown by the loss of TMRM fluorescence and diffusion of cytosolic calcein into the mitochondria. Both calcein and TMRM fluorescence completely vanished at 50 minutes and PI labeled the nuclei (arrows) due to the loss of the plasma membrane integrity.

cytoplasmic contents through lysosomal digestion [21]. Autophagy can be divided into five key stages: initiation, nucleation, elongation, fusion, and degradation [22] (Figure 2). Thus far, over thirty autophagy-related (ATG) proteins have been identified in mammalian cells with eighteen designated as core components essential to autophagosome formation [23]. The formation of a double-membrane autophagosome is initiated when core proteins (ULK1, ATG13, FIP200, Ambra, BECN1, ATG13, and UVRAG) act on the phagophore whose membranes are believed to originate from the endoplasmic reticulum, mitochondria, or Golgi [24]. ATG2, ATG3, ATG4, ATG5, ATG7, ATG8, ATG9, ATG10, ATG12, ATG16, and ATG18 guide the formation and subsequent elongation of the double-membrane phagophore. This autophagosome envelops target cellular constituents selectively or nonselectively before fusing with the lysosome to produce a single-membrane autolysosome for degradation. With the help of lysosomal enzymes, autolysosomal contents are degraded into amino acids.

Abnormal or dysfunctional mitochondria are cleared through selective autophagy, a process termed mitophagy. In addition to its integral role in the turnover of normal mitochondria, mitophagy prevents accumulation of damaged mitochondria and cytotoxic mitochondrial byproducts [25]. Impaired or insufficient mitophagy can amass damaged mitochondria, leading to uncontrolled increases in ROS, mutations in mitochondrial DNA, energetic failure, and ultimately cell death. There exist at least two distinct types of mitophagy in the cell: phosphatidylinositol-3-kinase- (PI3K-) dependent and phosphatidylinositol-3-kinase- (PI3K-) independent mitophagy. During PI3K-dependent mitophagy, mitochondria sequestered by autophagosomes remain polarized until delivery to the lysosome. Fusion of the autophagosome and lysosome creates the autolysosome [9] (Figure 3(a)). Depolarization occurs later as a result of either the onset of MPT or acidification of the mitophagosomal lumen [15]. This type of autophagy is observed during nutrient starvation and also occurs to hepatocytes after acute ischemic events [9].

PI3K-independent mitophagy becomes evident when the cell experiences a widespread mitochondrial depolarization [9]. This type of mitophagy requires BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)/BNIP3L (also known as NIX) and PTEN-induced putative kinase protein 1 (PINK1)/Parkin [26]. When the mitochondria are subjected to an uncoupler such as carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), PINK1 recruits Parkin to the outer membranes of dysfunctional mitochondria. Recruitment leads to ubiquitination of outer membrane proteins, thereby marking a mitochondrion for mitophagy.

Growing evidence indicates cytoprotective roles of autophagy in various diseases such as aging, diabetes, and neurodegenerative diseases [9]. Autophagy also plays a pivotal role in maintaining mitochondrial function and cell survival after hepatic I/R [8, 25, 27–29]. During ischemia,

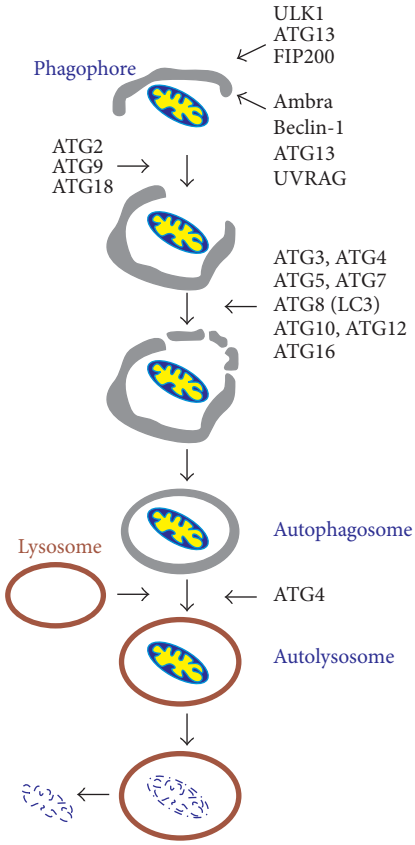

Figure 2: A schematic of autophagy process.

lack of blood flow exposes the liver to both nutrient depletion and ATP shortage. Although nutrient depletion is one of the most powerful stimuli for autophagy, a near-complete loss of cellular ATP after prolonged ischemia halts the execution of autophagy as this catabolic process relies highly on cellular energy. Moreover, ATP shortage in ischemic livers inhibits ATP-driven calcium pumps and other calcium exchangers, leading to calcium overloading in the cytosol [15]. We have shown that the increase in cytosolic calcium activates calpains which, in turn, hydrolyze key autophagy proteins, ATG7 and BECN1 [27]. Since both proteins are essential for successful formation and elongation of autophagosomes, autophagic machinery in ischemic hepatocytes becomes nonfunctional. Hence, a combination of ATP depletion and ATG loss during prolonged ischemia contributes directly to the reduction of autophagic flux in hepatocytes.

Not only does oxygen supply resume but also hepatocellular pH recovers to normal physiological value after reperfusion. During the early stage of reperfusion, the mitochondria of hepatocytes repolarize transiently (Figure 1). With a reestablishment of the proton motive force within the matrix, the electron transfer chain is reinstituted, albeit temporarily, leading to ATP generation. At this time, autophagy begins to eliminate abnormal proteins and organelles that are produced during ischemia. Consistent with this view, the induction of autophagy is often observed early after an inciting insult [27–31]. In contrast to normoxia or short ischemia where the number of damaged mitochondria is few so that the demand for mitophagy remains minimal, prolonged ischemia and reperfusion lead to substantial calcium and ROS accumulation in a subset of mitochondria. In order for hepatocytes to remain viable, this subset of injured mitochondria must be immediately eliminated via mitophagy. Yet autophagic capacity becomes impaired following prolonged ischemia and subsequent reperfusion due to a detrimental chain of mitochondrial calcium overloading, additional activation of calpains, and loss of autophagy proteins, such that the extent of mitochondrial injury surpasses the ability of autophagy to clear I/R damaged mitochondria. Therefore, at the late stage of reperfusion, autophagy fails to eliminate dysfunctional mitochondria, and widespread onset of the MPT ensues thereafter (Figure 3(b)). The protective role of autophagy in hepatic I/R injury is supported by findings that nutrient depletion prior to ischemia or overexpression of ATG7 and BECN1 all prevents the MPT and promotes hepatocyte survival after reperfusion [27]. It has also been reported that inhibition of autophagy during I/R enhances ROS-induced hepatocyte necrosis [32]. Because defective or insufficient autophagy causatively contributes to lethal I/R injury in the liver, approaches to enhance autophagy or suppress defective autophagy may provide new therapeutic strategies to ameliorate liver function after reperfusion. Indeed, numerous attempts have been made recently to augment autophagy in ischemic livers. For example, studies demonstrate that a low dose of cisplatin, a chemotherapic agent, increases cell viability after warm ischemia through inducing ATG7 and BECN1 expression and more pronounced mitophagy [33, 34]. Pretreatment with rapamycin, an mTOR inhibitor, augments autophagy and reduces hepatic damage in a warm I/R model [35, 36]. Chronic administration of lithium also confers cytoprotection against warm hepatic I/R injury through various mechanisms including enhanced autophagy [37]. Lithium treatment has also been shown to lower inflammatory cytokine production, neutrophil infiltration, and high motility group box 1 (HMGB1) levels in the liver [37]. Carbamazepine, an FDA-approved anticonvulsant drug, provides cytoprotection against I/R in hepatocytes through blockade of a sequential chain of calcium overloading, calpain activation, and depletion of ATG7 and BECN1 [29].

5. Nitric Oxide

Nitric oxide (NO), produced from a reaction between L-arginine and oxygen, is a gaseous signaling molecule that plays an important and complex role in hepatic I/R. Two main isoforms, inducible NO synthase (iNOS) and endothelial NOS (eNOS), synthesize NO in the liver during I/R [38]. Whereas eNOS is constitutively expressed on sinusoidal endothelium, iNOS is regulated by many cytokines (TNF-α and IL-1) [39]. Depending on cell types and milieu, NO can either promote or prevent cell injury.

Several studies on hepatocytes support a cytoprotective role for NO. In cultured hepatocytes, NO prevents TNF-α and Fas ligand-induced apoptosis [40]. During I/R, NO blocks MPT onset and necrotic hepatocellular death through a signaling pathway of guanylyl cyclase and cGMP-dependent

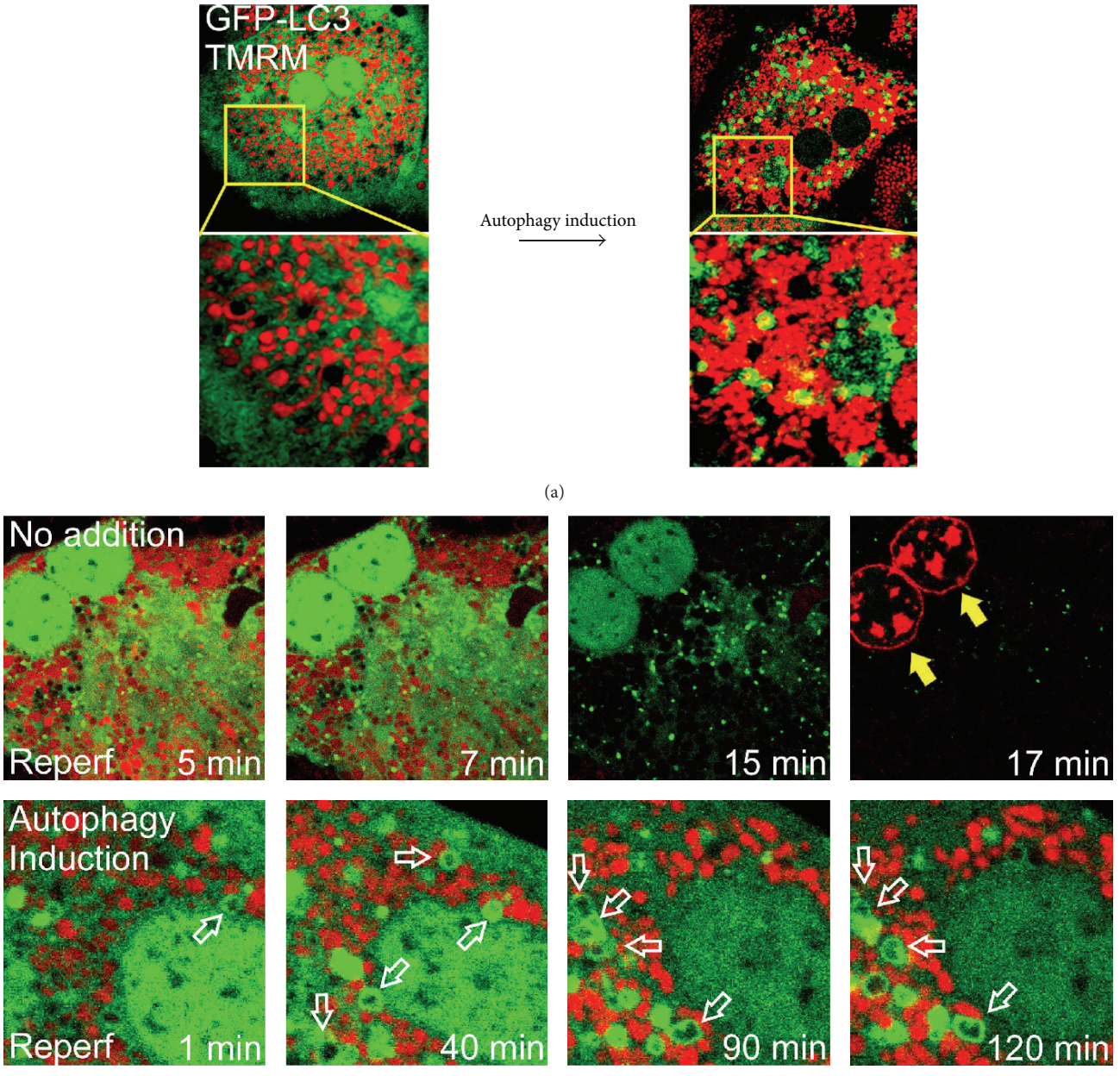

Figure 3: Autophagy in primary mouse hepatocytes. (a) Confocal microscopy with green fluorescent protein-labeled microtubule-associated protein 1 light chain 3 (GFP-LC3) and TMRM in normoxic hepatocytes. Under the basal condition of normoxia, GFP-LC3 predominantly localizes in the cytosol. After autophagy induction, hepatocytes show numerous punctate GFP-LC3, indicative of autophagosomes. Note that some red fluorescing mitochondria are entrapped by GFP-LC3, an event signifying the onset of mitophagy. The bottom panels represent magnified images of the square inserts at the top panels. (b) Loss of autophagy after I/R. Hepatocytes were labeled with GFP-LC3 and subjected to 4 hours of simulated ischemia. After 5 minutes of reperfusion, some autophagosomes (green fluorescing punctate structures) were evident but unable to sequester abnormal mitochondria (top panels). This cell was dead after 17 minutes, as indicated by PI labeling in the nuclei (yellow arrows). In striking contrast, when autophagy was stimulated prior to ischemia, hepatocytes executed a robust autophagy to clear abnormal mitochondria and remained viable after 2 hours of reperfusion. Empty arrows display the autophagosomes surrounding the mitochondria.

kinase (PKG) [41]. Studies demonstrating that NO plays a key role in ischemic preconditioning in cardiomyocytes [42] may also explain how ischemic preconditioning ameliorates I/R in the liver. In addition to its effects on parenchymal cells, NO exerts a protective impact on sinusoids during I/R. Under physiologic conditions, NO induces vasodilation and prevents platelet adhesion, thrombosis, and polymorphonuclear neutrophil accumulation, preventing sinusoidal obstruction [43]. Decreased NO production during ischemia and reperfusion can lead to constriction of the microvascular

bed and exacerbate I/R injury. Genetic manipulation of NOS has proposed that inhibition of eNOS exacerbates hepatic I/R [44], whereas eNOS overexpression is cytoprotective [45]. Cytoprotection by eNOS overexpression may be linked to both heme oxygenase-1 (HO-1) and guanylyl cyclase [45]. There are several reports demonstrating that pharmacological increase in NO can reduce hepatic I/R. In cultured hepatocytes, the addition of NO donors such as *S*-nitroso-*N*-acetylpenicillamine (SNAP), DETA NONOate, and spermine NONOate to the reperfusion medium decreases mitochondrial dysfunction and cell necrosis [41]. Mice injected with sildenafil, a cGMP-specific phosphodiesterase inhibitor, or 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), a guanylyl cyclase activator, also display lower serum levels of transaminases after I/R [45]. In the clinical setting, patients who have received NO gas during liver transplantation show a faster recovery of liver function and shorter hospital stay with lowered peak serum transaminase levels [46]. Hydrogen sulfide and 17-beta estradiol have been found to increase NO levels in serum and protect against hepatic I/R [47, 48]. The importance of NO is further substantiated by the observation that inhibition of NOS blunts 17-beta estradiol-dependent protection [47]. Beck-Schimmer et al. demonstrate that sevoflurane protects the liver against I/R by increasing mRNA levels of NOS [49].

Although NO can promote cell survival after I/R, cautions should be taken in the use of NO donors. Reactions between superoxide and NO can yield injurious peroxynitrite, a highly reactive oxidant, which can alter DNA and oxidize lipids and proteins [38]. Moreover, accumulation of ROS and inflammatory cytokines during the late stage of reperfusion are known to upregulate iNOS expression, leading to large quantities of NO and subsequent accumulation of reactive nitrogen species. Hence, whether NO promotes or prevents cell death is highly dependent on its concentration, the time and duration of administration, and the condition of the liver including baseline levels of antioxidants available to consume ROS prior to ischemic insult. While the addition of lower concentrations of NO during the early phase of reperfusion may be cytoprotective, high levels, especially at the late stage of reperfusion, may exacerbate tissue injury [50]. Of interest, a recent study suggests that NO impairs autophagy by inhibiting the synthesis of autophagosomes and activating mammalian target of rapamycin (mTOR) [51].

6. Surgical Methods to Reduce I/R Injury

6.1. Ischemic Preconditioning. During ischemic preconditioning (IPC) of the liver, hepatic inflow is occluded by placing a vascular loop or clamp around the portal triad for an interval of 10–15 minutes followed by removal of the clamp for an additional interval of 10–15 minutes of reperfusion before starting the actual procedure [52]. Various mechanisms have been proposed to explain how IPC provides cytoprotection. Both clinical and basic studies have noted that IPC is associated directly with preservation of ATP after I/R, corroborating the importance of mitochondria in reperfusion injury [53–55]. However, cytoprotective mechanisms underlying IPC are multifactorial, including inhibition of apoptosis and induction of autophagy [55–57]. Liu et al. recently reported that IPC confers protection via enhancing HO-1 mediated autophagy [58]. IPC also causes a mild increase in peroxides which stimulates protective pathways, suggesting a cellular adaptation after exposure to a sublethal oxidative stress [59]. It is noteworthy that inhibition of Kupffer cells with gadolinium chloride abolishes the protective effects of IPC, implying that IPC influences hepatocytes and nonparenchymal cells [60]. Multiple clinical studies have reported that IPC improves outcomes. Using an IPC protocol of 10 minutes of clamping followed by 10 to 15 minutes of reperfusion prior to an anatomic liver resection, Clavien et al. demonstrated that IPC reduced liver injury, indicated by reduced postoperative serum transaminase levels. Furthermore, IPC was associated with less sinusoidal apoptosis in comparison to gender and age matched controls [61]. The benefits of IPC have been substantiated by studies demonstrating that IPC prior to prolonged hepatic inflow occlusion reduced both peak postoperative transaminase levels and the use of intraoperative vasopressors [53, 62]. It is, however, noteworthy that there exist controversies with regard to its efficacy, particularly in surgical trials. While a meta-analysis of the clinical literature has concluded that IPC resulted in reduced hospital length of stay and decreased transfusion rates [63], mortality, morbidity, intraoperative blood loss, and peak transaminase levels were comparable in the two groups. Others have noted that there was no difference in blood loss [64], morbidity, mortality, or lab values [65] in noncirrhotic and cirrhotic livers [66]. The conflicting results seen in the literature may be attributed to differences in IPC protocols used and the heterogeneity of patient populations evaluated, including underlying liver disease prior to resection.

6.2. Intermittent Clamping. Like IPC, during intermittent clamping, hepatic inflow is occluded with use of a vascular loop or clamp. Rather than a single period of ischemia prior to one period of reperfusion, a prolonged ischemic period is interrupted by short periods of reperfusion throughout surgery [52]. Belghiti et al. evaluated whether intermittent clamping rather than prolonged, continuous clamping improved surgical outcomes after hepatic resection. In their prospective randomized study, intermittent clamping resulted in reduced peak transaminase levels and was associated with lower frequency of postoperative liver failure [64]. A study comparing IPC and intermittent clamping has concluded that either technique conferred comparable protection based on peak transaminase levels, ICU duration, hospital stay, and complication rate [67]. IPC, however, was associated with lower intraoperative blood loss and shorter transection time. In their murine model, Rüdiger et al. demonstrate that both surgical strategies provide a comparable protection during 75 minutes of hepatic ischemia. Beyond this time, intermittent clamping was superior to IPC [68]. It is, thus, assumed that intermittent clamping confers protection through mechanisms similar to those of IPC, including preservation of mitochondrial integrity and function, and cellular ATP [69].

6.3. Remote Ischemic Preconditioning (RIPC). Remote ischemic preconditioning (RIPC) is the phenomenon whereby brief episodes of I/R to distant tissues or organs such as limb or intestine render the liver resistant to a subsequent sustained I/R. Intestinal I/R prior to hepatic I/R in rats has been reported to improve survival after reperfusion through enhanced expression of HO-1 [70]. RIPC of the femoral bundle also provides protection against liver I/R by upregulating IL-10 and matrix metalloproteinase-8 [71]. Although it is plausible that cytoprotective factors released during this procedure may be delivered to the liver before I/R, future studies are warranted to elucidate the mechanisms and further validate the efficacy of RIPC.

7. Diseased Liver and I/R

7.1. Role of Aging in I/R. The increase in life expectancy over the past century has resulted in an equivalent rise in elderly patients. Aging is strongly associated with increased incidence and severity of diseases, accidents, and stress. In the liver, aging reduces hepatic blood flow and the number of mitochondria and endoplasmic reticulum (ER). As compared to other organs such as muscles and the brain, deficits in hepatic morphology and function with advancing age are less apparent clinically. The liver is indeed one of the least-studied organs in aging research. Livers from elderly patients, however, have a poorer recovery from surgical stresses during liver resection and transplantation, indicating reduced reparative capacity with aging [72]. The aged liver also responds differently to even minor stresses. For instance, clinical studies evaluating the IPC efficacy have shown that IPC provided greater protection to younger rather than older patients [53, 67]. Several mechanisms may account for this increased susceptibility. Mather and Rottenberg demonstrate that mitochondria isolated from aged livers undergo MPT onset at lower calcium concentrations than their young counterparts, suggesting a reduced calcium buffering capacity in aged mitochondria [73]. Our group has shown the importance of autophagy in the age-dependent mitochondrial injury [28, 74]. While the basal autophagy in aged hepatocytes was comparable to that in young hepatocytes, exposure of aged cells to short ischemia, a condition inducing a minimal injury to young cells, substantially decreased levels of ATG4B which plays an integral role in autophagosome formation and clearance [9]. As a consequence, the autophagy machinery halts, and aged cells fail to remove damaged mitochondria, leading to hepatocellular death later. In addition to autophagy, aging appears to reduce the capacity of proteasomal degradation. Decreased heat shock protein 70 expression and lowered NF-κB activation due to derangements in transport of ubiquitinylated proteins to the proteasome have been observed in senescent liver cells and may account for their enhanced sensitivity to I/R [72, 75, 76].

7.2. Role of Steatosis in I/R. Hepatic steatosis, lipid accumulation in the liver that exceeds 5% of wet weight, has become a common problem in industrialized countries caused by multiple comorbidities including obesity, ethanol toxicity, metabolic disorders, and certain drugs [77, 78]. Clinically, steatosis has been associated with increased morbidity and mortality during hepatic resection [79, 80]. The mechanisms underlying the increased susceptibility of steatotic livers to I/R injury may be multifactorial and involve both parenchymal and nonparenchymal dysfunction, including mitochondrial dysfunction, increased lipid peroxidation and ER stress, enhanced increased release of proinflammatory mediators, and increased neutrophil infiltration. In addition, increased cellular volume due to lipid accumulation may potentially obstruct the adjacent sinusoid space leading to poor delivery of oxygen and nutrients and reduction of mitochondrial ATP synthesis. Mitochondrial uncoupling and ATP depletion have been observed in steatotic livers where the expression of mitochondrial uncoupling protein-2 (UCP-2) substantially increases in hepatocytes [81]. As UCP-2 mediates a proton leakage across the mitochondrial inner membranes, steatotic hepatocytes tend to consume oxygen to generate heat rather than ATP. These short-circuited mitochondria encounter more challenges after I/R and become vulnerable to reperfusion injury. Furthermore, steatotic hepatocytes have reduced antioxidant activity, as evidenced by decreased GSH and thioredoxin [82, 83], and are prone to oxidative stress such as I/R injury [84].

While animal literature suggests that fatty livers are more susceptible to I/R injury than lean counterparts, caution should be taken before these studies are translated into clinical application. While genetic and dietary models exist to induce steatosis, no animal model induces both metabolic syndrome and the type of liver pathology seen in human nonalcoholic fatty liver disease (NAFLD) [85, 86].

8. The Challenge of Translating Animal Studies to Humans

Table 1 summarizes proposed therapeutic strategies against hepatic reperfusion injury. However, promising treatments in preclinical studies have not translated to significant clinical benefit in human trials. I/R injury is multifactorial. Although simultaneous inhibition of all individual factors triggering I/R injury has intuitive appeal as an ideal therapy, the identification of all these factors is far from complete. Furthermore, it is highly likely that these factors are mechanistically intertwined. There is also a striking disparity between animal models and clinical settings. While animal experiments are typically conducted in young, healthy animals with no comorbidities, the patients requiring liver resection and transplantation are critically ill with preexisting liver diseases. Moreover, the laboratory environment significantly influences experimental results. Thus, despite the importance of animal models in delineating basic principles of therapeutic strategies, they may not encompass all salient features of the human disease condition.

9. Concluding Remarks and Future Directions

Over the past years, much progress has been made in understanding the mechanisms involved in the development of I/R in the liver. Mitochondrial dysfunction is an important

Table 1: Summary of strategies to reduce I/R injury.

Therapeutic strategy	Proposed mechanism	Reference
Cyclosporin A	Inhibits MPT onset	[87]
ATG7, Beclin-1 overexpression	Increase autophagy	[27]
Carbamazepine	Blocks calpains and increases autophagy	[29]
Hemin	Increases HO-1 Decreases Ca overload and calpain activation Increases autophagy	[88]
Cisplatin	Increases ATG7 and Beclin-1 to increase autophagy Decreases HMGB1 secretion	[33] [34]
Rapamycin	Increases autophagy	[35, 36]
Chronic lithium	Increases autophagy Reduces inflammatory cytokines, neutrophil infiltration, and HMGB1 levels GSK-3β inhibitor	[37] [89]
Nutrient depletion	Increases autophagy Reduces circulating HMGB1	[9, 27] [90]
Glutathione	Antioxidant; reduces TNF-α levels	[91–93]
N-Acetylcysteine	Maintains glutathione	[91, 92]
Glycine	Hepatocyte plasma membrane stabilization	[13, 94]
Gadolinium chloride	Kupffer cell inhibition; reduces lipid peroxidation	[95]
Allopurinol	Xanthine oxidase inhibitor	[96–100]
Interleukin 6	Reduces TNF-α levels and attenuates inflammatory response	[101, 102]
Atorvastatin	TLR-4 downregulation and NF-κB downregulation	[103]
Butyrate	Decreases TLR-4 expression	[104]
SB216763	GSK 3β inhibitor, suppressing proinflammatory response Inhibits MPT onset	[105]
Oleanolic acid	GSK-3β inhibitor	[106]
Ulinastatin	Decreases HMGB1 expression	[107]
Eritoran	TLR-4 antagonist	[108]
ICAM-1 antibody	Blocks neutrophil infiltration	[109]
Mac-1 antibody	Blocks neutrophil activity	[110]
Carbon monoxide	GSK-3β inhibitor	[111]
Cobalt protoporphyrin	HO-1 inducer	[112]
Isoflurane	Increases HO-1 activity	[113]
Erythropoietin	Increases HO-1 activity	[114]
Heme oxygenase-1	Increases autophagy Decreases TNF-α mediated apoptosis Inhibits TLR4 Maintains sinusoidal diameter and decreases platelet adhesion to sinusoids	[88] [115] [116] [117]
Nitric oxide	Inhibits MPT onset Prevents TNF-α and Fas ligand-mediated apoptosis Vasodilator Prevents platelet adhesion	[87] [40] [41] [43]
S-Nitroso-*N*-acetylpenicillamine (SNAP)	NO donor	[41]
DETA NONOate	NO donor	[41]
Spermine NONOate	NO donor	[41]
Sildenafil	cGMP phosphodiesterase inhibitor	[45]
YC-1	Guanylyl cyclase activator	[45]
eNOS overexpression	Increases NO	[45]

Table 1: Continued.

Therapeutic strategy	Proposed mechanism	Reference
17-β estradiol	Increases serum NO	[47]
Hydrogen sulfide	Increases serum NO	[48]
Sevoflurane	Increases iNOS expression Decreases TNF-α, IL-1β, MCP-1, NF-κB liver expression, and caspase 3 activity	[49] [118]
Remifentanil	Decreases apoptosis and myeloperoxidase activity Increases superoxide dismutase and NO/iNOS expression	[119]
Ischemic preconditioning	Increases HO-1 Increases autophagy Reduces apoptosis Preserves and restores ATP	[58] [57] [59] [55]
Intermittent clamping	Limits mitochondrial damage Preserves and restores ATP	[69]
Remote ischemic preconditioning	Increases HO-1, IL-10, and MMP-8	[71]

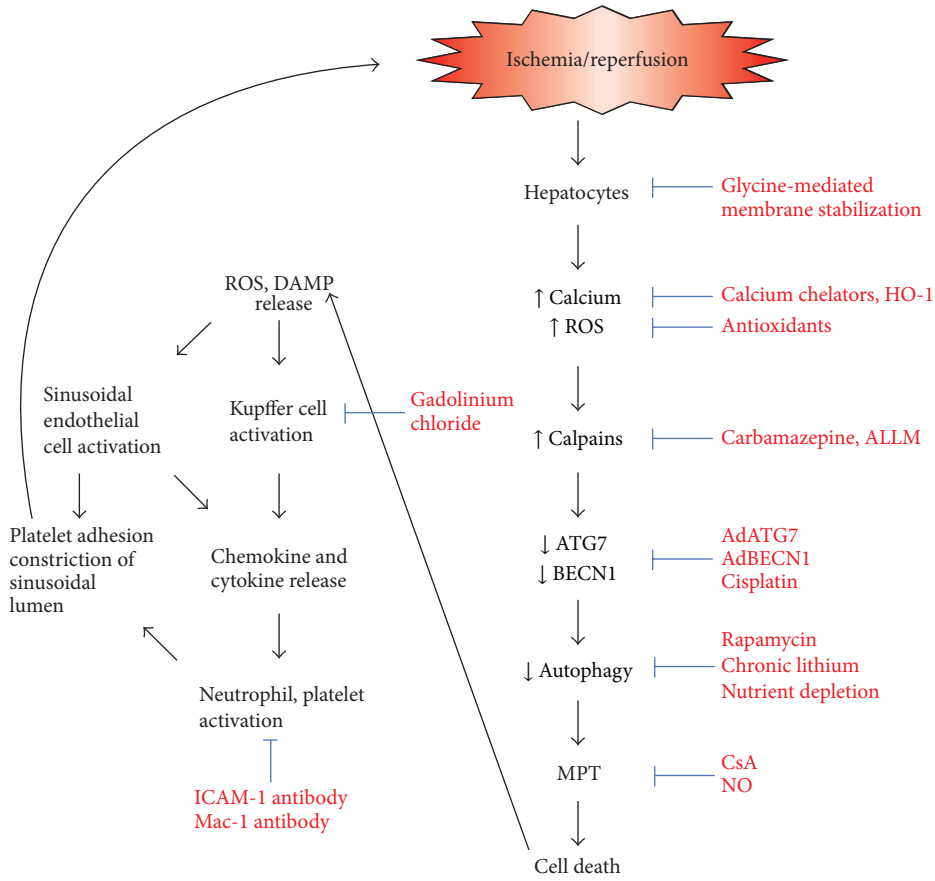

Figure 4: Scheme of I/R-induced impairment of autophagy. After reperfusion, hepatocytes become overloaded with ROS and calcium, which in turn stimulates calpains. These enzymes subsequently hydrolyze ATG7 and BECN1, causing defective autophagy. Since impaired autophagy fails to eliminate abnormal mitochondria, the mitochondria laden with ROS and calcium undergo the MPT and ultimately induce cell death. Suppression of calcium increase, inhibition of calpains with acetyl-leu-leu-methioninal, enhancement of autophagy, or blockade of the MPT with cyclosporin A or nitric oxide prevents reperfusion-induced cell death. Damaged hepatocytes subsequently release damage-associated molecular patterns and ROS. Kupffer cells and sinusoidal endothelial cells are then activated, leading to chemokine and cytokine release, neutrophil and platelet activation, and platelet adhesion to the sinusoidal lumen. Congestion and constriction of the sinusoid further aggravate reperfusion injury. AdATG7, adenovirus expressing ATG7; AdBECN1, adenovirus expressing Beclin-1; ALLM, acetyl-leu-leu-methioninal; CsA, cyclosporin A; DAMP, damage-associated molecular patterns; HO-1, heme oxygenase-1; ICAM-1, intercellular adhesion molecule-1; Mac-1, macrophage-1 antigen; NO, nitric oxide; ROS, reactive oxygen species.

cellular event contributing to I/R injury, and timely removal of abnormal and dysfunction mitochondria not only sustains the quality of the mitochondria but also provides cell and tissue survival (Figure 4). Cells employ mitophagy as an early adaptive response to facilitate a better response to various stresses, including I/R. The mechanisms underlying the onset and propagation of mitophagy remain elusive. Future studies are warranted to characterize detailed signaling pathways inducing lethal reperfusion injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Protective Effect of Intravenous High Molecular Weight Polyethylene Glycol on Fatty Liver Preservation

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Ischemia reperfusion injury (IRI) leads to significant tissue damage in liver surgery. Polyethylene glycols (PEGs) are water soluble nontoxic polymers that have proved their effectiveness against IRI. The objective of our study was to investigate the potential protective effects of intravenous administration of a high molecular weight PEG of 35 kDa (PEG 35) in steatotic livers subjected to cold ischemia reperfusion. In this study, we used isolated perfused rat liver model to assess the effects of PEG 35 intravenous administration after prolonged cold ischemia (24 h, 4°C) and after reperfusion (2 h, 37°C). Liver injury was measured by transaminases levels and mitochondrial damage was determined by confocal microscopy assessing mitochondrial polarization (after cold storage) and by measuring glutamate dehydrogenase activity (after reperfusion). Also, cell signaling pathways involved in the physiopathology of IRI were assessed by western blot technique. Our results show that intravenous administration of PEG 35 at 10 mg/kg ameliorated liver injury and protected the mitochondria. Moreover, PEG 35 administration induced a significant phosphorylation of prosurvival protein kinase B (Akt) and activation of cytoprotective factors e-NOS and AMPK. In conclusion, intravenous PEG 35 efficiently protects steatotic livers exposed to cold IRI.

1. Introduction

Organ preservation is a fundamental requirement in organ transplantation; it preserves the viability of the organ during its transport from the donor to the recipient so that the graft can maintain its function after transplantation [1]. Besides advances in organ preservation, the presence of steatosis remains a limiting factor for the suitable preservation of liver grafts, as steatotic livers are particularly vulnerable to hepatic ischemia reperfusion injury (IRI) [2]. Their use is accompanied by increased risk of primary failure and lowered success of liver transplantation [3]. Currently, the increasing needs of transplantation as well as the scarce of donors pool have obliged the physicians to take advantage of suboptimal liver grafts, as steatotic ones [4]. For this reason, there is an urgent need to explore new strategies that provide a more efficient preservation of steatotic liver grafts. Minimizing the deleterious effects of hypothermia could decrease the reperfusion injury and, consequently, assure an increased rate of graft survival after transplantation.

Polyethylene glycols (PEGs) are water soluble nontoxic polymers that have been employed in many biomedical applications such as gastrointestinal disorders and drugs pegylation [5, 6]. Besides their usefulness as oncotic agents in preservation solutions [7, 8], it has been shown that PEGs molecules protect against cold injury and ischemic

damage. Indeed, PEG used as cryoprotectant in supercooling technique was necessary to achieve successful liver transplantation [9]. Moreover, PEG suppressed hypothermic-induced cell swelling in hepatocyte preservation [10] and protected primary hepatocyte during supercooling preservation [11]. Also, PEG protected cardiac myocytes from hypoxia and reoxygenation-induced cell death [12], decreased oxidative stress [13], and protected injured mitochondria [14].

With this in mind, we hypothesized whether intravenous administration of PEG in the rat prior to organ procurement could protect fatty liver graft against hypothermic and hypoxic damage occurring during preservation and the subsequent reperfusion injury. Our results demonstrated that PEG 35 prevented the deleterious effects of cold IRI when administered intravenously in obese rats.

2. Materials and Methods

2.1. Animals. Male homozygous obese Zucker rats, aged 9 to 10 weeks, were purchased from Charles River (France) and housed at 22°C with free access to water and standard chow. All experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 697/14), University of Barcelona, and were conducted according to European Union regulations for animal experiments (Directive 86/609 CEE).

2.2. Liver Procurement and Ex Vivo Perfusion. All procedures were performed under isoflurane anesthesia inhalation. After laparotomy, the common bile duct was cannulated and livers were flushed with 40 mL of chilled UW preservation solution (4°C) by the mean of catheter insertion into the aorta. After cooling, a second catheter was inserted into the portal vein to complete liver rinsing with further 10 mL of UW solution. The whole liver was then excised and preserved at 4°C for 24 h in the same solution. This procedure implicates the death of the animal under isoflurane anesthesia, and thus the application of analgesia or euthanasia was unnecessary. After 24 h of cold preservation, steatotic livers were removed from preserved solution and flushed at room temperature with 20 mL of Ringer Lactate solution to eliminate the metabolite waste accumulated during liver storage. Then, livers were perfused at 37°C via the portal vein in a closed and controlled pressure circuit. Time point 0 was considered when the portal catheter was satisfactorily connected to the circuit. During the first 15 minutes of perfusion (initial equilibration period), the flow was progressively increased in order to stabilize the portal pressure at 12 mmHg (Pression Monitor BP-1; Pression Instruments, Sarasota, FL). The flow was controlled by a peristaltic pump (Minipuls 3; Gilson, France). The reperfusion liquid (150 mL for each perfusion) consisted of a cell culture medium (William's medium E; BioWhittaker, Barcelona, Spain) with a Krebs-Henseleit-like electrolyte composition enriched with 5% albumin as oncotic supply. The medium was continuously gassed with 95% O_2 and 5% CO2 gas mixture and subsequently passed through a heat exchanger (37°C) and a bubble trap prior to entering the liver. After 120 minutes of normothermic reperfusion, the effluent perfusion fluid was collected for biochemical determinations and hepatic tissues were sampled and stored at −80°C for further analysis.

2.3. Drug Treatment. PEG 35 was kindly provided by IGL-1 Company. PEG 35 was dissolved in physiological saline (5 g/L) and administrated 10 min before liver procurement by intravenous bolus through the penile vein at the concentration of 10 mg/kg.

For confocal microscopy study with PEG-FITC, PEG 35 was fused with fluorescein as previously described by Mero et al. [15].

2.4. Experimental Groups. All animals were randomly distributed into different experimental groups, as indicated below (Scheme 1).

Protocol 1: Effect of PEG 35 in Fatty Livers after Cold Storage. In order to study the effects of PEG 35 administration in cold preservation, rats were randomly divided into the following groups:

(1) Group 1: Control 1 (Ctr 1) ($n = 4$): control livers were flushed via the portal vein with Ringer's lactate solution immediately after laparotomy. Then liver samples were collected for posterior analysis.

(2) Group 2 ($n = 6$): UW: steatotic livers were preserved for 24 hours in UW solution at 4°C. Then, livers were flushed with Ringer's solution and the effluent liquid was collected for further biochemical determinations. Liver tissue was stored at −80°C.

(3) Group 3 ($n = 6$): PEG 35: livers were pretreated with PEG 35 intravenously at 10 mg/kg 10 min before liver procurement and then preserved for 24 hours in UW solution as in group 2.

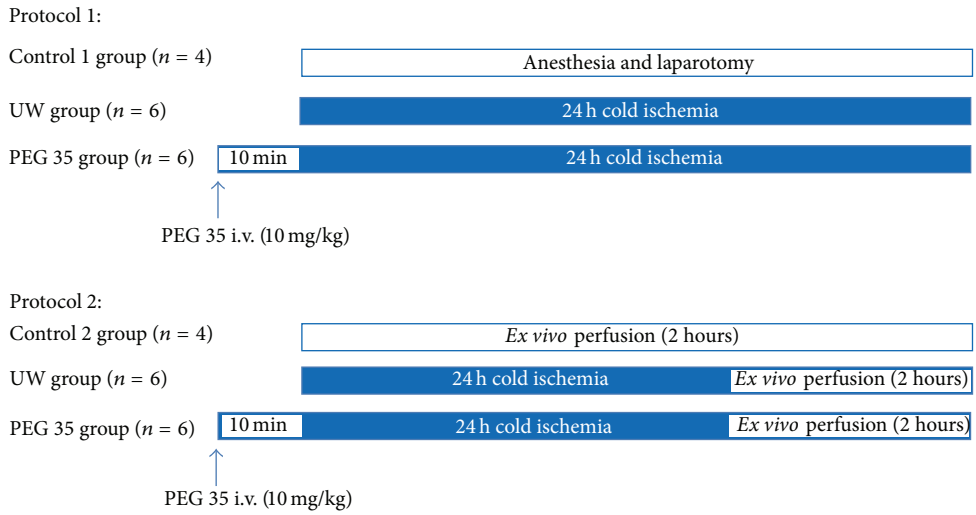
Protocol 2: Effect of PEG 35 in Fatty Livers after 24 h of Cold Storage and 2 h of Normothermic Reperfusion. To examine the effect of PEG 35 in liver injury after normothermic perfusion, fatty livers were randomized in the following groups:

(1) Control group (Ctr 2) ($n = 4$): after procurement, steatotic livers were *ex vivo* perfused for 2 h at 37°C as described above, without prior cold storage.

(2) UW group ($n = 6$): fatty livers were preserved in UW preservation solution for 24 hours at 4°C and then subjected to 2 h of normothermic reperfusion at 37°C.

(3) PEG 35 group ($n = 6$): Zucker Ob rats were pretreated with intravenous administration of PEG 35 at 10 mg/kg, 10 min before liver procurement. Then, livers were preserved for 24 h in UW solution and finally *ex vivo* perfused for 2 hours at 37°C.

2.5. Liver Injury: Transaminases Assay. Hepatic injury was assessed in terms of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels with commercial kits from RAL (Barcelona, Spain). Briefly, 100 μL of effluent



Scheme 1: Experimental designs to investigate PEG 35 effects on steatotic livers after cold ischemia at 4°C (Protocol 1) and after cold storage followed by normothermic *ex vivo* reperfusion (Protocol 2).

washout liquid or perfusate was added to 1 mL of the substrate provided by the commercial kit and then transaminases activity was measured at 340 nm with a UV spectrometer and calculated following the supplier's instructions. Results were normalized using a commercial calibrator (Biocal, RAL, Barcelona, Spain).

2.6. Mitochondrial Damage

2.6.1. Glutamate Dehydrogenase Activity. Glutamate dehydrogenase (GLDH) is a mitochondrial enzyme present predominantly in liver and contributes to the oxidative deamination of glutamate. Measurable increases in serum levels are indicative of mitochondrial damage. Serum concentrations of GLDH were determined using a commercial kit (GLDH, Randox laboratories Ltd., Crumlin, UK) by quantifying the decrease in absorbance at 340 nm according to the manufacturer's protocol.

2.6.2. Confocal Microscopy. After 24 h of hypothermic preservation, fatty livers pretreated with PEG conjugated to FITC (PEG-FITC) or saline were washed out via the portal vein with 20 mL of Ringer lactate solution containing fluorescent dyes. The fluorescent dyes were diluted in the washout liquid and injected to the preserved fatty liver at the following final concentrations: Hoechst 33342 trihydrochloride (12 mg/kg body weight, Invitrogen, H3570) for DNA-nuclei staining and rhodamine 123 (0.11 mg/kg body weight, Sigma, R8004) for mitochondrial membrane potential staining. Fatty livers were then carefully sectioned (0.5 cm^3 fragments) and the internal side of the liver was exposed on the glass coverslip mounted on the stage of a Leica TCS SP5 resonant scan multiphoton confocal microscope (Leica Microsystems Heidelberg GmbH) equipped with a HCX IR APO L 25x water immersion objective (Numerical Aperture 0.95), scanner at 400 lines/s, and a near infrared Titanium:Saphire laser (MaiTai, SpectraPhysics) for two-photon excitation running at 800 nm. Images were acquired with resonant scan at 8000 lines/second. Two-photon excitation was performed at 800 nm and emission of the different fluorescent dyes was captured at the following wavelength ranges: PEG-FITC (400–550 nm), Hoechst 33342 (400–470 nm), and rhodamine 123 (500–550 nm).

2.7. Vascular Resistance. Vascular resistance was defined as the ratio of portal venous pressure which was maintained at 12 mmHg during the reperfusion to flow rate and expressed in mmHg/min per gram of liver/mL. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min per gram of liver.

2.8. Western Blotting Technique. Liver tissue was homogenized in HEPES buffer and proteins were separated by SDS-PAGE and transferred to PVDF membranes. Then, membranes were immunoblotted over night at 4°C using the following antibodies: anti-p-AMPKα (Thr172, #2535), anti-AMPKα (#2603), anti-p-SAPK/JNK (Thr183/Tyr185), anti-p-p38 MAP kinase (Thr180/Tyr182, #9211), and anti-p-p44/42 MAPK (Erk1/2, Thr202/Tyr204, #9101); the above antibodies were all purchased from Cell Signaling (Danvers, MA); anti-eNOS (610296) was purshased form Transduction Laboratories (Lexington KY) and anti-b-actin (A5316) was purshased from Sigma Chemical (St. Louis, MO, USA). After washing, bound antibody was detected after incubation for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected and quantified by scanning densitometry.

2.9. Statistical Analysis. Statistical analysis was performed with GraphPad Prism version 4.02 for Windows (GraphPad Software). Quantitative data are reported as mean ± SEM and statistical comparison was performed with analysis of

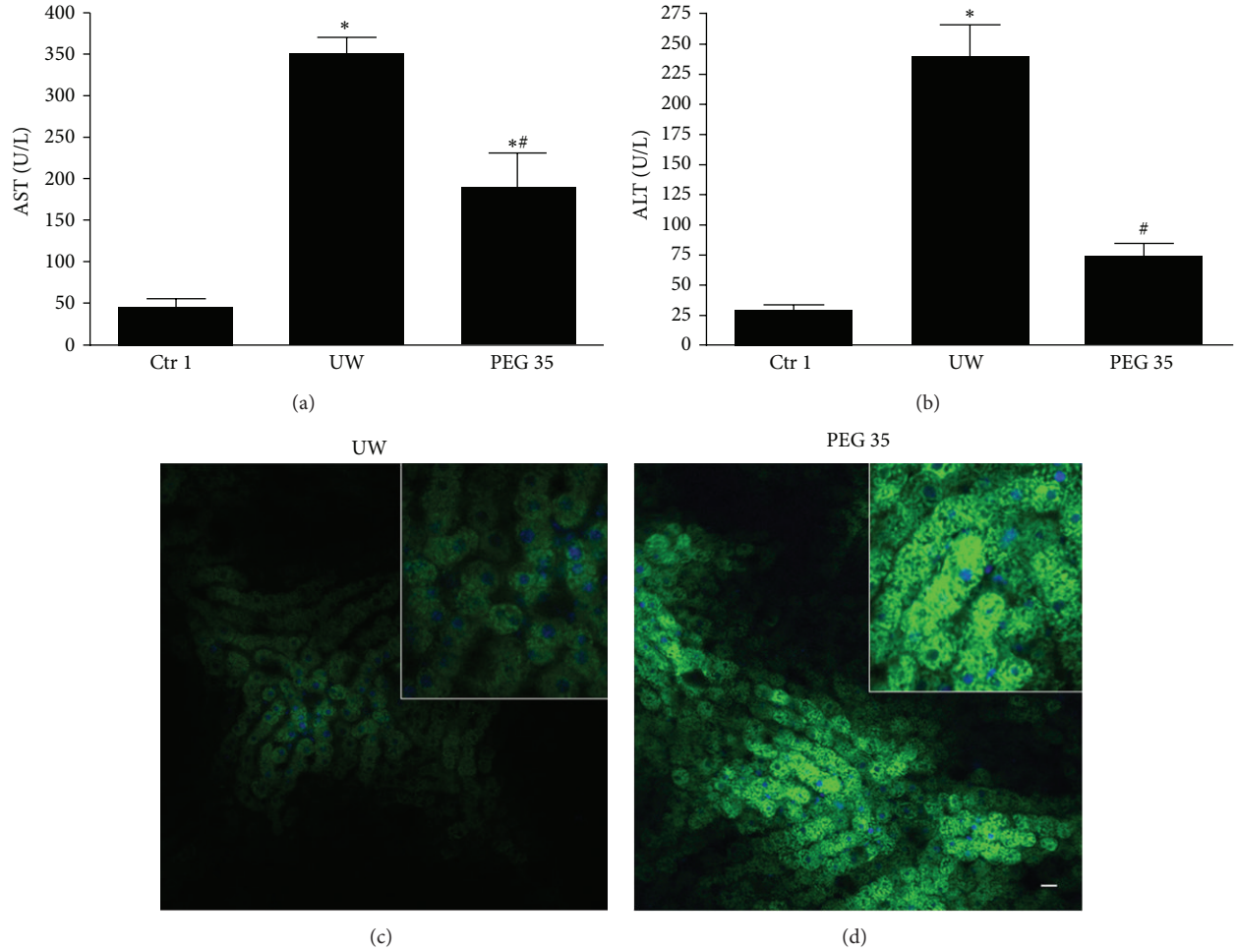


Figure 1: Hepatic and mitochondrial injuries after cold ischemia. PEG 35 administration decreases aspartate aminotransferase (AST) (a) and alanine aminotransferase (ALT) levels (b) after 24 h of cold storage. Confocal microscopy of mitochondrial membrane potential stained with rhodamine 123 (green) after cold storage: mitochondrial depolarization occurs after preservation (c); however, in rats pretreated with PEG 35 conjugated to FITC, we observed bright punctate fluorescence showing polarized mitochondria (d). No PEG fluorescence has been detected in liver sinusoids, neither in hepatocytes nor bound to cell membrane (d). Ctr 1: anaesthesia and laparotomy; UW: livers preserved in UW preservation solution for 24 hours at 4°C; PEG 35: Zucker obese rats treated intravenously with PEG 35 at 10 mg/kg and steatotic livers were then subjected to 24 h cold ischemia. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 1; $^{\#}p < 0.05$ versus UW.

variance, followed by Tukey tests. An associated probability of $p < 0.05$ was considered to be significant.

3. Results

3.1. Effect of Intravenous PEG 35 on Cold Storage of Steatotic Rat Livers. To investigate the protective effect of intravenous PEG 35 treatment on liver preservation, we measured transaminases levels in the effluent of washout liquid after 24 h of cold storage. As shown in Figures 1(a) and 1(b), liver preservation resulted in increased AST/ALT levels versus control group and the intravenous administration of PEG 35 at 10 mg/kg decreased significantly transaminases release indicating substantially less hepatocellular damage. Also, we explored mitochondrial polarization after fatty liver preservation using rhodamine 123 vital dye. In livers pretreated with PEG 35, we observed bright punctate fluorescence standing for the cells with polarized mitochondria. By contrast, in nontreated livers, we observed a cloudy diffuse cytosolic fluorescence standing for cells with depolarized mitochondria (Figures 1(c) and 1(d)). Moreover, our result shows that when obese rats were treated with PEG-FITC, no significant PEG fluorescence was detected in liver sinusoids neither into hepatocytes or other liver cells nor bound to cell membrane (Figure 1(d)).

3.2. Effect of Intravenous PEG 35 Administration on Fatty Liver Injury after Ischemia Reperfusion. In the following, we evaluated the reperfusion injury after 2 hours of *ex vivo* perfusion at 37°C (Protocol 2). We observed a significant decrease in transaminases levels in the perfusate from rats pretreated with PEG 35 when compared to the untreated ones (Figures 2(a) and 2(b)). Moreover, the evaluation of mitochondrial damage, measured by GLDH activity, showed

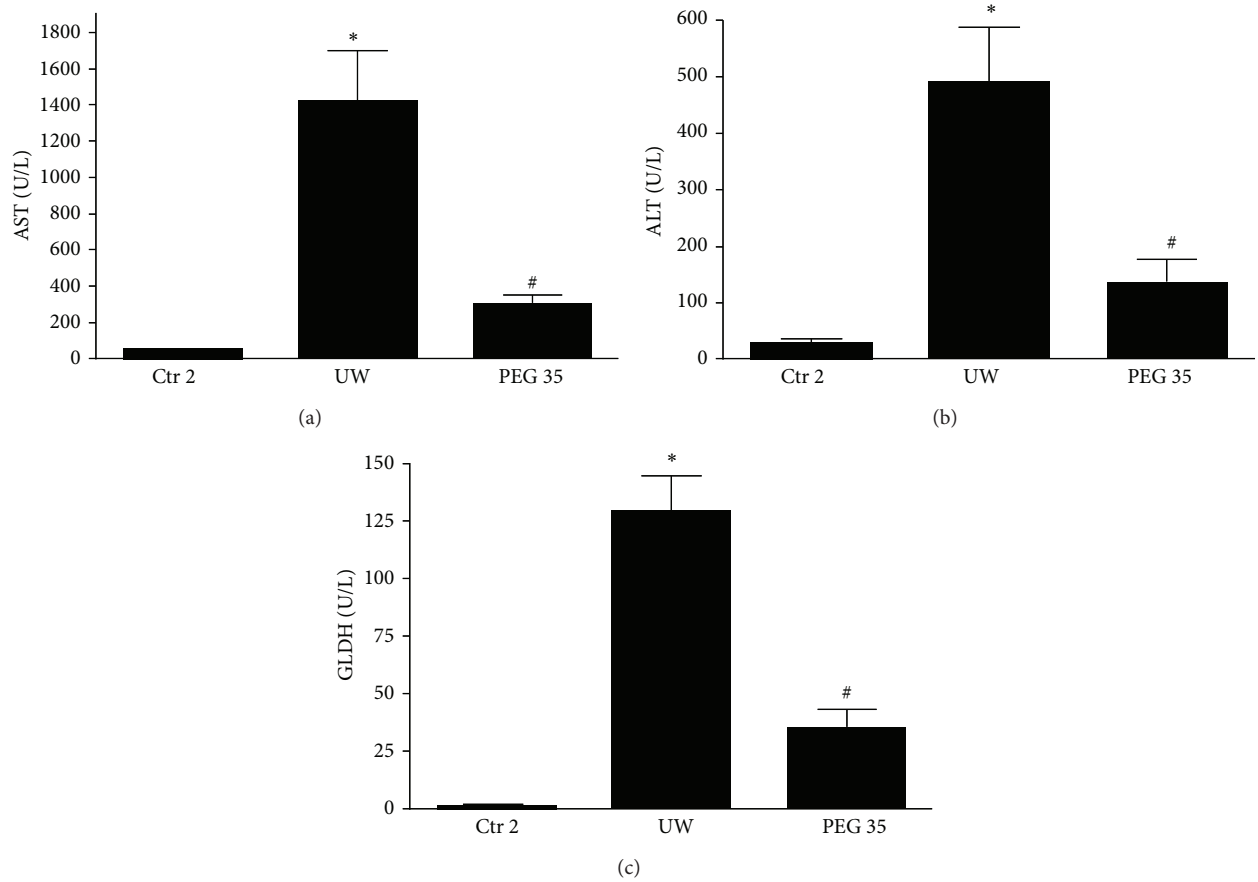


Figure 2: Hepatic and mitochondrial injuries after cold ischemia and reperfusion. PEG 35 administration decreases aspartate aminotransferase (AST) (a), alanine aminotransferase (ALT) (b), and glutamate dehydrogenase (GLDH) levels after 2 hours of *ex vivo* perfusion. Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

significant decreases when rats were pretreated with PEG 35 (Figure 2(c)).

Steatotic livers present fat accumulation in the cytoplasm of the hepatocytes which causes disturbance of the sinusoidal flow during reperfusion [2, 16]. Given that, we explored vascular resistance and endothelial nitric oxide synthase (eNOS) activation after reperfusion. Figure 3(a) shows that eNOS protein expression decreased after 2 h of *ex vivo* perfusion in UW compared to control group. In contrast, pretreatment with PEG 35 clearly induced eNOS expression which was concomitant with decreased vascular resistance (Figure 3(b)).

Next, we explored whether the hepatoprotective effect of PEG 35 could be attributed to well-known cell signaling pathways associated with IRI such as adenosine monophosphate activated protein kinase (AMPK) and protein kinase B (Akt). As shown in Figure 4(a), liver preservation followed by 2 hours of *ex vivo* perfusion promoted Akt phosphorylation, which was further enhanced when rats were pretreated with PEG 35. Regarding AMPK, PEG 35 administration prior to liver procurement induced a significant activation in AMPK in comparison to non-PEG 35-treated rats (Figure 4(b)).

It is well known that mitogen activated protein kinase (MAPK) signaling pathway regulates inflammation and cell survival during IRI [17, 18]. We therefore assessed the possible involvement of MAPK regulation in the protective effect of PEG 35. As indicated in Figure 5, all MAP kinases (p-p38, p-JNK, and p-Erk) levels were increased at 2 h reperfusion. A significant reduction in p-p38 activation was evident after PEG 35 treatment (Figure 5(a)). On the contrary, no changes for JNK and Erk activity were found (Figures 5(b) and 5(c), resp.).

4. Discussion

The beneficial effects of PEG in tissue injury are well documented [5, 12–14, 19]. However, because PEG molecules are not absorbed in the gastrointestinal tracts, their use against ischemic damage was limited to their addition to preservation solutions as oncotic agents. The present study was thus designed to investigate if the intravenous PEG 35 administration by a unique and nontoxic dose of 10 mg/kg could protect steatotic liver grafts against the deleterious

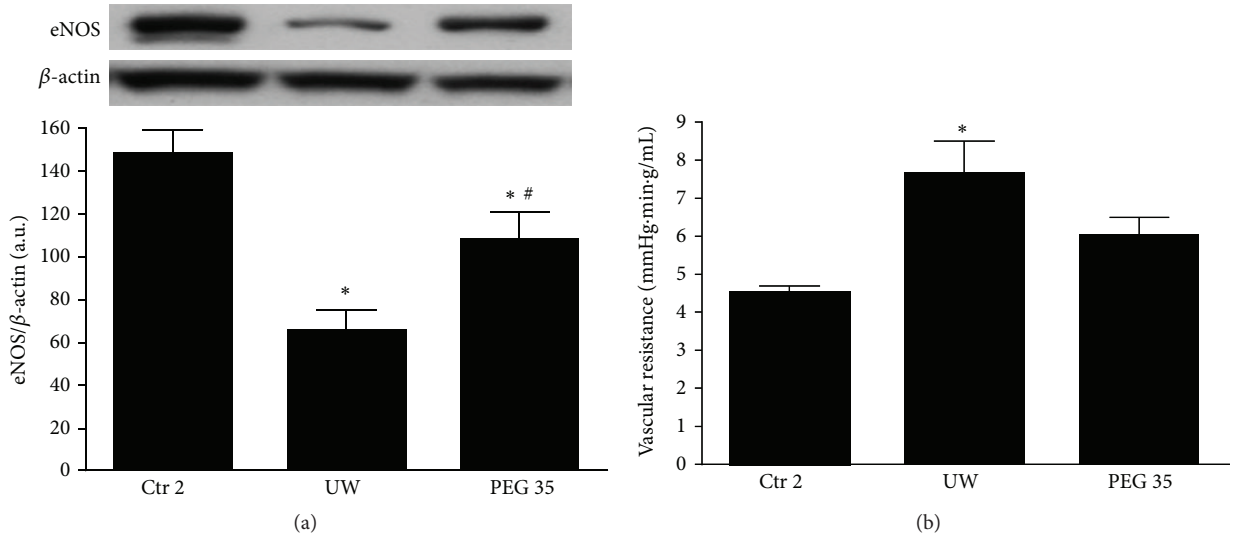


Figure 3: Effects of PEG 35 on eNOS activation and vascular resistance. PEG 35 pretreatment activates eNOS and decreases vascular resistance: densitometric analysis of eNOS/β-actin (a) and vascular resistance (b) after 120 min of normothermic reperfusion. Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

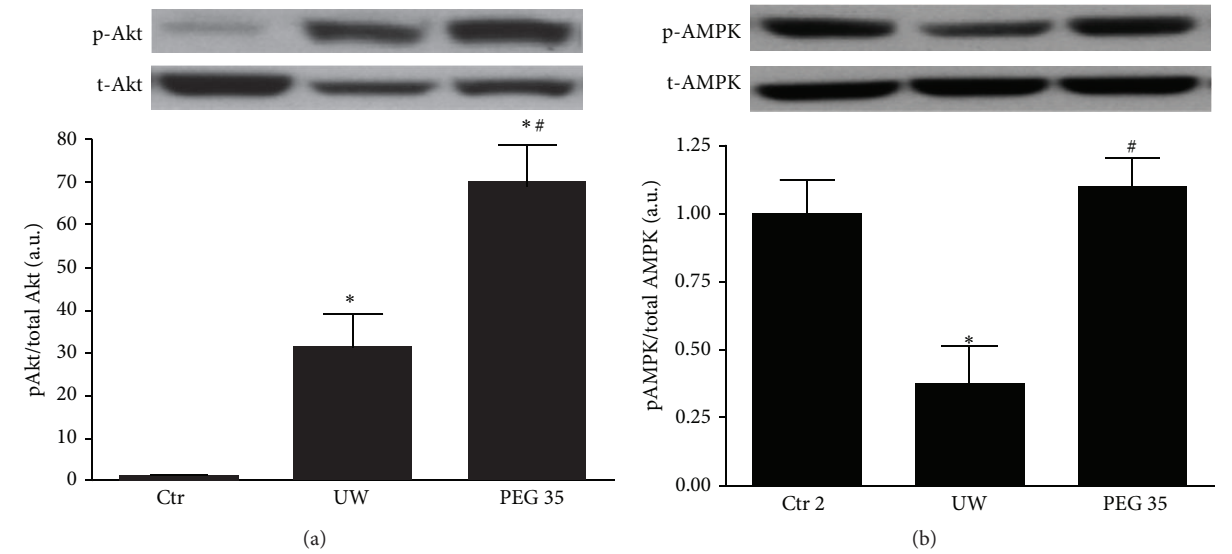


Figure 4: Effects of PEG 35 on Akt and AMPK. PEG 35 administration activates Akt and AMPK: densitometric analysis of phosphorylated Akt/total Akt (a) and phosphorylated AMPK/total AMPK (b). Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

effects of cold storage and the subsequent reperfusion. Our data demonstrated that pretreatment of rats with PEG 35 lessened liver injury associated with ischemia reperfusion.

In our study, we have used the isolated perfused rat liver (IPRL) model, a widely used and appreciated method to assess cellular injury and liver function in an isolated setting. In comparison to other *in vitro* models, the IPRL-model does have considerable advantages, such as the use of the entire intact organ instead of only single cells or several layers of cells (i.e., isolated hepatocytes or the liver slice model) and an intact cellular architecture. Furthermore, the use of an acellular perfusion solution (Krebs solution) prevents alloreactivity and permits a conclusive focus on IRI effects. Regarding liver transplantation, IPRL model presents the advantages of minimizing the use of laboratory animals as well as the suppression of the immunological

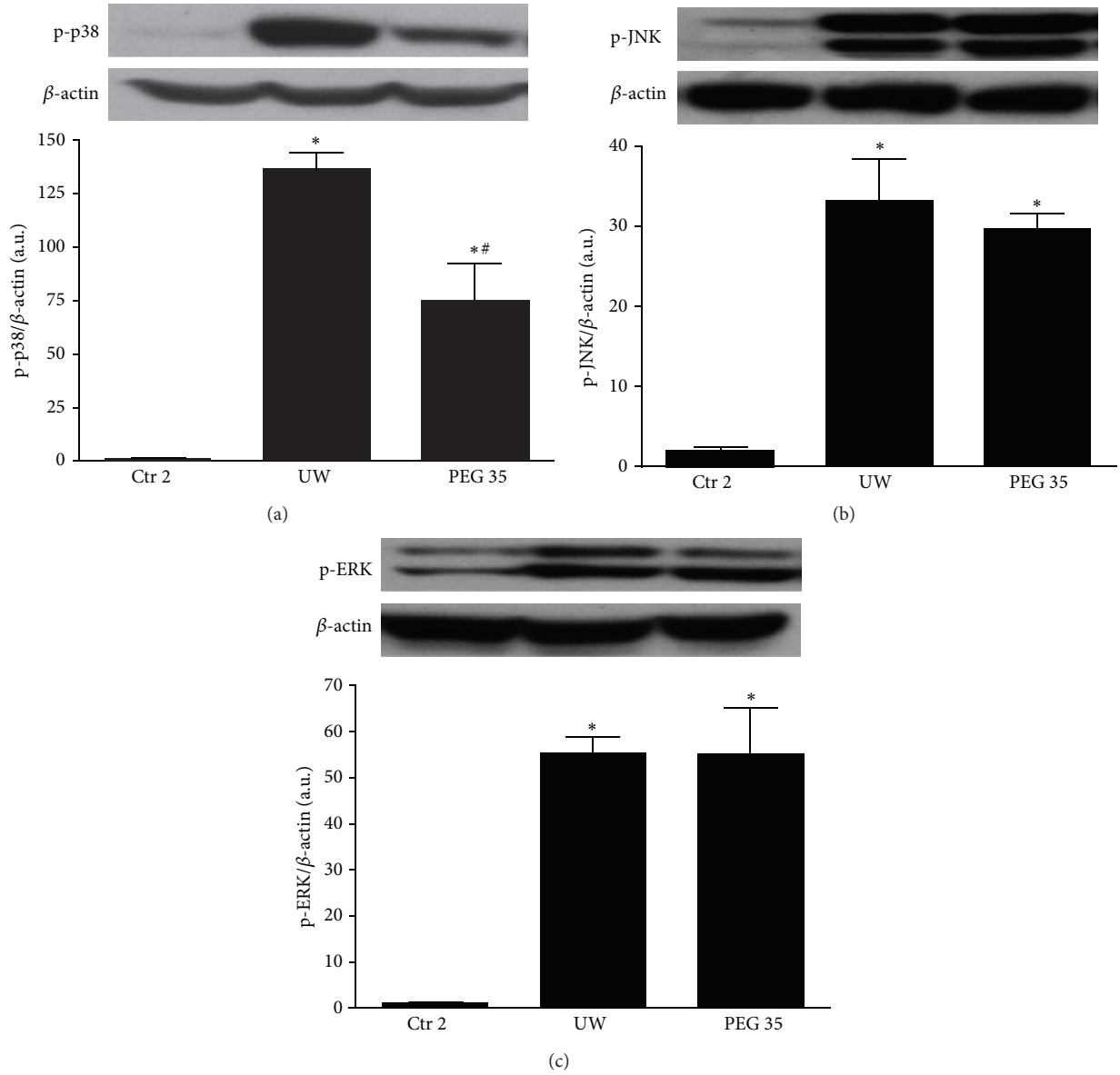


Figure 5: Effect of PEG 35 on MAPKs. PEG 35 reduces p38-MAPK activation whereas it has no effect on JNK and ERK phosphorylation. Densitometric analysis of phosphorylated p38/β-actin (a), phosphorylated JNK/β-actin (b), and phosphorylated ERK/β-actin (c). Ctr 2: liver procurement and *ex vivo* perfusion; UW: livers preserved in UW preservation solution for 24 h at 4°C and then subjected to 2 h of normothermic *ex vivo* perfusion; PEG 35: rats treated intravenously with PEG 35 (10 mg/kg) and then subjected to 24 h cold ischemia followed by 2 h of normothermic *ex vivo* perfusion. Data represent mean ± SEM. $^{*}p < 0.05$ versus Ctr 2; $^{\#}p < 0.05$ versus UW.

reactions and the influences of other organs occurring during transplantation. The disadvantage of the IPRL-model is the duration of reperfusion, which is limited to 90–120 minutes and the fact that it remains an *in vitro* tool that merely simulates the initial phase after liver transplantation. In this sense IPRL model could be considered as a pre-screening model before liver transplantation especially in ischemia reperfusion research [20, 21].

In contrast to the current pharmacological strategies used against IRI, PEG administration presents the advantages of being a multitarget strategy. In fact, IRI is a multifactorial disease including oxidative stress, inflammation, proteasome activation, endoplasmic reticulum stress, mitochondrial damage, and cytoskeleton alterations which lead to cell death and organ dysfunction [22–24]. PEG has been associated with the majority of these events as it has been shown that PEG reduces reactive oxygen species, prevents cell death, maintains mitochondrial integrity, and reduces inflammation and endoplasmic reticulum stress [12, 14, 19, 25, 26].

The half-life and biodistribution of the polymer and consequently its activity mainly depend on its molecular weight. Based on our experience in organ preservation, we used PEG with a molecular weight of 35 kDa. Indeed, we have previously demonstrated that PEG 35 addition to washout

solution protected the liver against reperfusion injuries [27]. Moreover, PEG 35 addition to IGL-1 preservation solution protects kidney and liver grafts against ischemic damage [7, 8, 28, 29]. PEG with a molecular weight of 20 kDa has also been used as an additive to HTK and SCOT preservation solutions and was associated with protective effects against IRI in pancreas [30], kidney [8], intestine [26], and liver grafts [31]. In addition, PEG20 has been shown to protect against cardiomyocyte apoptosis induced by hypoxia [12]. However, PEG 35 was more effective than PEG20 in protecting porcine proximal tubular epithelial cell line against cold storage at the same doses used [32].

Mitochondrial protection is essential for graft survival after transplantation [33]. Thus, we further explored mitochondrial depolarization after cold preservation and we evidenced that PEG 35 prevented fatty liver mitochondria depolarization after prolonged cold ischemia. Also, mitochondrial injury was lessened after liver reperfusion as indicated by the decrease in GLDH release. These results are in accordance with previous published data showing that PEG 2 kDa improved mitochondrial function *in vitro* and *in vivo* after acute spinal cord injury [25]. Moreover, PEG of 4 kDa inhibited mitochondrial pore transition (MPT) and cytochrome C release in rat liver mitochondria [34]. Also, PEG (1.5 and 2 kDa) was able to cross the cytoplasmic membrane and directly interact with neuronal mitochondria to preserve its structure and restore function [14]. Interestingly, PEG with higher molecular weight (4 kDa) failed to exert significant improvement in neuronal injured mitochondria indicating that PEG-mediated mitochondrial protection is dependent on the size of PEG [14]. In our study, we did not detect any PEG fluorescence after cold storage in liver sinusoids, neither in hepatocytes nor bound to cell membrane. In this sense, the mechanism by which PEG 35 decreases mitochondrial damage and exerts its protective effects needs more profound investigation.

Our results show that PEG 35 activated eNOS, the enzyme responsible for nitric oxide (NO) generation, and consequently decreased vascular resistance. This could also explain the protective mechanism of PEG toward mitochondria as it has been showed that NO protects rat hepatocytes against reperfusion injury through the inhibition of MPT [35]. Previous study from Bertuglia et al. has shown that PEG 15–20 kDa reduced vasoconstriction and the altered capillary perfusion after ischemia reperfusion [36]. However, in that case, the decreased vascular resistance of PEG were not mediated by eNOS activation [36].

In order to explore whether the beneficial effects of PEG 35 are associated with other well-known cell signaling pathways involved in IRI, we further evaluated the activation of AMPK and Akt and the regulation of MAPKs. AMPK is a metabolic fuel gauge and energy regulator activated during ischemia in order to induce an energy-saving state preventing thus the lactate accumulation and cell death [37–39]. Here, we showed that PEG 35 enhanced AMPK levels after reperfusion, which could contribute to assuring energy levels sufficient to cell survival. Another cytoprotective marker is Akt, a serine-threonine protein kinase that is linked to cell survival during reperfusion [40–42]. Data reported here revealed that PEG 35 increased Akt levels, as similarly observed with PEG 20 in cardiac myocyte submitted to IRI [12]. Regarding MAPKs signalling, we observed that PEG 35 was capable of preventing p38 activation, while no changes were found on JNK and ERK pathways. The data reported here are consistent with previously reported works showing that the inhibition of p38 prevented preservation-induced graft injury and improved the outcome of liver transplantation [43–45]. Other studies as well reported that PEG 35 decreased p38 activation while it activated JNK in cold stored porcine proximal tubular cell line [32].

The rationale of PEG 35 intravenous administration was to induce a pharmacological preconditioning against the subsequent cold storage and reperfusion injury. PEG presents the advantages of being safe and multifactorial agent and may constitute a novel strategy to increase liver graft preservation. This could be relevant in clinical situation of brain-dead donors or steatotic livers, both being risk factors in liver transplantation. Until now, PEG has been used clinically for ischemia reperfusion purpose as additive to preservation solution due to its oncotic properties. In this study, we used UW solution which contains hydroxyethyl starch as an oncotic support in order to demonstrate that the protective mechanisms of PEG are not only related to its oncotic effect, but also to other properties such as the preservation of mitochondria and the induction of protective cell signaling pathway (eNOS, Akt, and AMPK). In a previous study we have shown that PEG addition to rinse solution protected preserved liver against the subsequent reperfusion injury (PEG postconditioning). Interestingly, when liver grafts were preserved in IGL-1 solution which contains PEG 35, the rinse solution does not show any additional protective effect [46]. In this sense, PEG pre- and postconditioning would be considered as a safe and protective strategy applicable to all preservation solutions.

5. Conclusions

PEG 35 represents a potential pharmacological agent for preventing the deleterious effects of cold IRI and may constitute a novel clinical strategy to increase liver graft preservation, especially for "marginal" organs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

PPARα Agonist WY-14643 Induces SIRT1 Activity in Rat Fatty Liver Ischemia-Reperfusion Injury

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Ischemia-reperfusion injury (IRI) remains a frequent complication in surgery, especially in case of steatotic livers that present decreased tolerance towards IRI. Apart from its major role in metabolism, activation of peroxisome proliferator-activated receptor α (PPARα) has been related with positive effects on IRI. In addition, the deacetylase enzyme sirtuin 1 (SIRT1) has recently emerged as a promising target for preventing IRI, through its interaction with stress-related mechanisms, such as endoplasmic reticulum stress (ERS). Taking this into account, this study aims to explore whether PPARα agonist WY-14643 could protect steatotic livers against IRI through sirtuins and ERS signaling pathway. Obese Zucker rats were pretreated or not pretreated with WY-14643 (10 mg/kg intravenously) and then submitted to partial (70%) hepatic ischemia (1 hour) followed by 24 hours of reperfusion. Liver injury (ALT levels), lipid peroxidation (MDA), SIRT1 activity, and the protein expression of SIRT1 and SIRT3 and ERS parameters (IRE1α, peIF2, caspase 12, and CHOP) were evaluated. Treatment with WY-14643 reduced liver injury in fatty livers, enhanced SIRT1 activity, and prevented ERS. Together, our results indicated that PPARα agonist WY-14643 may exert its protective effect in fatty livers, at least in part, via SIRT1 induction and ERS prevention.

1. Introduction

Ischemia-reperfusion injury (IRI) is a limiting factor for the outcome of many clinical conditions and although the extensive investigations, the underlying mechanisms remain largely unclear [1]. Moreover, the increased rates of obesity have resulted in the augmented number of livers with severe steatosis [2]. Steatotic livers present an exaggerated accumulation of lipids which contributes to the activation of various cellular stress signaling pathways and finally to increased vulnerability against IRI [3]. Consequently, there is an augmented interest for identifying mechanisms able to reduce IRI in steatotic livers.

Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor that is highly expressed in metabolically active tissues, such as liver, and functions as a lipid sensor. Upon the binding of various lipids, it forms heterodimers with the retinoid X receptor (RXR) and activates the transcription of various genes that regulate lipid homeostasis and metabolism, including genes involved in mitochondrial β-oxidation, fatty acid uptake and binding, and lipoprotein transport [4]. In fact, PPARα activation is associated with reduced hepatic steatosis [5, 6] through the regulation of a wide variety of genes involved in peroxisomal, mitochondrial, and microsomal fatty acid β-oxidation systems in the liver [7].

Various studies have evidenced the antioxidant and anti-inflammatory effects of PPARα agonists against IRI in various organs; WY-14643 efficiently decreased neutrophil infiltration and proinflammatory cytokine expression (TNF-α and IL-1β) and prevented the formation of ROS [8–10]. In addition, PPARα has also been associated with the prevention of endoplasmic reticulum stress (ERS), a common feature of IRI [11]. Pretreatment with PPARα agonist WY-14643 protected liver HepG2 cells against ERS-induced apoptosis by downregulating the expression of BiP and C/EBP homologous protein (CHOP), two components of the ERS-mediated apoptosis pathway. Moreover, ERS has been linked to a number of downstream pathways that contribute to the pathogenesis of nonalcoholic fatty liver disease [12].

Sirtuin 1 (SIRT1), NAD^+-dependent protein deacetylase, is involved in numerous physiological processes including cellular stress response, glucose homeostasis, and immune response. In accordance with its role as a metabolic mediator, SIRT1 is known to regulate genes involved in fatty acid oxidation and lipolysis [13]. Among them, PPARα is a well-known factor that is activated by SIRT1 [14, 15]. SIRT1 deletion in hepatocytes impaired the activity of PPARα, resulting in development of hepatic steatosis, whereas SIRT1 hepatic overexpression suppressed the expression of gluconeogenic genes and attenuated obesity-induced ERS [16, 17]. Furthermore, sirtuin 3 (SIRT3), another member of the sirtuin's family, has also been involved in metabolic regulation [18, 19] and both SIRT1 and SIRT3 have emerged as potential targets to diminish IRI [20].

This study aims to explore new mechanisms by which a PPARα agonist, WY-14643, exerts its beneficial effects against hepatic IRI in a genetic model of obese rats. SIRT1 and ERS signaling appear to be potential targets of WY-14643.

2. Materials and Methods

2.1. Experimental Animals. Homozygous obese (Ob) Zucker rats (Charles River, France) aged 16 weeks were used; Ob rats lack the cerebral leptin receptor and are characterized by severe macro- and microvesicular fatty infiltration in hepatocytes. Animals had free access to water and standard laboratory food *ad libitum* and were kept under constant environmental conditions with a 12-hour light-dark cycle. All procedures were performed under isoflurane inhalation anesthesia. This study was performed in accordance with European Union regulations (Directive 86/609 EEC). Animal experiments were approved by the Ethics Committees for Animal Experimentation (CEEA, Directive 396/12), University of Barcelona.

2.2. Experimental Design. Rats were randomly divided into three experimental groups: (1) Sham, $n = 6$; (2) ischemia-reperfusion (IR), $n = 6$; and (3) WY-14643 + IR, $n = 6$. A model of partial (~70%) hepatic warm ischemia was applied. Briefly, a midline laparotomy was performed and the portal triad was dissected free of surrounding tissue. Then, an atraumatic clip was placed across the portal vein and hepatic artery to interrupt the blood supply to the left lateral and median lobes of the liver. After 60 min of partial hepatic ischemia, the clip was removed to recover hepatic reperfusion for 24 hours. Sham control rats underwent the same protocol without vascular occlusion. In the group of WY-14643 + IR, rats were treated with WY-14643 (10 mg/kg intravenously) 1 hour before the induction of IR [21]. After 24 h of reperfusion, rats were sacrificed; blood samples were drawn from aorta and ischemic lobes were collected and stored at −80°C until assayed.

2.3. Biochemical Determinations

2.3.1. Transaminases Assay. Hepatic injury was assessed in terms of transaminases levels with a commercial kit from RAL (Barcelona, Spain). Briefly, blood samples were centrifuged at 4°C for 10 min at 3000 rpm and then were kept at −20°C. In order to assay transaminase activity, 200 μL of the supernatant was added to the substrate provided by the commercial kit. ALT levels were determined at 365 nm with an UV spectrometer and calculated following the supplier instructions.

2.3.2. Lipid Peroxidation Assay. Lipid peroxidation in liver was used as an indirect measurement of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction. MDA in combination with thiobarbituric acid (TBA) forms a pink chromogen compound whose absorbance at 540 nm was measured. The result was expressed as nmols/mg protein.

2.3.3. SIRT1 Activity Assay. SIRT1 activity was determined according to the method described by Becatti et al. with some modifications [22]. Protein extracts were obtained using a mild lysis buffer (50 mM Tris-HCl pH 8, 125 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 0.1% NP40). SIRT1 activity was measured using a deacetylase fluorometric assay kit (CY-1151, CycLex, MBL International Corp.), following the manufacturer's protocol. A total of 25 μL of assay buffer containing the same quantity of protein extracts (10 μg/μL) was added to all wells, and the fluorescence intensity was monitored every 2 min for 1 h using the fluorescence plate reader Spectramax Gemini, applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results are expressed as the rate of reaction for the first 30 min, when there was a linear correlation between the fluorescence and this period of time.

2.3.4. ATP Quantification. Tissue samples (20 mg) were pulverized in liquid N_2 and homogenized in ice-cold 25 μL of KOH buffer (KOH 2.5 M, K_2HPO_4 1.5 M). Homogenates were then vortexed and centrifuged at 14,000 ×g at 4°C for 2 min. The supernatants were collected and dissolved in 100 μL of K_2HPO_4 1 M. Following this, pH was adjusted to 7 and samples were frozen at −80°C for posterior use. Finally, adenosine nucleotides were quantified with an ATP bioluminescent assay kit (Sigma-Aldrich) on a Victor 3 (PerkinElmer, Waltham, MA) plate reader.

Table 1: Effect of WY-14643 administration in hepatic injury in steatotic livers subjected to ischemia-reperfusion.

ALT		
Sham	IR	WY-14643 + IR
97 ± 27,85	2675 ± 277,03*	380 ± 86,02*,#

Alanine aminotransferase (ALT) levels in plasma after 24 h of reperfusion. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. * $P < 0.05$ versus Sham; # $P < 0.05$ versus IR.

Table 2: Effect of WY-14643 on lipid peroxidation in steatotic livers subjected to ischemia-reperfusion.

MDA		
Sham	IR	WY-14643 + IR
0,32 ± 0,07	1,22 ± 0,13*	0,76 ± 0,17*,#

Photometric analysis of malondialdehyde levels (MDA). Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. * $P < 0.05$ versus Sham; # $P < 0.05$ versus IR.

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

2.4. Western Blotting Analysis. Liver tissue was homogenized in RIPA buffer (Tris-HCl pH = 7.5 50 mM, NaCl 150 mM, SDS 0.1%, $C_{24}H_{39}O_4Na$ 1%, NP-40 1%, EDTA 5 mM, Na_3VO_4 1 mM, NaF 50 mM, and DTT 1 mM, 1 complete tablet/100 mL). Fifty μg of proteins was electrophoresed on 8–15% SDS-PAGE gels and transblotted on PVDF membranes (Bio-Rad). Membranes were then blocked with 5% (w/v) nonfat milk in TBS containing 0.1% (v/v) Tween 20 and incubated overnight at 4°C with anti-SIRT1 (number 07-131, Merck Millipore, Billerica, MA); anti-SIRT3 (number 2627), anti-p-eIF2a (Ser51, number 9721), anti-IRE1α (number 3294), and anticaspase 12 (number 2202) were purchased from Cell Signaling (Danvers, MA), NAMPT (AP22021SU, Acris Antibodies GmbH, Germany), anti-GADD 153 (sc-575, Santa Cruz Biotechnology), and anti-GADPH (G9545, Sigma Chemical, St. Louis, MO, USA). After washing, bound antibody was detected after incubation for 1 h at room temperature with the corresponding secondary antibody linked to horseradish peroxidase. Bound complexes were detected using Western Bright ECL-HRP substrate (Advansta) and were quantified using the Quantity One software for image.

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

3. Results

3.1. WY-14643 Administration Decreased Hepatic Injury and MDA Levels in Obese Rats. First of all, we aimed to investigate the effect of WY-14643 pretreatment on hepatic injury in obese rats. As shown in Table 1, IR group was associated with increased ALT levels, which was prevented after treatment with WY-14643 (Table 1). In addition, pretreatment with the PPARα agonist decreased the release of lipid peroxidation products as observed for the low MDA levels (Table 2).

3.2. WY-14643 Treatment Increased SIRT1 Activity, While No Effects Were Found on SIRT1 and SIRT3 Protein Expression. It is known that hepatic deletion of SIRT1 alters PPARα signaling, but we then explored whether PPARα activation could affect the protein expression of SIRT1 and SIRT3. No changes on SIRT3 protein expression were observed among all the experimental groups (Figure 1(b)). By contrast, although SIRT1 protein expression increased during ischemia-reperfusion, its levels were not significantly different between IR and WY-14643 pretreated rats (Figure 1(a)). In addition, WY-14643 treatment resulted in enhanced SIRT1 activity in comparison to both Sham and IR group (Figure 1(c)).

3.3. WY-14643 Administration Enhanced NAD^+ Levels. Due to the fact that SIRT1 depends on NAD^+ levels, we determined the NAD^+/NADH levels and the protein expression of nicotinamide phosphoribosyltransferase (NAMPT), a well-known mediator of NAD^+ biosynthetic pathways. As evidenced in Figure 2(a), both IR and WY-14643 + IR groups showed augmented NAMPT levels when compared to Sham group. Moreover, obese rats submitted to IR presented significant decreases of NAD^+/NADH levels in contrast to untreated animals, but WY-14643 contributed to more elevated NAD^+ levels than IR group (Figure 2(b)).

3.4. WY-14643 Pretreatment Augmented ATP Levels. As PPARα induces fatty acid oxidation which is a source of ATP production, we then measured ATP levels. We observed that IR significantly decreased ATP levels when compared to Sham group, whereas WY-14643 administration previous to IR provoked an overwhelming increase in ATP levels (Figure 3).

3.5. PPARα Enhancement Decreased ERS. Excessive lipid accumulation in the tissues has been associated with ERS induction [23]. Thus, possible alterations in protein expression of ERS parameters were evaluated. As shown in Figure 4, expression of IRE1α, p-eIF2, caspase 12, and CHOP was exacerbated by IR and restored by pretreatment with the PPARα agonist WY-14643.

4. Discussion

PPARα has gained special interest for its implication in metabolism and its protective effects in IRI models. However, the underlying interactions beyond its activation in obese livers subjected to IRI are not fully understood. In this study, we aimed to investigate the hepatoprotective mechanisms of PPARα agonist WY-14643 in a genetic rat model of obesity.

To begin with, pretreatment of obese rats with WY-14643 proved to be protective against hepatic IRI. This result

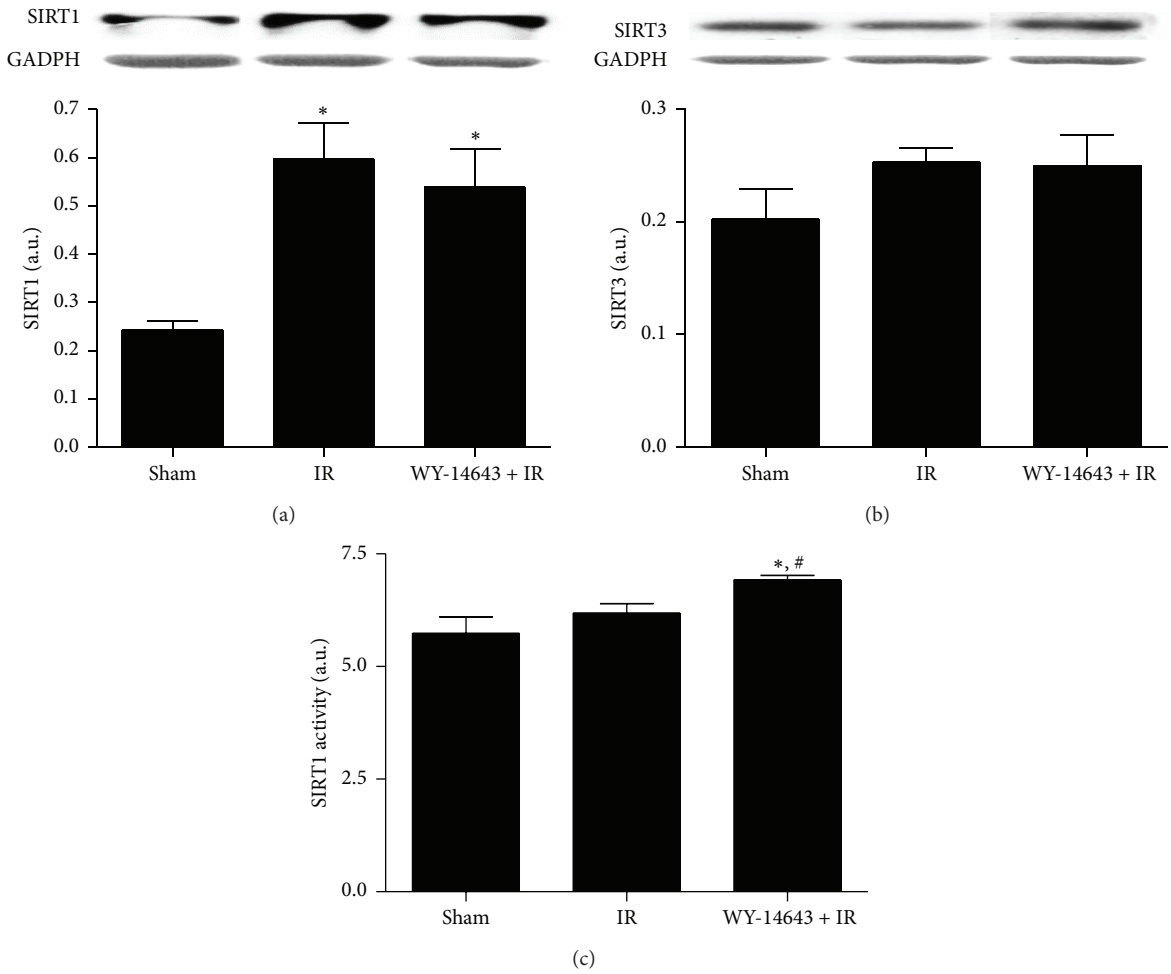

Figure 1: WY-14643 pretreatment and sirtuins protein expression and activity in steatotic livers after ischemia-reperfusion. Western blot and densitometric analysis of (a) SIRT1 and (b) SIRT3. (c) SIRT1 enzymatic activity. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

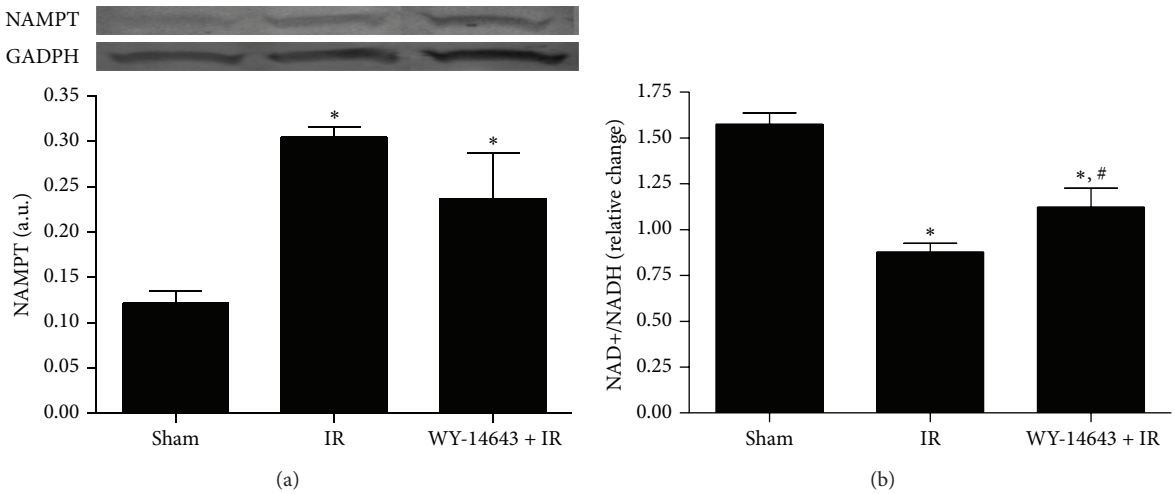

Figure 2: Effect of WY-14643 administration in NAMPT protein expression and NAD^{+}/NADH levels. (a) Western blot and densitometric analysis of NAMPT. (b) Photometric analysis of NAD^{+}/NADH levels in steatotic livers after 24 hours of reperfusion. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

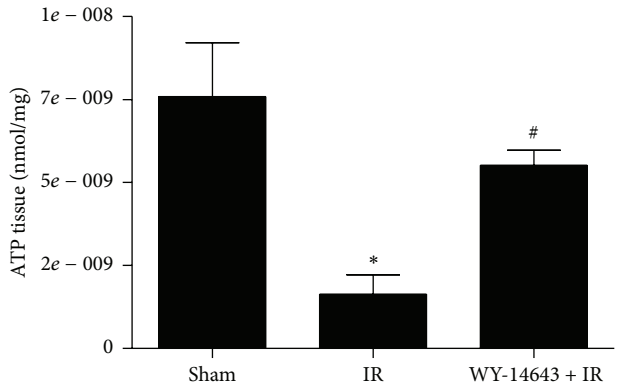

Figure 3: Role of WY-14643 treatment in ATP levels in steatotic livers subjected to ischemia-reperfusion. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

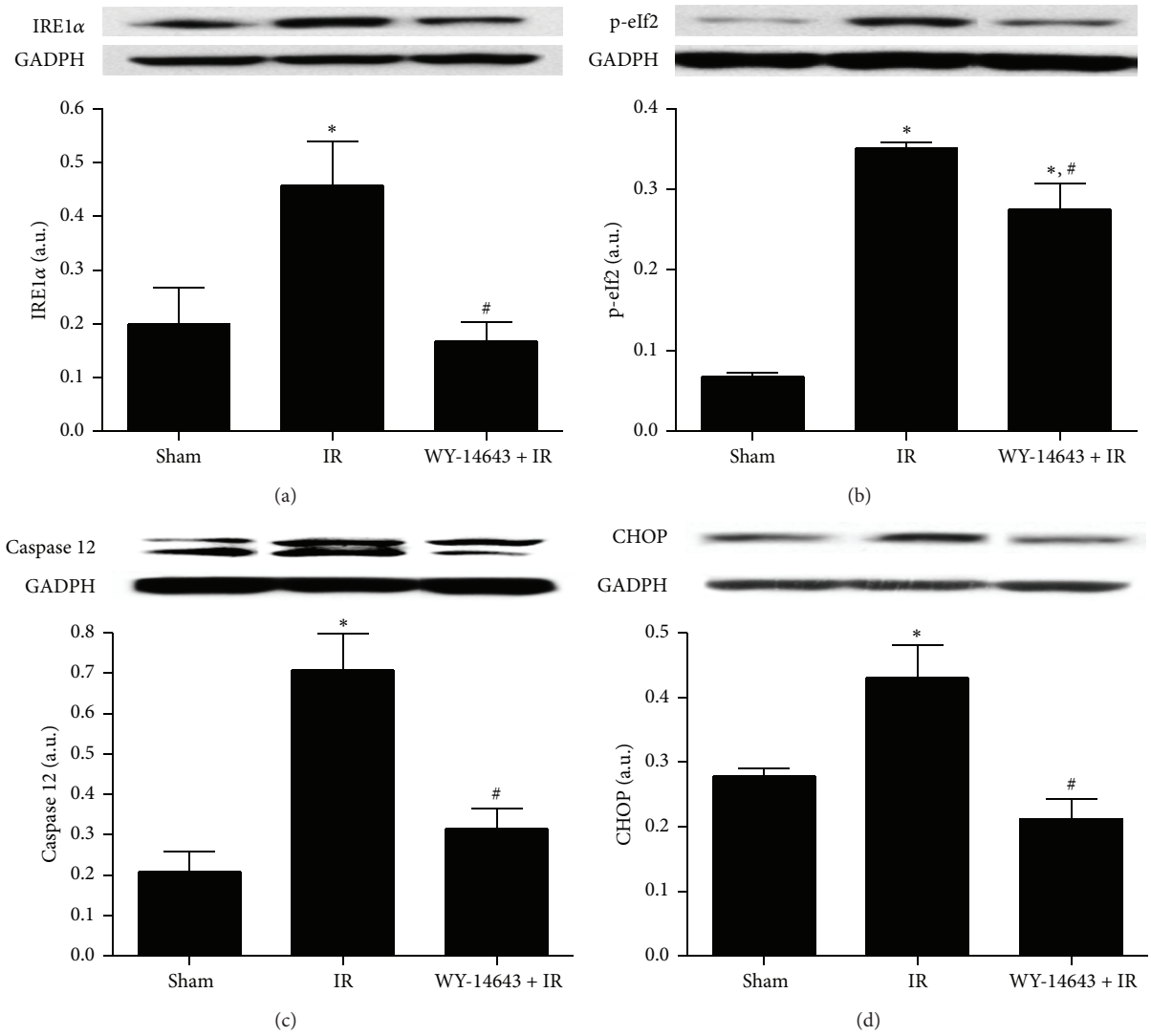

Figure 4: Implication of WY-14643 administration in ERS parameters in steatotic livers subjected to ischemia-reperfusion. Western blot and densitometric analysis of (a) IRE1α, (b) p-eIf2, (c) caspase 12, and (d) CHOP. Sham: anesthesia and laparotomy, IR: 60 min partial ischemia and 24 h of reperfusion, and WY-14643 + IR: iv administration of WY-14643 (10 mg/kg) 1 hour before IR. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

is consistent with other studies using genetic and dietary models of steatohepatitis. In nonalcoholic steatohepatitis (NASH) and simple steatosis, treatment of mice with WY-14643 protected steatotic livers against IRI [24]. Given the fact that oxidative injury is known to be more exaggerated in fatty livers [3, 25], we also determined lipid peroxidation and we found a significant reduction in MDA formation in the liver of rats pretreated with WY-14643. In this sense, PPARα beneficial effects against oxidative stress have also been demonstrated in other models [26, 27].

Various studies have evidenced the protective role of SIRT1 against IRI, which in most cases has been associated with attenuation of oxidative stress [28, 29]. In the liver, a major target of SIRT1 is the PPARα signaling pathway. Loss of hepatic SIRT1 impairs PPARα mediated fatty acid metabolism and decreases fatty acid β-oxidation [17]. Additionally, cell culture experiments suggested that PPARα may also positively regulate SIRT1 expression [30, 31]. In our study, we observed that WY-14643 administration did not affect either SIRT1 or SIRT3 protein expression, whereas it strengthened SIRT1 activity.

As SIRT1 requires NAD^+ for its enzymatic activity, the augmented NAD^+ levels that we observed in WY-14643 treated group led us to suggest that SIRT1 enhanced activity may be, at least in part, a result of the increased levels of its cofactor NAD^+. However, the fact that NAD^+ levels were not in accordance with NAMPT proteins levels may be attributed to the existence of other NAD^+ precursors, like tryptophan and nicotinic acid [32].

It has been shown that PPARα stimulates the β-oxidative degradation of fatty acids, provoking thus a high yield of ATP production [33, 34]. Indeed, in our study, we observed that administration of PPARα agonist WY-14643 resulted in overwhelming ATP increases. Fatty livers are characterized by a reduced ATP content, which limits the resistance and the survival of hepatocytes against stress conditions, including IRI [3]. Thus, PPARα activation diminished IRI in fatty livers by enhancing energy production. Furthermore, ATP forms part of the NAD^+ biosynthesis pathways [35, 36] and in this way may promote SIRT1 activity.

Various studies have associated steatotic livers with ERS [12, 37]. IR affects the ability of the endoplasmic reticulum to synthesize and fold proteins, leading to the exaggerated accumulation of unfolded proteins and the initiation of ERS. Upon ERS, various proteins localized in the endoplasmic reticulum are activated, such as inositol requiring enzyme 1α (IRE1α) and pancreatic ER kinase- (PKR-) like ER kinase (PERK). Activated PERK phosphorylates eukaryotic initiation factor 2 (eIF2) in order to block protein synthesis and activated IRE1α controls genes involved in protein degradation. This consists of a cell attempt to restrain the accumulation of newly synthesized proteins in the ER lumen but can also initiate proapoptotic events, including the activation of caspase 12 and enhanced protein expression of CHOP [38]. Here, we observed that the IRE1α, p-eIF2, and CHOP signaling pathways of the ERS, as well as caspase 12 levels, were significantly abrogated after WY-14643 treatment. Due to the fact that ROS impairs the protein folding, the attenuation of oxidative stress by PPARα activation could contribute to a more proper folding of the proteins and thus to lessened ERS. ERS prevention by PPARα activation has also been evidenced *in vitro* in H_2O_2-treated HepG2 cells [11]. Another potential mechanism to prevent ERS might be based on the SIRT1 augmented activity, as SIRT1 hepatocyte loss has been shown to be crucial for the development of ERS in a high-fat diet [16].

5. Conclusion

In conclusion, our study gives a new insight into the hepatoprotective mechanisms of the PPARα agonist WY-14643 in steatotic livers, implying that SIRT1 might be an important mediator of these beneficial effects. However, more efforts are required to elucidate the exact mechanisms that define the observed interactions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Pharmacological Preconditioning by Adenosine A2a Receptor Stimulation: Features of the Protected Liver Cell Phenotype

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Ischemic preconditioning (IP) of the liver by a brief interruption of the blood flow protects the damage induced by a subsequent ischemia/reperfusion (I/R) preventing parenchymal and nonparenchymal liver cell damage. The discovery of IP has shown the existence of intrinsic systems of cytoprotection whose activation can stave off the progression of irreversible tissue damage. Deciphering the molecular mediators that underlie the cytoprotective effects of preconditioning can pave the way to important therapeutic possibilities. Pharmacological activation of critical mediators of IP would be expected to emulate or even to intensify its salubrious effects. *In vitro* and *in vivo* studies have demonstrated the role of the adenosine A2a receptor (A2aR) as a trigger of liver IP. This review will provide insight into the phenotypic changes that underline the resistance to death of liver cells preconditioned by pharmacological activation of A2aR and their implications to develop innovative strategies against liver IR damage.

1. Ischemia/Reperfusion Injury of the Liver

Inflow occlusion during liver surgery with consequent reperfusion causes ischemia/reperfusion (I/R) injury of the liver. I/R injury is recognised as a main risk factor after major hepatic surgery and liver transplantation since it may affect patients recovery and carries a risk of poor postoperative outcome [1].

Hepatic I/R injury is a complex, multifaceted process that occurs during the ischemic period as well as during the reperfusion phase. During ischemia, mitochondrial deenergization, ATP depletion, and ionic and volume alterations lead to liver cell necrosis. Upon oxygen readmission, reactive oxygen species (ROS) production by uncoupled mitochondria promotes oxidative stress and mitochondrial permeability transition and is associated with a decreased capacity to synthesize ATP. Caspase activation, necrosis, and apoptosis of liver cells and activation of the inflammatory reactions follow these events. Resident Kupffer cells and infiltrating neutrophils and lymphocytes release ROS, proteases, and cytokines and further contribute to the progression of hepatic injury [2–4]. Preclinical studies have shown several strategies able to reduce hepatic damage by individually targeting the different alterations that contribute to I/R injury [2–6]. Their potential adverse effects and their focused approach have inhibited, however, their translation to patients and, to date, no definitive methods have become part of the clinical practise [1, 2].

2. Hepatic Ischemic Preconditioning

The term ischemic preconditioning (IP) refers to the increase in tissue tolerance to ischemia/reperfusion (I/R) damage that can be induced by the preexposure to brief periods of ischemia followed by reperfusion [7]. This phenomenon was first described by Murry et al. in the myocardium [8], but it was subsequently observed in many other tissues [7]. In liver, studies in rodents have shown that 10 min interruption

of blood flow followed by 10 min reperfusion reduces hepatic injury, oxidative stress, microvascular disturbances, and inflammation during a subsequent extended period of I/R [1–8].

The demonstration of the pleiotropic protective effects of IP in the experimental models has raised hopes that it could be a useful and easy technique to reduce I/R injury in human liver surgery. IP surgical application, however, has the disadvantage of inducing trauma to major vessels and stress to the target organ [9]. Moreover the contrasting outcomes of the first clinical studies, the different protocols of IP application in humans, and the variable clinical settings have not allowed a definitive demonstration of the benefit of the clinical application of IP [9–13].

This observation has inhibited, by now, the routine use of IP in human liver surgery and has indicated the need of more efficient approaches to activate IP in patients. In this regard, the pharmacological induction of liver preconditioning by targeted activation of one or more of the critical molecular mediators of IP may represent a more reliable technique to activate the intrinsic system of hepatoprotection in patients.

3. Adenosine A2a Receptor Activation: A Main Trigger of Hepatic Preconditioning

The nearly 25 years' research on liver IP has demonstrated that its applications induce deep modifications of liver tissue that make liver cells resistant to damage. The knowledge of the molecular changes responsible for the production of such protected liver cell phenotypes is however still incomplete. To date one of the established notions is the role of the adenosine A2a receptor (A2aR) activation as an inductor of liver preconditioning. Adenosine mainly originates by the breakdown of adenine nucleotides and even a transient damage of cell membranes, like that induced by the brief ischemic stress of IP, leads to massive ATP increase in extracellular space with rapid formation of adenosine [14]. Since the early reports of Peralta et al. [15, 16], *in vivo* and *in vitro* studies have shown that IP increases extracellular adenosine levels that in turn triggers IP protective effects upon stimulation of A2aR of liver cells. Consistently pretreatment with adenosine A2 receptor agonists enhances liver tolerance against hypoxia and I/R damage, while pharmacological or genetic inhibition of A2aR activation prevents the beneficial effects of IP [15–25].

The mechanisms responsible for A2aR-mediated hepatoprotection during IP are both indirect and direct. The indirect mechanisms depend on the maintenance of nitric oxide (NO) synthesis [15, 16] induced by preventing the downregulation of NO synthase of liver endothelial cells induced by I/R [26]. The direct effects are due to the activation of intracellular survival pathways as a consequence of the stimulation of the A2aR expressed on liver cells.

4. Adenosine A2a Receptor Activation Protects Hepatocyte Hypoxic Damage

In the past years, we have employed the *in vitro* model of primary rat hepatocytes preconditioned with a brief hypoxia-reoxygenation period to investigate the intracellular mechanisms responsible for the direct hepatoprotective action of A2aR stimulation. These studies have shown that A2aR stimulation activates a complex array of protective signals that contribute to the induction of hepatocytes resistance to hypoxic damage (Figure 1). Upon A2aR stimulation, with adenosine or pharmacological agonists, the activation of Gs protein and consequently of adenylate cyclase and protein kinase A (PKA) occurs [19, 20, 27]. PKA phosphorylates A2aR and shifts its coupling to Gi protein and Src kinase thus activating the surviving mediator phosphatidylinositol-3-kinase (PI3K) and its downstream effector Akt [21]. This allows the stimulation of phospholipase C, the recruitment of the specific isoforms δ and ε of protein kinase C (PKC), and the activation of p38 MAPK [19, 20, 27]. Full activation of preconditioning responses also needs downmodulation of inhibitory enzymes of PKC and PI3K. Hypoxic preconditioning as well as A2aR stimulation induces, in fact, a RhoA-GTPase-dependent inhibition of the diacylglycerol kinases θ, thus increasing diacylglycerol (DAG) and sustaining activation of the DAG-dependent PKC δ and ε [28]. Consistently recent "*in vivo*" studies with specific PKC δ inhibitors confirmed the critical role of PKC and, particularly, of PKC δ in mediating the protective effect of IP [25]. A2aR stimulation also induces the degradation of the PI3K inhibitor, phosphatase and tensin homologue deleted from chromosome 10 (PTEN), through a NADPH oxidase-dependent mechanism, thus allowing the maintenance of the PI3K-dependent signals [29]. The above observations indicate a key role played by PI3K and p38 MAPK in hepatocyte preconditioning as also confirmed by *in vivo* studies that reported a marked increase in the dual phosphorylation of hepatic p38 MAPK [30] and demonstrated the implication of PI3K in mediating hepatoprotection in preconditioned liver [31].

Biochemical studies shed light on mechanisms by which these protective signal networks induce the increased resistance of preconditioned hepatocytes to hypoxic injury.

As illustrated in Figure 1, hepatocytes death upon ATP depletion is precipitated by the deregulation of Na^+ homeostasis [32]. An irreversible increase of intracellular Na^+ promotes, in fact, hepatocytes killing induced by several insults including oxidative stress, mitochondrial toxins, and warm and cold hypoxia and at the first phases of reoxygenation [32–35]. Na^+ alterations that follow ATP depletion are the result of a combined block of the ATP-dependent Na^+ efflux through the Na^+/K^+ ATPase and of the activation of Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter in response to cytosolic acidification [32]. In the metastable phase that precedes death, hepatocytes respond to the progressive increase of intracellular Na^+ with the stimulation of the volume regulatory decrease mechanisms, that is, activation of the K^+ channels and K^+ efflux. The decrease of intracellular K^+ under a critical threshold definitively impairs the volume regulatory systems and leads to a sudden increase of hepatocytes volume, with osmotic lysis and death of hepatocytes [35].

Interestingly hypoxic preconditioning and A2aR activation prevent the irreversible Na^+ increase that precedes hypoxic hepatocytes damage. As shown in Figure 1, A2aR stimulation allows the maintenance of intracellular pH and

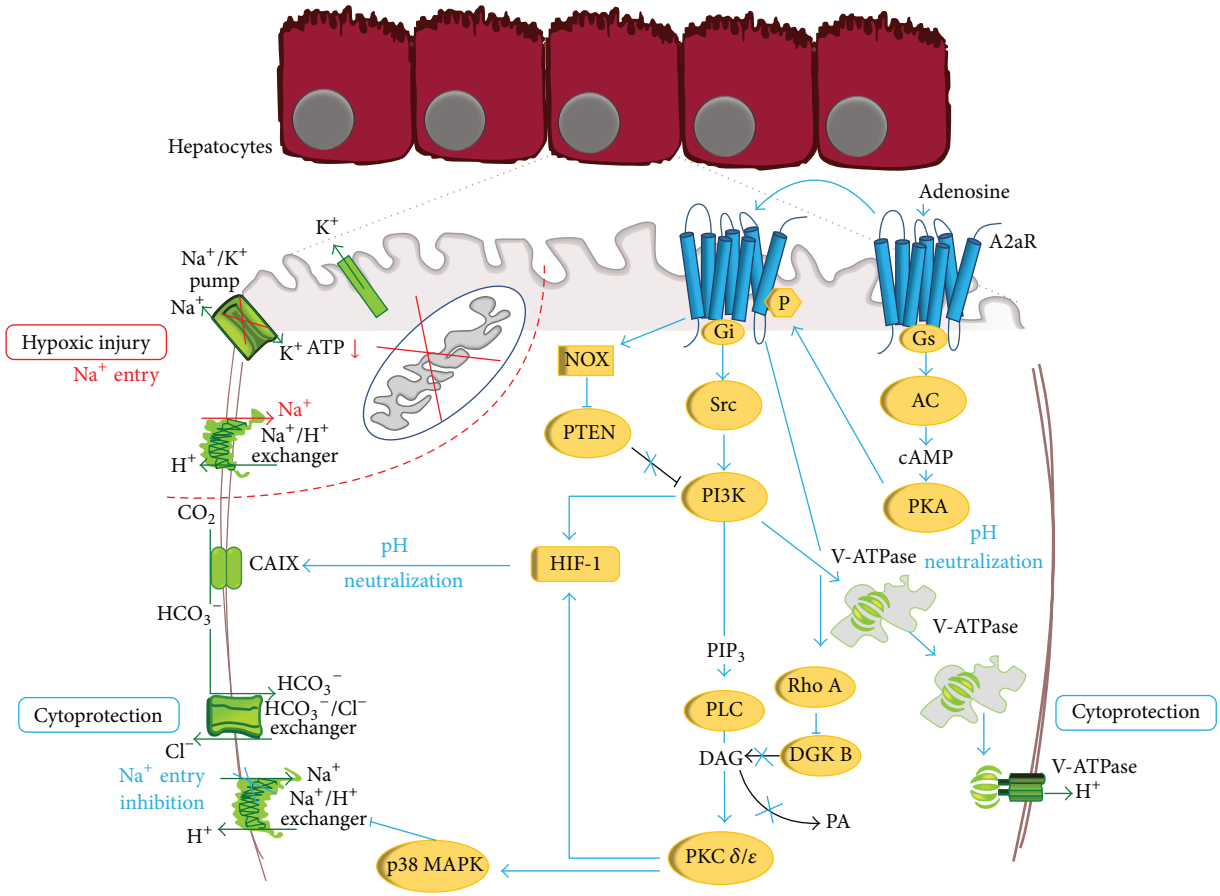

Figure 1: Molecular mechanisms involved in hypoxic injury of primary rat hepatocytes and in their protection upon A2aR stimulation. Hypoxic damage: ATP depletion causes intracellular acidosis, inhibition of the Na^+/K^+ ATPase, and activation of the Na^+/H^+ exchanger with an increase in intracellular Na^+ content and activation of the K^+ channel. A2aR protection: A2aR stimulation induces the sequential activation of PKA, Gs and Gi protein, Src, PI3K, PLC, PKC δ, and ε and p38 MAPK. A2aR also inhibits the negative regulators of PKC and PI3K, DGK, and PTEN. PI3K activates V-ATPase that maintains intracellular pH avoiding the activation of the Na^+/H^+ exchanger and Na^+ overload. PI3K and PKC δ and ε activate HIF with production of CAIX. CAIX converts CO_2 into bicarbonate that enters into hepatocyte through the Cl^-/HCO_3^- exchanger. This neutralizes intracellular pH without activation of the Na^+/H^+ exchanger and the consequent Na^+ increase. (See also text and [19, 20, 27, 28, 36, 37, 40].)

prevents the activation of the Na^+-dependent systems of pH regulation [19, 36]. Such effect is p38 MAPK- and PI3K-dependent and is due to the activation and translocation to the plasma membrane of the vacuolar ATPase (V-ATPase). V-ATPase acts as alternative pH buffering system and allows proton extrusion avoiding the irreversible Na^+ accumulation that precipitates hypoxic hepatocytes death [36, 37].

The protective effects of A2aR stimulation can be either immediate (early preconditioning) or delayed (late preconditioning). Early preconditioning allows hepatocytes to respond to a pathogenic stress that immediately follows the preconditioning stimulus and involves the activation of constitutive molecular systems. Late preconditioning is, instead, able to increase hepatocytes resistance to hypoxia up to 24 hours after the preconditioning stimulus and involves DNA transcription and *de novo* protein synthesis. Hypoxia-inducible factor 1 (HIF-1) is the main regulator of tissue adaptation to oxygen deprivation [38] and it is found to be increased in human transplanted livers exposed to IP [39]. Consistently we found that late hypoxic preconditioning of primary cultured hepatocytes is mediated by an A2aR-dependent nonhypoxic HIF-1 activation and the consequent production of its target protein carbonic anhydrase IX (CAIX) [34]. As shown in Figure 1, A2aR induces a PI3K- and PKC-dependent nuclear translocation, DNA binding, and activation of the nuclear transcription factor HIF-1. In turn, HIF-1 induces the expression of CAIX that converts CO_2 into bicarbonate in the extracellular milieu. Bicarbonate then is transported into the hepatocytes through the Cl^-/CO_3 exchanger and neutralizes the intracellular acids, thus maintaining the physiological cytosolic pH and preventing Na^+ accumulation [40].

5. Adenosine A2a Receptor Activation Protects Hepatocytes Lipotoxicity

The shortage of organs for liver transplantation has led to expansion of the criteria for the acceptance of marginal

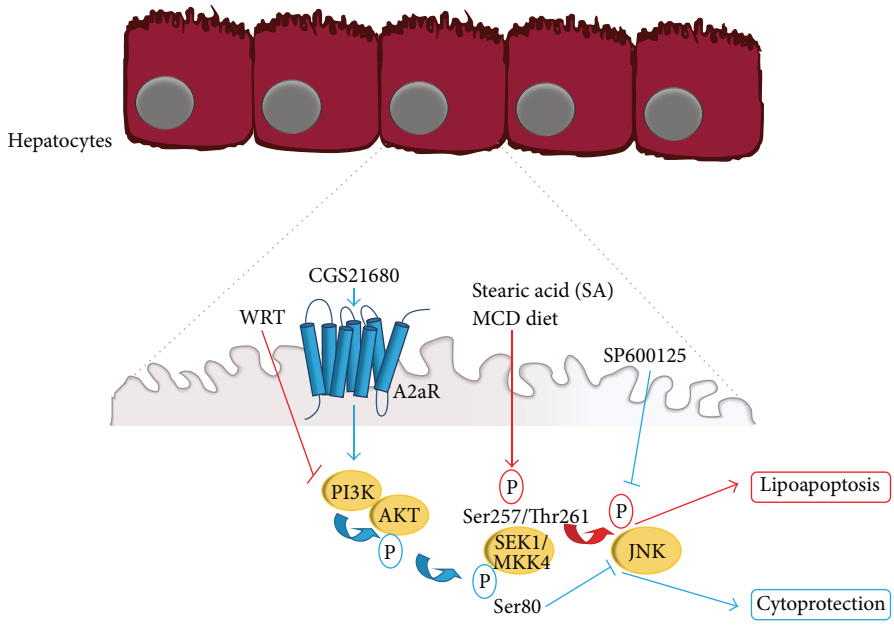

Figure 2: Molecular mechanisms involved in the lipotoxic effects of free fatty acids and in their protection upon A2aR stimulation. Stearic acid (SA) induces primary rat hepatocyte apoptosis by activating JNK-1/2 through the stimulation of MKK4/SEK1. A2aR activation prevents apoptosis by a PI3K/Akt-dependent inhibition of MKK4/SEK1. (See also text and [51, 67].)

donors, including the use of steatotic grafts [41]. Steatosis is characterized by accumulation of excess fat, that is, when the lipid content in cell exceeds 5% of lipid of total liver weight. Steatosis is the most frequent hepatic lesion in western countries with prevalence in the general population ranging from 3% to 15% but reaching up to 70% among overweight individuals [41, 42]. Importantly the presence of fatty infiltration dramatically reduces the tolerance of the liver to I/R injury in experimental models [43] increasing pathological consequences of I/R upon human liver surgery. Indeed clinical meta-analysis shows that patients with steatosis have an up to twofold increased risk of postoperative complications, and those with excessive steatosis have an almost threefold increased risk of death [44]. Several factors such as an increase of oxidative stress, mitochondrial alterations, and ATP depletion can participate in the decreased tolerance of steatotic liver to I/R injury compared with normal livers [45–48]. The accumulating lines of evidence on the phenomenon known as lipotoxicity [49] indicate that the hepatotoxic effects of free fatty acids may represent further attractive mediators of this process. The pathophysiological picture of steatosis is, in fact, characterized by an increase of circulating nonesterified free fatty acids and their metabolites [50] which have been shown to induce hepatic cell apoptosis through JNK activation [45].

The application of IP to fatty livers has demonstrated interesting results. IP, in fact, almost halved transaminase release and the histological evidence of liver cell death showing a greater efficacy of IP in steatotic liver compared to normal liver [48]. The mechanisms responsible for these beneficial effects are, however, unclear.

In recent studies, we evaluated the capacity of A2aR stimulation to prevent lipoapoptosis of primary rat hepatocytes and to inhibit the development of nonalcoholic steatohepatitis in rat fed with MCD (methionine choline-deficient) diet [51]. The treatment of primary rat hepatocytes with the free fatty acid, stearic acid (SA), promoted apoptosis by inducing MKK4 (mitogen activated protein kinase kinase-4)/SEK1 (stress-activated protein kinase/extracellular-signal regulated kinase kinase-1) and JNK-1/2 (c-Jun N-terminal kinase-1/2) activation (Figure 2). The pharmacological A2aR stimulation prevented JNK-1/2 activation by a PI3K/Akt-mediated block of MKK4/SEK1 and also protected lipoapoptosis *in vitro* (Figure 2) and the progression of steatosis to steatohepatitis *in vivo* [45]. These findings may have multiple implications. First, A2aR activation is able to exert separate protective effects against lipotoxicity associated steatosis and against I/R. This may account for additive protective action of A2aR activation and for the increased efficacy of IP in preventing I/R injury in fatty liver (researches are in progress to investigate this point). In addition, the capacity of a molecular inductor of IP to protect against hepatic insults also different from I/R injury potentially broadens the field of clinical application of IP. The activation of IP by pharmacological stimulation of one or some of its mediators would allow, in fact, its employment in all the clinical settings where the chirurgical IP is not applicable.

6. Proteome Reveals Protection Mechanisms in Preconditioned Hepatocytes and LSECs

An important approach to identify new protein mediators of liver preconditioning is the use of the proteomic analysis.

In a recent research we evaluated the proteomic patterns of primary hepatocytes and sinusoidal endothelial cells (LSECs) isolated from mice liver following I/R with or without pretreatment with the A2aR agonist CGS21680 [52]. Hepatocytes and LSECs are the main targets of I/R injury and of the beneficial effects of IP. In comparison to hepatocytes, the knowledge of the molecular mechanisms responsible for the effects of I/R and IP on LSECs is very limited [53]. LSECs, however, have been demonstrated to be largely sensitive to ischemic preservation and I/R [54]. Early studies showed that cultured LSECs exposed to hypoxia-reoxygenation produce high level of oxidative species that can lead to LSECs damage [54]. More recently, ischemic preservation of LSECs demonstrates the downregulation of the transcription factor Kruppel-like factor 2 (KLF2) [55] that is involved in the induction of a number of protective genes including the transcription factor Nrf2 that controls the expression of several antioxidant enzymes such as NAD(P)H dehydrogenase, quinone 1 (NQO1), glutathione peroxidase (GPX), or heme-oxygenase 1 (HO-1) [56]. Consistently recent reports show that remote or intestinal preconditioning prevents hepatic I/R injury via HO-1 mediated mechanisms [57, 58]. In addition the microcirculatory disturbances are a hallmark of hepatic I/R injury [59] and IP application was demonstrated to prevent both LSECs damage and microcirculatory alteration [60, 61].

The employment of proteomic analysis allowed us to evidence profound changes of hepatocytes and LSECs proteome, providing new insights into some critical aspects of I/R injury and IP-induced hepatoprotection. In particular, we observed the modulation of several proteins involved in response to apoptosis and in regeneration and cell signalling and, more importantly, we found major modifications in enzymes involved in oxidative stress protection and energy production, two fundamental processes affected by I/R and IP.

Previous studies clearly showed an increased production of oxidative species during hepatic I/R as well as the capacity of IP to prevent such damaging process [1, 4, 5, 56, 62]. Consistently we evidenced the modulation of several proteins involved in cell response to oxidative stress such as catalase, glutathione transferases GSTP1, GSTP2, and GSTM1, and peroxiredoxin 6. Notably we observed that I/R depressed the antioxidant enzymes content in LSECs exclusively, while A2aR stimulation generally increased the antioxidant defences in both LSECs and hepatocytes. These findings provide a rational base to the greater susceptibility of LSECs to oxidative stress [54] and are consistent with the possible downmodulation of Nrf2 [56]. Our observations indicate, moreover, that the ability of preconditioning to protect against I/R-induced oxidative stress can be explained by an increased antioxidant enzymes expression.

Another critical process is the decrease of ATP content in liver exposed to I/R and its prevention upon preexposure to IP [1–5]. Consistently the proteomic analysis shed light on large modification of enzymes involved in the transport and catabolism of metabolites necessary for energy production. We have observed that I/R induces in hepatocytes and LSECs a decrease of enzymes involved in carbohydrate and lipid catabolism. On the contrary, A2aR stimulation not only rescued the enzymes downregulated by I/R, but even increased enzymes associated with carbohydrate and aminoacids and lipid supply and catabolism. In the specific case of the glycolytic metabolism we found that almost the entire pathway was upregulated in both hepatocytes and LSECs.

The severe ATP depletion of liver tissue exposed to I/R is generally ascribed to the lack of O_2 and glycolytic substrates supply consequent to blood interruption during ischemia [1–3]. Our results indicate that the decrease in the efficiency of the pathways involved in the anaerobic ATP production can significantly exacerbate this process. On the other hand, the rescue or increase of the same pathways by A2aR stimulation can explain the maintenance of the ATP content of preconditioned liver. Another critical aspect is the inability of I/R-injured liver to recover aerobic ATP production at blood flow reestablishment during reperfusion and, on the other hand, the ability of IP to prevent such alteration [1–3]. We observed that I/R inhibited in both hepatocytes and LSECs ATP synthesis downmodulating the regulatory subunit B of ATP synthase and also affecting the catalytic subunit A that is essential for completion of the synthase activity. On the other hand, CGS21680 upregulated in hepatocytes and LSECs both ATPA and ATPB and also, in LSECs, the additional catalytic subunit D (ATPH5). Furthermore, in both cells, CGS21680 increased the electron transfer flavoprotein subunit alpha (ETFA), active in oxidative phosphorylation, and, in hepatocytes, S2542, a carrier mediating the transport of CoA in mitochondria that will then enter in the Krebs cycle to produce ATP. This indicated that I/R, by decreasing the enzymes of the mitochondrial metabolism, affects the capacity to synthesize ATP also in presence of O_2 and that A2aR activation restores this process by rescuing or even increasing these enzymes.

Altogether, these results showed that hepatic cells isolated from liver exposed to I/R develop a "pathological phenotype" characterized by a decrease of the metabolic enzymes involved in the aerobic and anaerobic ATP production and, in the specific case of LSECs, an additional decrement of antioxidant defences (Figure 3). On the contrary, A2aR stimulation induces the expression of a "protected phenotype" characterized by an enhancement of enzymes necessary for energy production and ROS detoxification (Figure 3). This gives a sort of metabolic and antioxidant advantage to preconditioned compared to nonpreconditioned cells and can account for the increased resistance to death of preconditioned hepatic tissue during I/R exposure.

7. Clues for Novel Pharmacological Approaches to Minimize Ischemia/Reperfusion in Patients

The analysis of the molecular changes induced by A2aR stimulation suggests novel potential pharmacological strategies to be applied in human hepatic surgery. First, the findings of the multiple mechanisms of liver cell protection induced by A2aR activation strongly enforce the idea to translate A2aR agonists

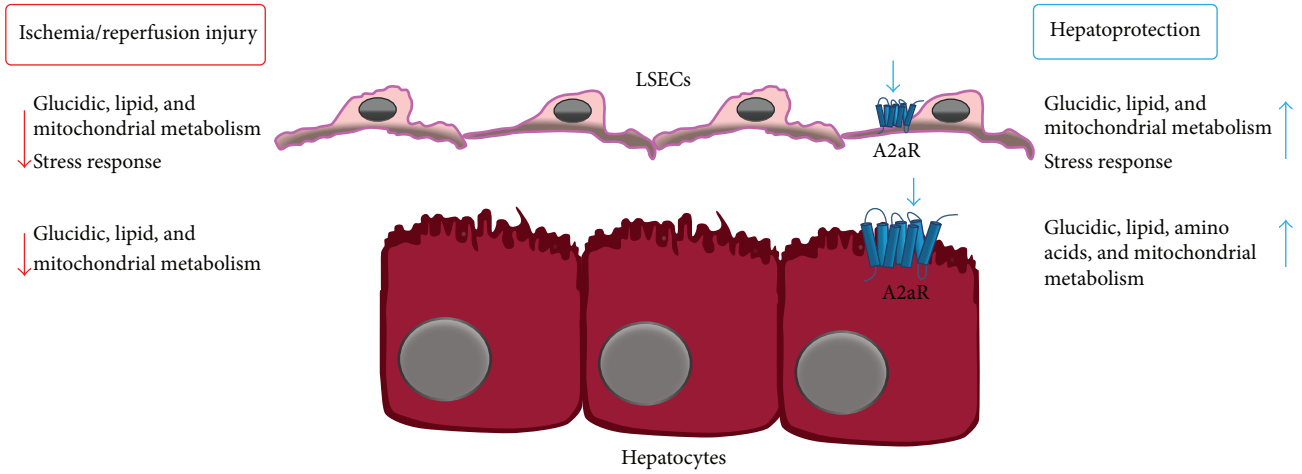

Figure 3: Main proteomic changes of hepatocytes and LSECs upon A2aR stimulation and/or hepatic ischemia/reperfusion. Mice liver exposure to ischemia/reperfusion (I/R) downmodulates proteins associated with glucidic, lipid, and mitochondrial metabolism in hepatocytes and LSECs and, specifically in LSECs, with stress response. These proteins or proteins associated with the same processes are restored or upregulated in both hepatocytes and LSECs, upon A2aR stimulation after mice *in vivo* treatment with the A2aR agonist CGS21680. (See also text and [52].)

to the clinical practise as hepatoprotective tool. In addition to the chemical A2aR agonists such as CGS21680, apadenoson (ALT-146), and ATL-313 largely employed in the preclinical models (see [63] for review), pharmacological agents leading to A2aR activation are already available for clinical purpose in humans. For example, the compound known as regadenoson (CVT-3146) is already approved by the U.S. Food and Drug Administration and it is in use as coronary vasodilator [64, 65].

Additionally, the molecular identification of pleiotropic effects of A2aR stimulation implicates the possibility to intensify these beneficial effects by a concomitant stimulation of their mediators. Moreover, in relation to the needed clinical setting, it might be of interest to achieve a focused stimulation of specific protective signals. For example, in case of short surgical hepatic interventions, it might be favourable to stimulate the protective network of early preconditioning. The choice could be then a simultaneous treatment with A2aR agonists and DGK and PTEN inhibitors in order to sustain the A2aR-induced repression of the negative regulators of PKC and PI3K that are activated within the first hour after stimulation of A2aR. In case of prolonged interventions, like those necessary for major liver surgery and transplantation, the cocktail treatment could additionally include items able to sustain HIF activation such prolyl hydroxylase inhibitors that appear to be well tolerated in patients [66]. Critical would be also the exploitation of antioxidant and metabolic advantages of preconditioned liver cells. In particular, the increased antioxidant enzymatic efficiency of A2aR preconditioned liver cells could be improved by the inclusion in liver graft conservation solutions of natural or synthetic antioxidants [67]. On the other hand, the increased metabolic activities of preconditioned liver cells can take a further advantage by the supplementation with energy-linked metabolites to sustain the glucidic, aminoacids, and lipid catabolism and thus anaerobic and aerobic ATP production.

Abbreviations

IP: Ischemic preconditioning
I/R: Ischemia/reperfusion
A2aR: Adenosine A2a receptor
ROS: Reactive oxygen species
PKA: Protein kinase A
PI3K: Phosphatidylinositol-3-kinase
PKC: Protein kinase C
DGK: Diacylglycerol kinases
DAG: Diacylglycerol
PTEN: Phosphatase and tensin homologue deleted from chromosome 10
V-ATPase: Vacuolar ATPase
HIF-1: Hypoxia-inducible factor 1
CAIX: Carbonic anhydrase IX
SA: Stearic acid
MKK4: Mitogen activated protein kinase kinase-4
SEK1: Stress-activated protein kinase/extracellular-signal regulated kinase kinase-1
JNK 1/2: c-Jun N-terminal kinase-1/2
LSECs: Sinusoidal endothelial cells
KLF2: Kruppel-like factor 2
GST: Glutathione transferase
NQO1: NAD(P)H dehydrogenase quinone 1
GPX: Glutathione peroxidase
HO-1: Heme-oxygenase 1
ETFA: Electron transfer flavoprotein subunit alpha.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Comparative Study on the Cytoprotective Effects of Activated Protein C Treatment in Nonsteatotic and Steatotic Livers under Ischemia-Reperfusion Injury

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Activated protein C (APC) has cytoprotective effects on liver ischemia-reperfusion injury (IRI). However, it is unclear whether APC is beneficial in steatotic liver IRI. We compared the cytoprotective effects of APC in nonsteatotic and steatotic liver IRI. *Methods.* Mice fed either normal diets (ND mice) or high fat diets (HF mice), were treated with APC or saline (control) and were performed 60 min partial IRI. Moreover, primary steatotic hepatocytes were either untreated or treated with APC and then incubated with H_2O_2. *Results.* APC significantly reduced serum transaminase levels and the inflammatory cells infiltration compared with control at 4 h in ND mice and at 24 h in HF mice. APC inhibited sinusoidal endothelial injury in ND mice, but not in HF mice. In contrast, APC activated adenosine monophosphate-activated protein kinase (AMPK) phosphorylation in HF mice, but not in ND mice. In the in vitro study, APC significantly increased AMPK phosphorylation, ATP concentration, and survival rates of hepatocytes compared with control. *Conclusion.* During IRI in normal liver, APC attenuated initial damage by inhibiting inflammatory cell infiltration and sinusoidal endothelial injury, but not in steatotic liver. However, in steatotic liver, APC might attenuate late damage via activation of AMPK.

1. Introduction

Hepatic steatosis is a major risk factor for liver resection and transplantation. Recently, the epidemic of obesity in developed countries has increased, along with its attendant complications, including metabolic syndrome and hepatic steatosis. Between 6 and 33% of individuals in the general population [1] and 70–80% of obese patients have hepatic steatosis. Liver transplants using steatotic liver grafts are associated with a high primary graft nonfunction rate compared with nonsteatotic livers [2, 3]. Along with transplantation, steatotic livers have a negative impact in other clinical situations, such as hepatectomy, shock, and cardiac arrest, which are all subject to warm hepatic ischemia-reperfusion injury (IRI) [4].

Although it is generally accepted that steatotic livers are particularly vulnerable to hepatic IRI, results from animal experiments indicate that the mechanisms underlying IRI are different in nonsteatotic and steatotic livers [5]. Cellular hypoxia persists in fatty hepatocytes during IRI because the hepatic sinusoidal space is obstructed due to fat droplet accumulation in the cytoplasm of hepatocytes. This causes a reduction in sinusoidal blood flow [6] and a decrease in ATP synthase and increase in reactive oxygen species (ROS) production within the steatotic liver is induced by the increased level of mitochondrial uncoupling protein-2, a mitochondrial inner membrane protein that mediates proton leakage across the inner membrane by uncoupling substrate oxidation from ATP synthesis [7–9]. However, the reason why hepatic IRI is increased in steatotic liver has not yet been fully elucidated.

Among a large number of pharmacological agents to protect against IRI in animal models, activated protein C (APC), an anticoagulant, has been shown to have cytoprotective

effects against IRI in several organs [10, 11]. Previously, we reported that APC administration had a cytoprotective effect on hepatic IRI in rat models, by preventing recruitment of inflammatory cells, ameliorating sinusoidal endothelial cell injury, and maintaining sinusoidal blood flow [11]. Previous studies provide evidence supporting the idea of direct cytoprotective actions of APC in which endothelial protein C receptor- (EPCR-) bound APC activates protease-activated receptor 1 (PAR1) to initiate signaling on endothelial cells [12, 13]. In contrast, a recent study using a myocardial IRI model elucidated another mechanism of APC, namely, triggering adenosine monophosphate-activated protein kinase (AMPK) signaling: phosphorylation of AMPK mediates dramatic changes in cell metabolism, cell survival, and other functions [14]. In steatotic liver, mitochondria in hepatocytes produce excessive amounts of reactive oxidative species (ROS) leading to damage of mitochondrial inner membrane proteins and a consequent decrease of mitochondrial adenosine triphosphate (ATP) production [15]. Moreover, hepatic ATP stores are reduced in steatotic livers which are more vulnerable to necrosis after transient hepatic ischemia [16]. To the best of our knowledge, there have been no previous studies investigating whether APC treatment is beneficial against steatotic liver IRI, compared with nonsteatotic liver. On the basis of evidence from these previous studies, we speculate that APC might have a different mechanism in steatotic liver compared with nonsteatotic liver, namely, that APC attenuates I/R injury by preventing the depletion of ATP via AMPK activation, and this effect is specific to steatotic liver.

In the present study, we compared the cytoprotective effects of APC administration between nonsteatotic and steatotic liver IRI in a mouse model, in an attempt to elucidate the theoretical mechanism by which APC attenuates liver damage specifically in steatotic liver.

2. Materials and Methods

2.1. Animals. Five-week-old male mice (C57BL/6; Japan SLC, Inc.) were fed either a normal diet (ND mice) or a high fat diet (60% calories from fat; Research Diets number D12492) (HF mice) for 9 weeks (Figure 1(a)). All experiments were conducted in compliance with the Guideline for Animal Experiments in Mie University Graduate School of Medicine.

2.2. Activated Protein C. Human plasma-derived APC was kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). ND and HF mice were randomly assigned to either APC treatment or control groups, resulting in the following four groups being established: (1) ND-APC, (2) ND-Control, (3) HF-APC, and (4) HF-Control. The number of animals used in each group was 12. APC (0.2 mg/kg of body weight) or saline solution (the volume equivalent to APC solution) was intravenously administered just prior to surgery and at 8 h and 16 h after reperfusion [11, 14].

2.3. Model of Partial Lobar Liver IRI. A warm hepatic IRI model was established in 14-week-old male mice (i.e., after 9 weeks of diet feeding). Mice were anaesthetized with isoflurane and livers were exposed through a midline laparotomy. The arterial and portal venous blood supplies were interrupted to the cephalad lobes of the liver for 60 min using an atraumatic clip. The right hepatic lobe and the caudate lobe were perfused to prevent intestinal congestion. After 60 min of ischemia, the clip was removed, thus initiating hepatic reperfusion. Mice were sacrificed 4 h or 24 h after reperfusion ($n = 6$ in each group; Figure 1(b)). Body weight was measured before the operation.

2.4. Serum Transaminases. Serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels were measured using a commercially available kit (Wako Pure Chemical Industries Ltd., Osaka, Japan), following manufacturer's instructions.

2.5. Histology and Immunohistochemistry. Liver histology and immunohistochemistry were performed as previously reported [17, 18]. Liver specimens embedded in paraffin were processed for hematoxylin and eosin (H&E) staining. The histological damage due to hepatic IRI was assessed using the modified Suzuki score [19], as well as by the extent of the necrotic area. In the modified Suzuki score, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration were graded from 0 to 4. No necrosis, congestion, or centrilobular ballooning was given a score of 0, whereas severe congestion and ballooning degeneration and 60% lobular necrosis were given a score of 4. The degree of hepatic necrosis 24 h after IRI was assessed in H&E-stained paraffin sections; H&E stains were digitally photographed and the percent of necrosis was quantified using NIH Image J software in a manner blinded to the different experimental groups, as previously described [20]. Liver steatosis was evaluated using Oil Red O staining. Immunohistochemistry was performed using Ly6G (1A8) from BioLegend (San Diego, CA), MAC-1 (M1/70) and PECAM-1 (MEC13.3) from BD Biosciences (San Jose, CA), and phospho-AMPK (p-AMPK) (40H9) from Cell Signaling Technology (Beverly, MA), with all antibodies used at optimal dilutions. The results were evaluated by an average of 10 times' counting in 40 high-power (×400 magnification) fields per section.

2.6. Western Blot Analysis. Western blots were performed as described previously [17]. PVDF membranes were incubated with antibodies against PECAM-1 (epitope within extracellular domain; SC-28188; Santa Cruz Biotechnology, Santa Cruz, CA) and p-AMPK (40H9; Cell Signaling Technology). After development, membranes were stripped and reblotted with antibodies against AMPK (D5A2; Cell Signaling Technology) and actin (Cell Signaling Technology). Prestained molecular weight markers (Protein MultiColor III; BioDynamics Laboratory Inc., Tokyo, Japan) served as standards. Relative quantities of protein were determined using a densitometer (NIH Image J software).

2.7. Cell Culture. Primary steatotic hepatocytes were isolated from HF mice. To isolate primary murine hepatocytes,

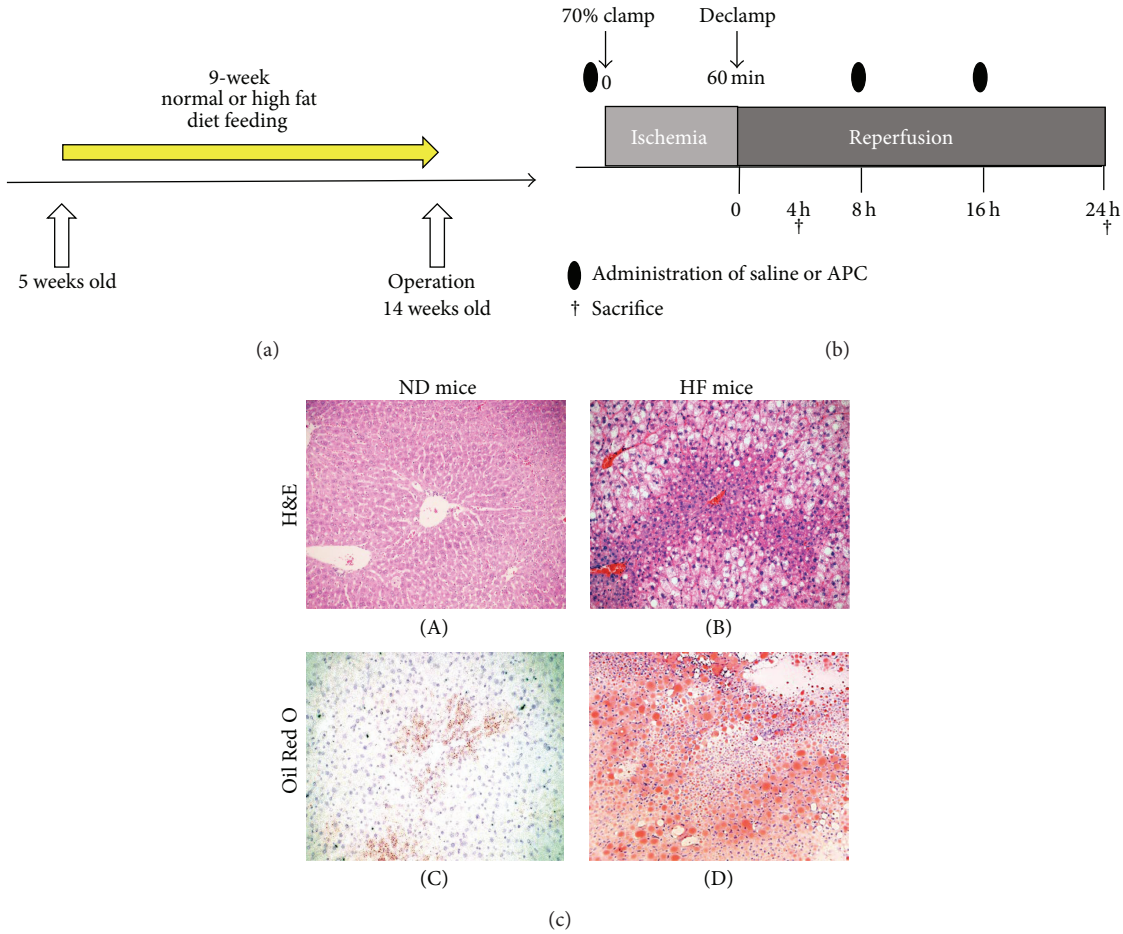


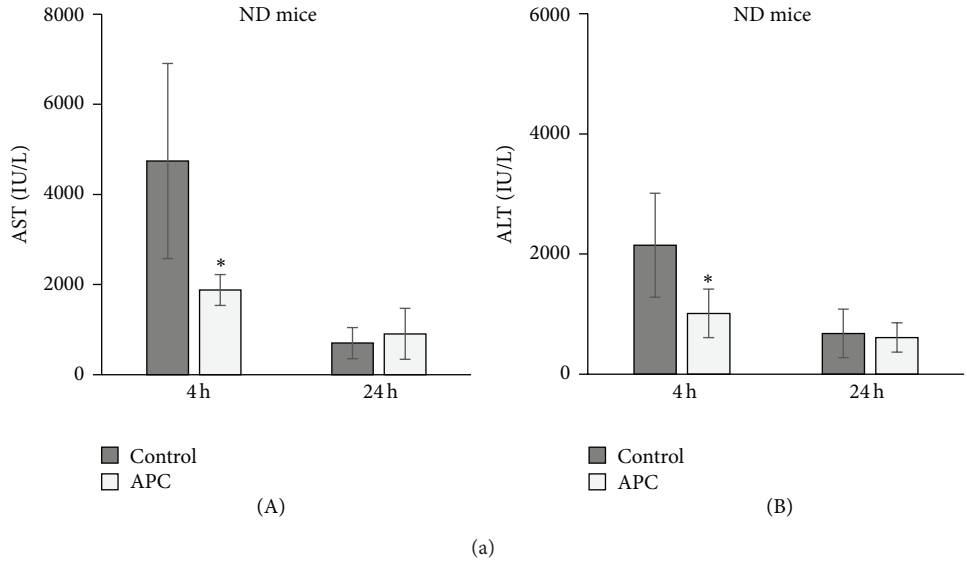
Figure 1: Development of steatotic mice and method of IRI. Five-week-old C57BL/6 male mice were fed either a normal diet (ND mice) or a high fat diet (HF mice) for 9 weeks (a). Blood supply to the cephalad lobes of the liver was interrupted for 60 min using an atraumatic clip. After 60 min of ischemia, the clip was removed. APC or saline solution was administered just prior to surgery and at 8 h and 16 h after reperfusion. Mice were sacrificed 4 h or 24 h after reperfusion (b). Representative H&E ((c)-(A), (c)-(B)) and Oil Red O staining ((c)-(C), (c)-(D)) of liver tissue after 9 weeks on the experimental diets. There was no steatosis in ND mice ((c)-(A), (c)-(C)). After 9 weeks of HF diet feeding, macrosteatosis was observed using H&E staining and Oil Red O staining ((c)-(B), (c)-(D)). The original magnification was ×100.

anesthetized mice were subjected to a midline laparotomy and cannulation of the portal vein followed by liver perfusion with an EGTA-chelating perfusion buffer (EGTA: 190 mg, glucose: 900 mg, HEPES: 10 mL of 1 M stock solution, KCL: 400 mg, Na_2HPO_4-$2H_2O$: 151 mg, NaCl: 8 g, NaH_2PO_4-H_2O: 7 mg, and $NaHCO_3$: 350 mg, made up to 1 L with dH_2O). After perfusion with 0.4% collagenase buffer ($CaCl_2$-$2H_2O$: 560 mg, HEPES: 10 mL of 1 M stock solution, KCL: 400 mg, Na_2HPO_4-$2H_2O$: 151 mg, NaCl: 8 g, NaH_2PO_4-H_2O: 7 mg, $NaHCO_3$: 350 mg, and collagenase P: 400 mg, made up to 1 L with dH_2O), livers were minced and cells dispersed in culture medium; hepatocyte and nonparenchymal cells were separated using low-speed centrifugation methods. Isolated mouse steatotic hepatocytes (2×10^5/well) were cultured in DMEM with 10% FBS on 24-well collagen-coated plate at 37°C with 5% CO_2 for 12 h. Hepatocytes were incubated in the presence or absence of H_2O_2 (500 nM) and/or APC (300 nM) and/or compound C (10 μM) (Tocris Bioscience, Bristol, UK), which is an inhibitor of AMPK. After 24 h culture, the cell lysates were prepared for protein evaluation, and the supernatants were collected for cytotoxicity assays. Cell viability was assessed by counting an aliquot in the presence of 0.4% Trypan blue. Cell cytotoxicity was assessed by AST levels in culture media. ATP levels of hepatocytes were measured using a commercially available kit (BioVision, Palo Alto, CA), according to the manufacturer's instructions.

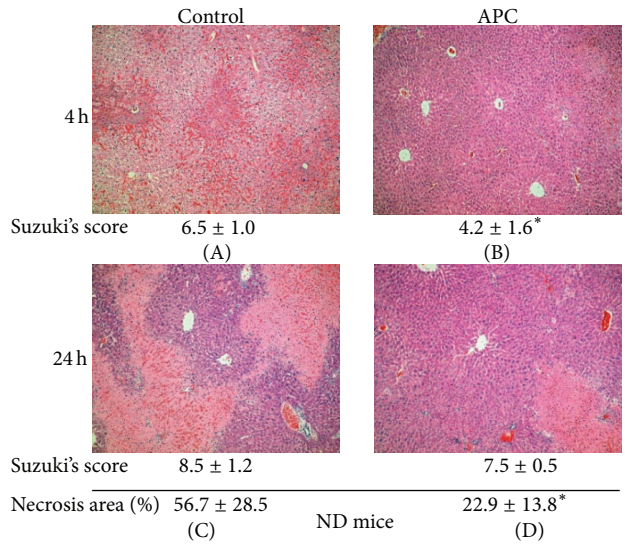
2.8. Data Analysis. The results of continuous variables are expressed as the mean value ± standard deviation. Statistical comparisons between groups of normally distributed data were performed using the Mann-Whitney U test with SPSS software (SPSS Inc., Chicago, IL). P values less than 0.05 were considered statistically significant.

3. Results

3.1. HF Mice Develop Macrosteatosis. There was no observation of steatosis in ND mice (Figures 1(c)-(A) and 1(c)-(C)). HF mice developed fatty livers which resembled those



(a)



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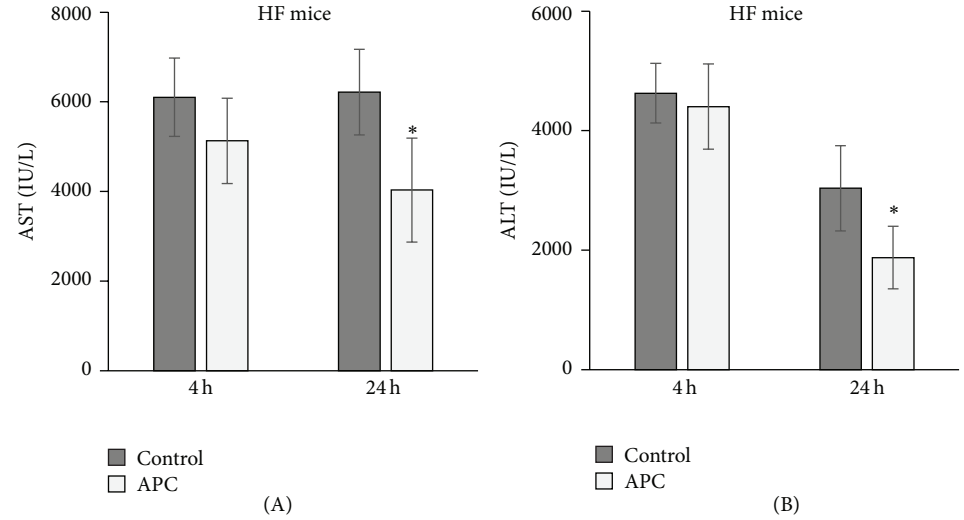


(c)

Figure 2: Continued.

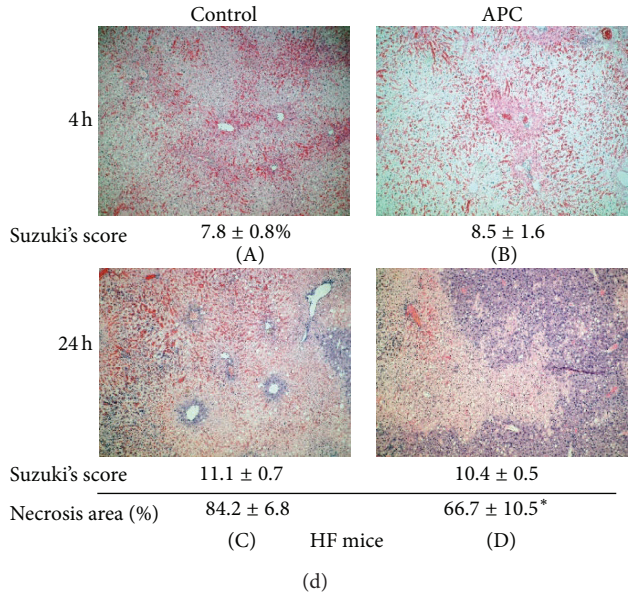


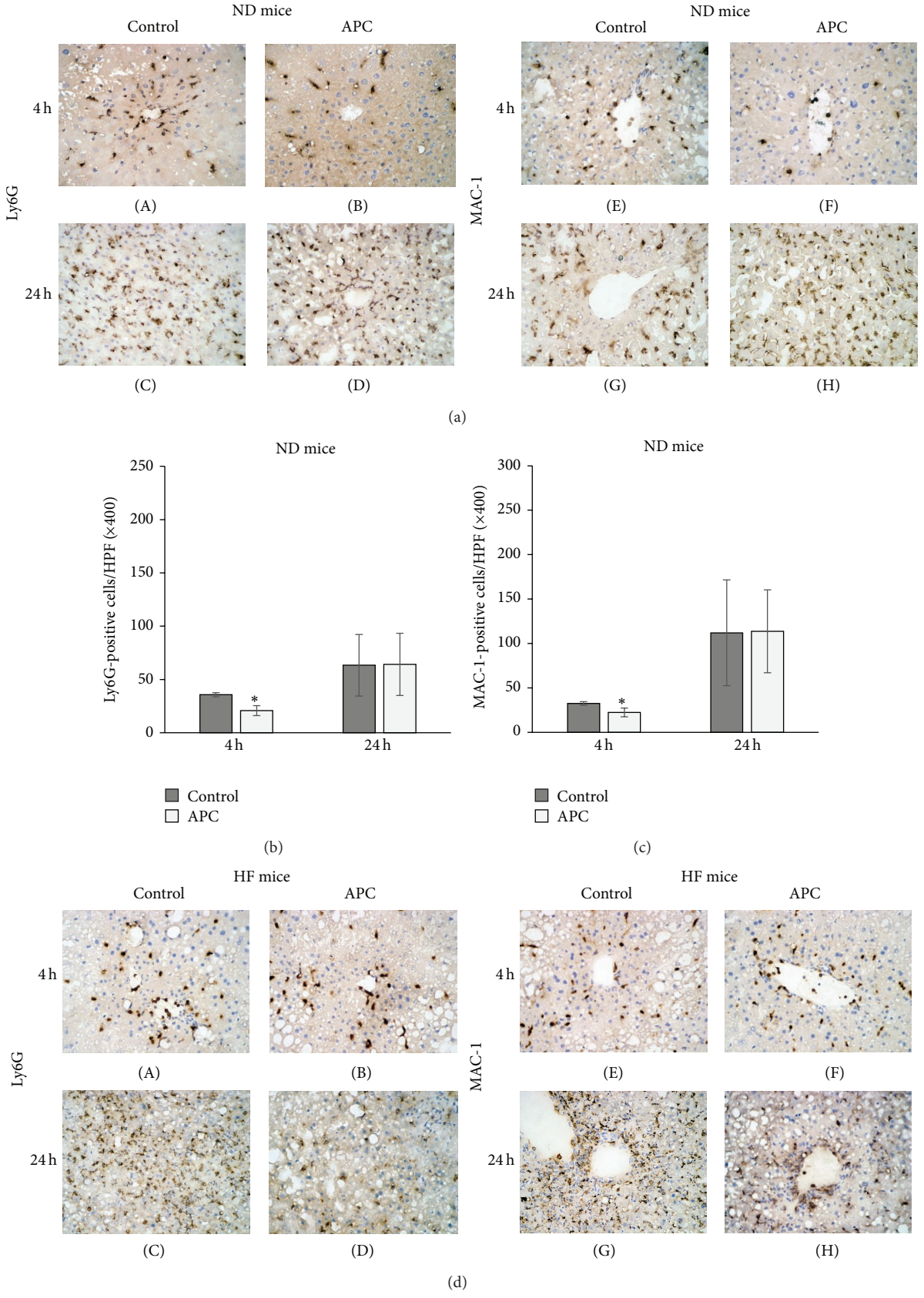
Figure 2: Transaminase levels and histology in ND and HF mice. Serum AST and ALT levels at 4 h were significantly decreased in ND-APC compared with ND-Control mice ($^*P < 0.05$). There was no significant difference at 24 h between ND-APC and ND-Control mice ((a)-(A), (a)-(B)). H&E staining showed that the ND-APC had significantly preserved lobular architecture and reduced intrasinusoidal/vascular congestion compared with ND-Control at 4 h ((b)-(A), (b)-(B)). The necrotic area within livers was significantly reduced in ND-APC compared with ND-Control mice at 24 h ($^*P < 0.05$) ((b)-(C), (b)-(D)). While serum AST and ALT levels at 24 h were significantly decreased in HF-APC compared with HF-Control mice ($^*P < 0.05$), there was no significant difference at 4 h between HF-APC and HF-Control mice ((c)-(A), (c)-(B)). In HF mice, however, liver tissues in both the HF-APC and HF-Control groups showed marked changes in vacuolization and intrasinusoidal/vascular congestion ((d)-(A), (d)-(B)). Although there was more severe necrosis in HF mice than in ND mice, the necrotic area of hepatocytes was significantly reduced in HF-APC compared with HF-Control mice at 24 h ($^*P < 0.05$) ((d)-(C), (d)-(D)). The numbers under the pictures show the modified Suzuki score and the percentage of necrotic area (%). The original magnification was ×100 (b, d).

of human obesity [21]. In our experimental setting, HF mice were characterized by 50% liver steatosis, with macrovesicular fatty infiltration, as assessed by H&E (Figure 1(c)-(B)) and Oil Red O staining (Figure 1(c)-(D)). Moreover, the body weight of 9-week HF mice was significantly higher than that of ND mice (41.3 ± 2.1 versus 27.7 ± 1.5 g, $P < 0.05$).

3.2. APC Ameliorates Hepatocellular Injury in ND and HF Mice at Different Time Points. In ND mice, APC treatment significantly reduced serum AST and ALT levels at 4 h (AST: 1,879 ± 344 versus 4,741 ± 2,167 IU/L; ALT: 1,012 ± 403 versus 2,146 ± 866 IU/L, $P < 0.05$). However, there were no significant differences at 24 h between ND-APC and ND-Control mice (AST: 903 ± 565 versus 700 ± 344 IU/L; ALT: 610 ± 243 versus 678 ± 403 IU/L) (Figures 2(a)-(A) and 2(a)-(B)). In the assessment of histological damage, necrotic area could not be accurately assessed at 4 h because development of necrosis was scarce. The modified Suzuki score at 4 h was significantly lower in ND-APC than in ND-Control mice: 4.2 ± 1.6 versus 6.5 ± 1.0, $P < 0.05$ (Figures 2(b)-(A) and 2(b)-(B)), while at 24 h there was no significant difference between the two groups: 7.5 ± 0.5 versus 8.5 ± 1.2 (Figures 2(b)-(C) and 2(b)-(D)). The necrotic area at 24 h was significantly lower in ND-APC than in ND-Control mice: 22.9 ± 13.8 versus 56.7 ± 28.5%, $P < 0.05$ (Figures 2(b)-(C) and 2(b)-(D)).

In HF mice, APC treatment did not significantly reduce serum AST or ALT levels at 4 h compared with HF-Control mice (AST: 5,130 ± 954 versus 6,103 ± 873 IU/L; ALT: 4,403 ± 715 versus 4,627 ± 499 IU/L), while it significantly improved steatotic liver function at 24 h (AST: 4,032 ± 1,160 versus 6,218 ± 954 IU/L; ALT: 1,876 ± 523 versus 3,037 ± 715 IU/L, $P < 0.05$) (Figures 2(c)-(A) and 2(c)-(B)). In the assessment of histological damage, the modified Suzuki score was very difficult to assess in steatotic liver due to the large number of fatty droplets, and necrotic area could not be evaluated at 4 h because development of necrosis was scarce. The modified Suzuki scores at 4 h and 24 h were not significantly different between HF-APC and HF-Control mice: 8.5 ± 1.6 versus 7.8 ± 0.8 at 4 h (Figures 2(d)-(A) and 2(d)-(B)); 10.4 ± 0.5 versus 11.1 ± 0.7 at 24 h (Figures 2(d)-(C) and 2(d)-(D)). In contrast, the necrotic area at 24 h was significantly lower in HF-APC than in HF-Control mice: 66.7 ± 10.5 versus 84.2 ± 6.8% ($P < 0.05$) (Figures 2(d)-(C) and 2(d)-(D)).

3.3. APC Prevents Intrahepatic Leucocyte Infiltration in ND and HF Mice. To determine whether APC affects local leucocyte infiltration, we assessed Ly6G-positive cells and MAC-1-positive cells using immunohistochemical staining. The number of Ly6G-positive cells in the liver was significantly decreased in ND-APC mice compared with ND-Control mice at 4 h (20.9 ± 4.6 versus 35.7 ± 1.9, $P < 0.05$) (Figures 3(a)-(A), 3(a)-(B), and 3(b)). However, at 24 h, there were no significant differences in the number of Ly6G-positive cells that had infiltrated when comparing the two groups



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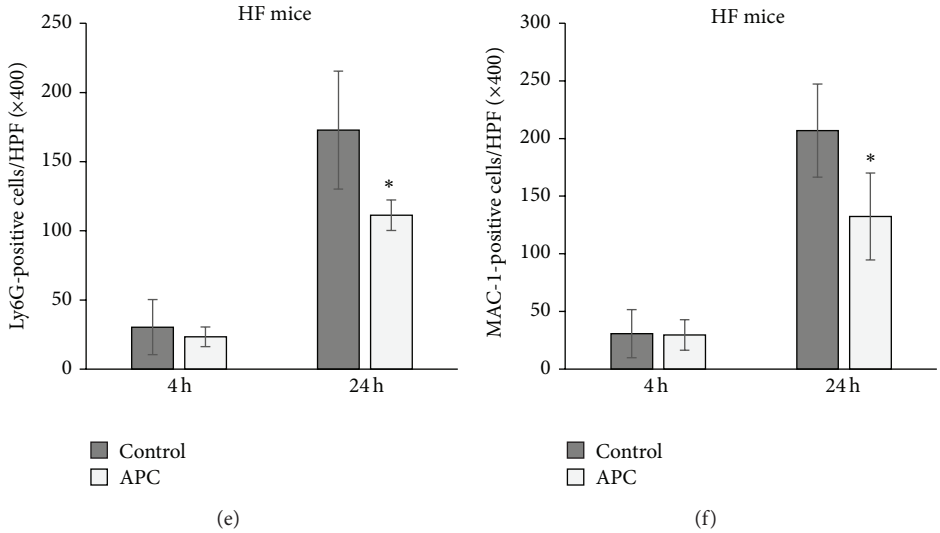

Figure 3: Leukocyte infiltration 4 h and 24 h after reperfusion. Infiltration of Ly6G-positive cells ((a)-(A), (a)-(B), and (b)) and MAC-1-positive cells ((a)-(E), (a)-(F), and (c)) in the liver was significantly decreased in ND-APC compared with ND-Control mice at 4 h ($^*P < 0.05$). However, in HF mice, infiltration of Ly6G-positive cells ((d)-(C), (d)-(D), and (e)) and MAC-1-positive cells ((d)-(G), (d)-(H), and (f)) was significantly decreased in HF-APC livers compared with HF-Control livers at 24 h ($^*P < 0.05$). There was no significant difference at 4 h between HF-APC and HF-Control mice. The original magnification was ×400 (a, d).

(64.1 ± 29.2 versus 63.4 ± 28.8) (Figures 3(a)-(C), 3(a)-(D), and 3(b)). The number of MAC-1-positive cells in the liver was significantly decreased in ND-APC compared with ND-Control mice at 4 h (22.3 ± 4.9 versus 32.5 ± 1.9, $P < 0.05$) (Figures 3(a)-(E), 3(a)-(F), and 3(c)). However, at 24 h, there were no significant differences in the number of MAC-1-positive cells between the two groups (113.8 ± 46.7 versus 111.9 ± 59.6) (Figures 3(a)-(G), 3(a)-(H), and 3(c)).

In HF mice, there were no significant differences in the number of Ly6G-positive cells at 4 h between the two groups (23.4 ± 7.1 versus 30.4 ± 19.9) (Figures 3(d)-(A), 3(d)-(B), and 3(e)), while at 24 h the number was significantly decreased in HF-APC mice compared with HF-Control mice (111.4 ± 11.0 versus 172.8 ± 42.6, $P < 0.05$) (Figures 3(d)-(C), 3(d)-(D), and 3(e)). Although there were no significant differences in the number of MAC-1-positive cells at 4 h between the two groups (29.5 ± 13.2 versus 30.7 ± 20.8) (Figures 3(d)-(E), 3(d)-(F), and 3(f)), their number was significantly decreased in HF-APC mice compared with HF-Control mice at 24 h (132.4 ± 37.7 versus 206.8 ± 40.4, $P < 0.05$) (Figures 3(d)-(G), 3(d)-(H), and 3(f)).

3.4. APC Prevents Sinusoidal Endothelial Cell Damage in ND Liver, but Not in HF Liver. PECAM-1 expression is readily detected on the intact vascular endothelium of naïve livers without IRI [18]. However, while PECAM-1 expression was relatively preserved on the vascular endothelium of ND-APC livers after IRI, it was largely absent from the vasculature of control livers, particularly at 4 h and 24 h (Figures 4(a)-(A)–4(a)-(D)). Indeed, the full length PECAM-1 (132 kDa) was detected in ND-APC livers and markedly depressed in ND-Control livers at 4 h and 24 h (4 h: 0.77 ± 0.06 versus 0.51 ± 0.28; 24 h: 1.45 ± 0.50 versus 0.96 ± 0.18, $P < 0.05$) (Figures 4(b)-(A) and 4(b)-(B)). In contrast, sinusoidal endothelial structures were severely disrupted regardless of the APC administration at 4 h and 24 h in HF mice (Figures 4(c)-(A)–4(c)-(D)). There were no significant differences in relative quantities of PECAM-1 between HF-APC and HF-Control livers (4 h: 1.02 ± 0.26 versus 0.84 ± 0.13; 24 h: 0.57 ± 0.19 versus 0.50 ± 0.14) (Figures 4(d)-(A) and 4(d)-(B)). Taken together, these data suggest that APC reduced sinusoidal damage due to hepatic IRI in ND livers, which is cytoprotective effect of APC, but this effect was abolished in steatotic livers, meaning that the other effects of APC needed to be considered, except in the case of endothelial cells.

3.5. APC Administration Activates AMPK Phosphorylation at 4 h in HF Livers, but Not in ND Livers. According to previous reports, APC is thought to potentiate the phosphorylation of AMPK, a serine-threonine kinase which maintains cellular energy stores and prevents energy depletion [14, 22, 23]. When we focused on the phosphorylation of AMPK in ND mouse livers, there were no significant differences at 4 h or 24 h between ND-APC and ND-Control mice (4 h: 0.58 ± 0.11 versus 0.67 ± 0.10; 24 h: 1.45 ± 0.47 versus 1.09 ± 0.34) (Figures 5(a) and 5(b)). In contrast, AMPK phosphorylation was significantly increased in HF-APC mice compared with HF-Control mice at 4 h (1.21 ± 0.21 versus 0.83 ± 0.13, $P < 0.05$) (Figure 5(c)). However, at 24 h, there were no significant differences between the two groups in relative quantities of AMPK phosphorylation (0.82±0.33 versus 0.62± 0.22) (Figure 5(d)). We then focused on the localization of p-AMPK in HF livers. Immunohistochemical staining of the liver revealed that p-AMPK was predominantly expressed by the steatotic hepatocytes in the periportal area (zone I) 4 h after reperfusion (Figure 6).

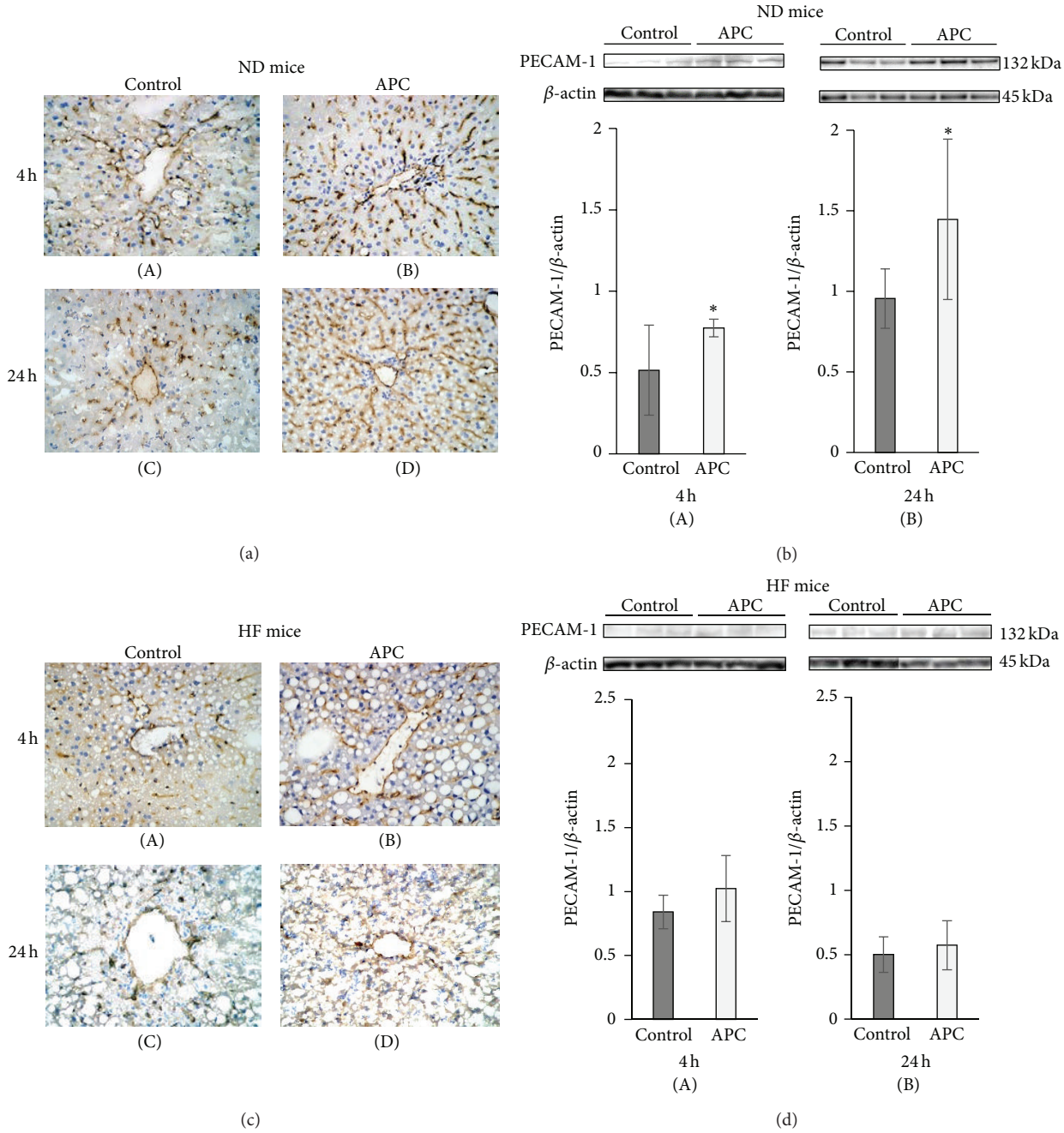

Figure 4: Sinusoidal endothelial cell damage in ND and HF mice. IRI disrupted sinusoidal vasculature regardless of APC administration in HF mice. In the liver tissue of ND mice, there was significant large number of sinusoidal endothelial cells, which were stained with PECAM-1 antibody in ND-APC than in ND-Control mice at 4 h ((a)-(A), (a)-(B), and (b)-(A)) and 24 h ((a)-(C), (a)-(D), and (b)-(B)). In liver tissue of HF mice, sinusoidal endothelial cells which were stained with PECAM-1 antibody were disrupted regardless of APC administration at both 4 h ((c)-(A), (c)-(B), and (d)-(A)) and 24 h ((c)-(C), (c)-(D), and (d)-(B)).

3.6. APC Improves Hepatocyte Survival via Upregulation of AMPK Phosphorylation In Vitro. According to the data from the in vivo AMPK analysis and evidence that p-AMPK was mainly detected in surviving hepatocytes at 4 h after IRI, we isolated primary steatotic hepatocytes, which are considered the main site of energy storage in steatotic liver, and evaluated the levels of AMPK phosphorylation and the degree of energy depletion in the presence or absence of APC administration.

The survival rates of steatotic hepatocytes were 75.4±1.0% in the sham group and 69.5 ± 5.6% in response to APC alone, while they were only 56.6 ± 10.1% in response to H_2O_2 alone. In response to H_2O_2 + APC, they were significantly increased (to 73.3 ± 5.4%) compared with H_2O_2 treatment alone. By adding an inhibitor of AMPK (compound C), the survival rate was significantly decreased to 63.0 ± 2.3% (Figure 7(a)). AST levels in the culture medium, an index of

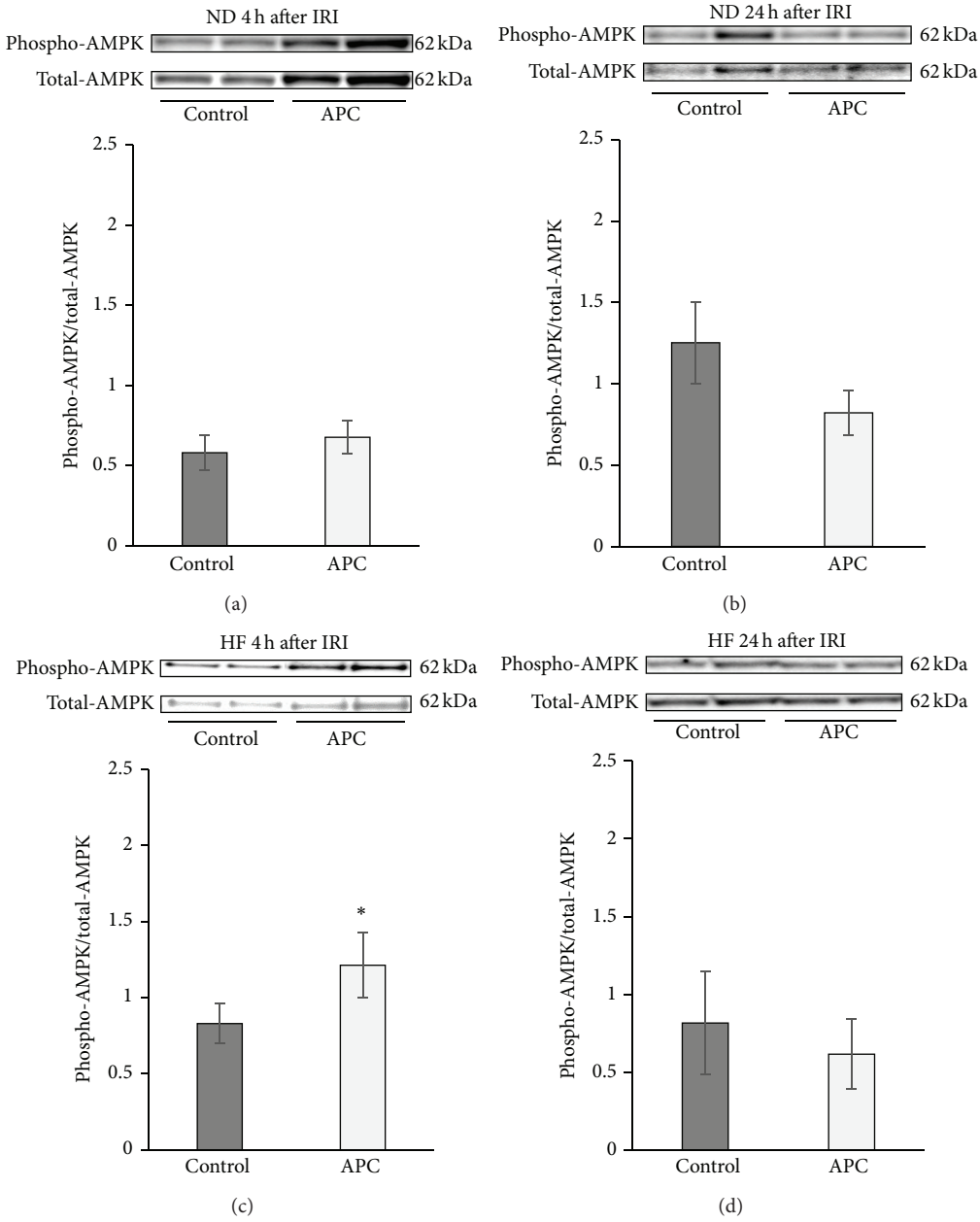

Figure 5: Phosphorylation of AMPK in steatotic liver tissue. In ND mice, there was no significant difference in phosphorylation of AMPK regardless of the APC administration at 4 or 24 h (a, b). APC significantly increased phosphorylation of AMPK at 4 h in HF mice ($^{*}P < 0.05$) (c). At 24 h, however, there was no significant difference in phosphorylation of AMPK between HF-APC and HF-Control mice (d).

cell cytotoxicity, were 41.5 ± 0.7 IU/L in the sham group and 45.7 ± 6.8 IU/L in response to APC alone, while they were increased to 87.5 ± 15.7 IU/L in response to H_2O_2 alone. In the H_2O_2 + APC treatment, they were significantly decreased (to 53.1 ± 19.7 IU/L) compared with H_2O_2 treatment alone. By adding compound C, the AST levels were significantly increased (to 73.9 ± 17.9 IU/L; Figure 7(b)). ATP levels of steatotic hepatocytes, after 24 h incubation with H_2O_2, were significantly preserved in the presence of APC, compared with the respective controls (6.64 ± 1.96 versus 4.51 ± 1.32 pmol/μg protein, $P < 0.05$; Figure 7(c)). The relative quantities of AMPK phosphorylation in steatotic hepatocytes (as assessed by western blot analysis) were significantly higher in the presence of APC compared with controls (2.08 ± 0.75 versus 1.07 ± 0.26, $P < 0.01$; Figure 7(d)).

4. Discussion

In the present study, both nonsteatotic and steatotic mice treated with APC showed significant improvements with respect to serum transaminase levels, liver histological damage, and leukocyte recruitment, and these effects were mainly

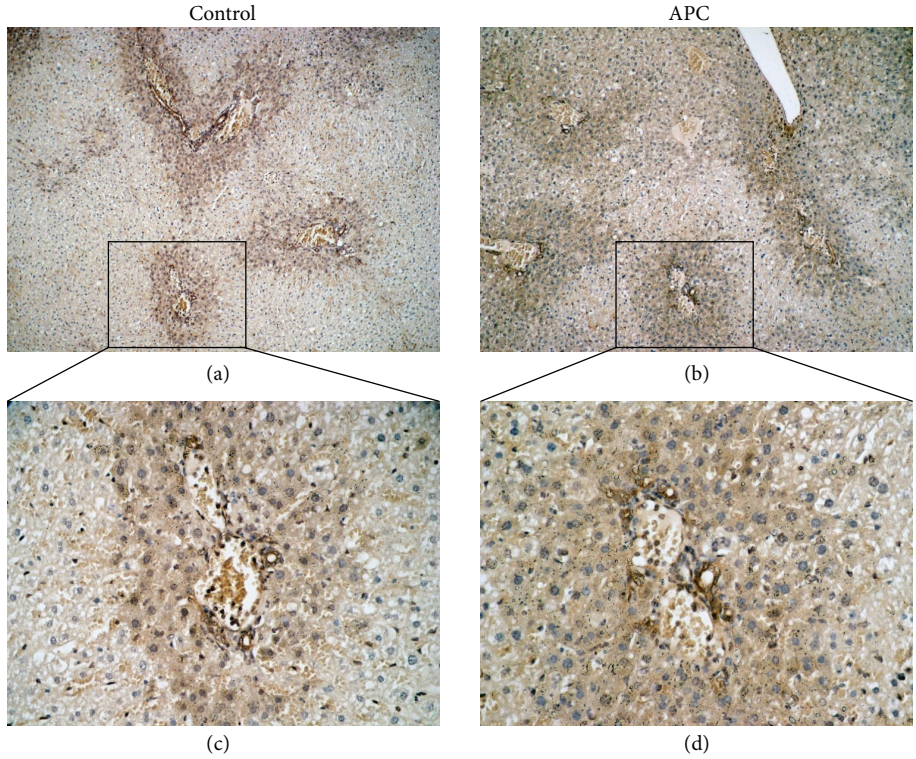

Figure 6: Immunohistochemical staining of phospho-AMPK in steatotic liver. Phospho-AMPK was predominantly expressed by the surviving steatotic hepatocytes particularly around the portal triad. The original magnification was ×100 (a, c) or ×400 (b, d).

observed at 4 h in nonsteatotic livers and at 24 h in steatotic livers. In nonsteatotic liver, the transaminase levels which were markedly increased at 4 h could be attenuated by APC treatment, while these levels were significantly decreased at 24 h in both groups, showing no significant difference. The modified Suzuki score of liver damage, which was derived from a combination of congestion, centrilobular ballooning, and necrosis, was significantly reduced by APC treatment at 4 h, but not at 24 h. The level of inflammatory cell infiltration assessed by immunochemical staining of Ly6G and MAC-1 was significantly attenuated at 4 h by APC treatment, while at 24 h it was significantly increased, showing no significant difference between the two groups. In contrast, necrotic area was significantly attenuated at 24 h by APC treatment. Taken together, these results suggest that APC treatment for IRI in nonsteatotic (normal) liver attenuated initial liver damage by inhibiting inflammatory cell infiltration, which in turn significantly reduces necrosis at 24 h.

In contrast, in steatotic liver, APC treatment did not attenuate transaminase levels at 4 h, which were markedly increased and significantly higher than those in nonsteatotic liver, although it did attenuate the levels at 24 h. Histological assessment showed that APC treatment reduced necrotic area at 24 h, although it did not affect the modified Suzuki score at 4 h or 24 h. The number of inflammatory cells infiltrating, which was not significantly reduced at 4 h by APC treatment, was significantly increased at 24 h in both groups but this effect was significantly attenuated by APC treatment. These results show that APC treatment for IRI in steatotic liver does not affect initial liver damage, but it may attenuate late damage, suggesting that APC might act through a pathway in addition to an anti-inflammatory cytoprotective effect. In previous reports, the presence of macrosteatosis significantly increased the incidence in primary graft dysfunction [24, 25] and decreased patient survival after orthotopic liver transplantation. Moreover, livers with macrovesicular fat deposition have lower tolerance to IRI compared with microsteatotic livers [26, 27]. Our steatotic mice had significantly increased IRI, shown by higher transaminase levels and disrupted liver histology compared with nonsteatotic liver, although serum aminotransferase levels and liver histological outcomes were improved by APC treatment in both nonsteatotic and steatotic mice. Particularly when we focused on the time point of 24 h, post-IRI livers of the steatotic mice showed prolonged parenchymal injury, compared with nonsteatotic mice whose liver injury was already settled down from IRI.

APC treatment in nonsteatotic liver prevented sinusoidal endothelial injury at 4 h and at 24 h, as shown by the expression of PECAM-1 (which is a major constituent of the endothelial cell intercellular junctions and a negative regulator of inflammatory responses [28, 29]). However, APC treatment in steatotic liver did not show any effect on injury until 24 h. APC provides a direct cytoprotective effect on sinusoidal endothelial cells through PAR1 signaling via EPCR-bound APC activation [11]. When we compared the structure of endothelial cells positively stained by PECAM-1 between nonsteatotic and steatotic livers (as shown in

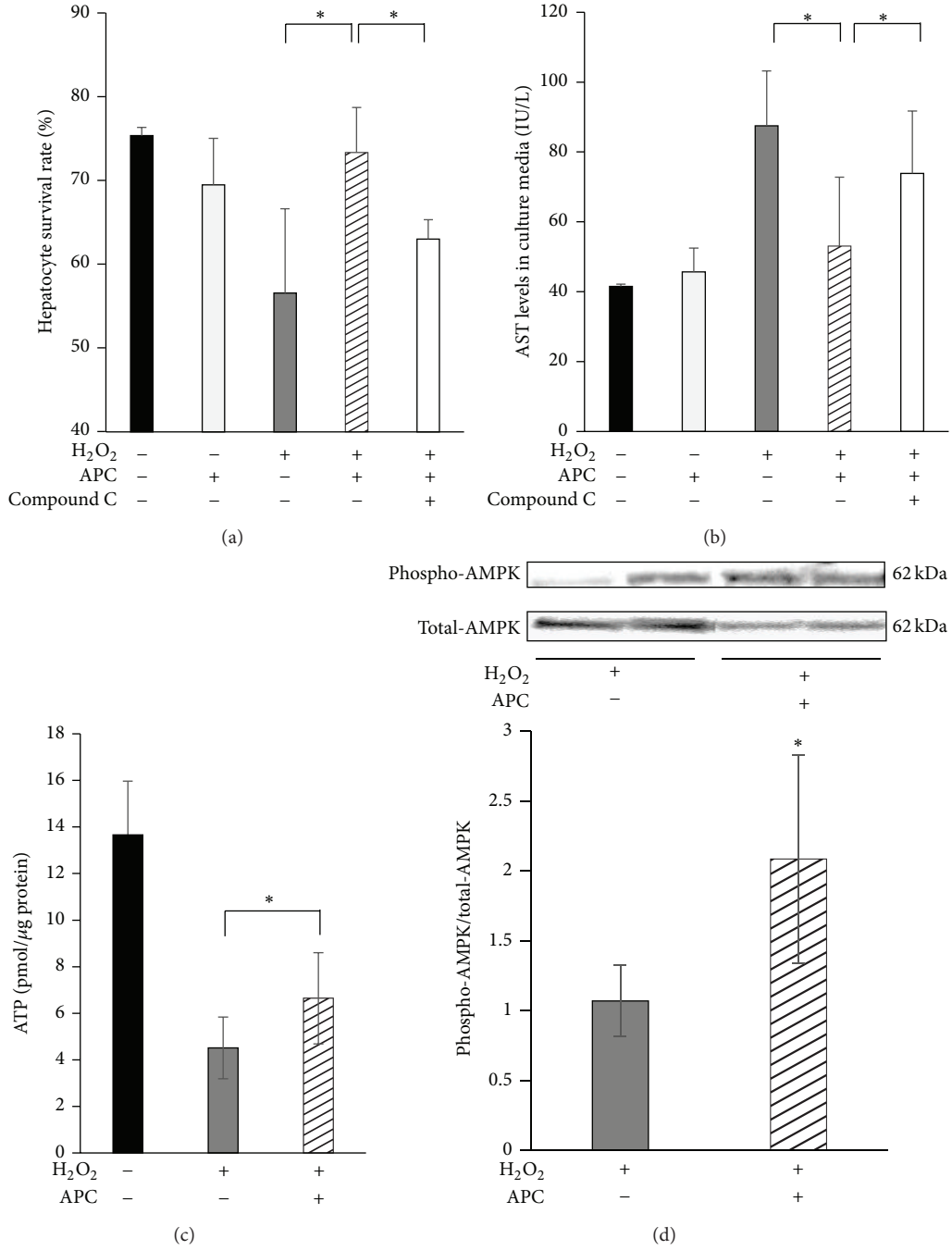

Figure 7: Direct effects of APC on steatotic hepatocytes in vitro. In cells treated with H_2O_2 + APC, the survival rates of steatotic hepatocytes were significantly increased compared with those of cells treated with H_2O_2 alone ($^*P < 0.05$). By adding an inhibitor of AMPK (compound C), the survival rate was significantly decreased ($^*P < 0.05$) (a). Cell cytotoxicity, which was calculated by the AST level in the culture media, was significantly decreased in the H_2O_2 + APC treatment, compared with H_2O_2 treatment alone. By adding compound C, the AST levels were significantly increased (b). APC increased the ATP concentration in hepatocytes which were cultured for 24 h with H_2O_2 (c). Representative western blots for phospho-AMPK (p-AMPK) and total-AMPK (t-AMPK) in hepatocytes which were cultured for 24 h with H_2O_2 are shown; APC increased AMPK phosphorylation ($^*P < 0.05$) (d).

Figures 4(a) and 4(c)), sinusoidal endothelial structures were severely disrupted regardless of the APC treatment in steatotic liver, while these structures in nonsteatotic liver were well preserved even in mice without APC treatment. Consequently, we propose that APC treatment could not exert a cytoprotective effect on endothelial vasculature in steatotic liver because it was too severely damaged.

Recently, Wang et al. demonstrated that APC strongly attenuated acute myocardial injury by activating AMPK, an effect that was largely independent of its anticoagulant

function. This was shown in both in vivo and ex vivo mouse model systems: cardiomyocytes expressed EPCR and APC directly triggered AMPK phosphorylation in cardiomyocytes by enhancing the Ca2+/CaMKKβ activity by EPCR- and PAR1-dependent mechanisms [14]. AMPK activates ATP-generating pathways and downregulates ATP-consuming anabolic pathways. We therefore focused on AMPK phosphorylation as a pathway additional to the anti-inflammatory cytoprotective effect of APC. In the present study, APC administration in steatotic liver enhanced its phosphorylation at 4 h, but not at 24 h. In contrast, in nonsteatotic liver, APC administration did not influence AMPK phosphorylation. Histologically, APC treatment did not attenuate liver damage at 4 h, while it significantly reduced the percentage of liver necrosis at 24 h. According to these results, we consider that APC might prevent ATP depletion at 4 h via activation of AMPK phosphorylation, which in turn might partly attenuate the necrosis at 24 h in I/R injury. In our in vitro study, which included a specific inhibitor of AMPK (compound C), the survival rates of steatotic hepatocytes were significantly increased when APC was added in the culture media, while steatotic hepatocyte survival rates were significantly decreased when compound C was added in the culture media containing APC. Nevertheless, in the in vivo study, APC administration significantly attenuated serum transaminase levels, liver histological damage, and leukocyte recruitment in nonsteatotic liver at 4 h and in steatotic liver at 24 h. Therefore, we propose that the main pathway through which APC exerts its cytoprotective effect is the suppression of neutrophil recruitment, although we believe that APC might also exert cytoprotective effects via activation (phosphorylation) of AMPK in steatotic liver.

Target receptors of APC that might directly act on hepatocytes are still unknown. There have been several reports demonstrating that there are receptors of APC other than EPCR, such as the sphingosine-1-phosphate receptor 1 in intestinal epithelial cells [30] and lung epithelial cells [31], several integrins in macrophages [32], PAR3 in neural cells [33], and the apolipoprotein E receptor 2 in platelets [34]. Further study is required to elucidate which of these specific receptors could be the targets of APC in steatotic hepatocytes.

In conclusion, during IRI in normal liver, APC treatment attenuated initial liver damage by inhibiting inflammatory cells infiltration and sinusoidal endothelial injury, while under IRI in steatotic liver APC did not affect initial liver damage but attenuated late damage, suggesting the existence of a pathway additional to the anti-inflammatory cytoprotective effect of APC which may be via activation of AMPK.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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