Estrategias para proteger el injerto esteatósico en el trasplante hepático

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Anexos

Los artículos originados directamente a partir de la presente Tesis son los siguientes:

- **Casillas-Ramírez A**, Mosbah IB, Ramalho F, Roselló-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. Life Sci. 2006;79(20):1881-1894.

- Alfany-Fernandez I*, **Casillas-Ramirez A***, Bintanel-Morcillo M, Brosnihan KB, Ferrario CM, Serafin A, Rimola A, Rodés J, Roselló-Catafau J, Peralta C. Therapeutic targets in liver transplantation: angiotensin II in nonsteatotic grafts and angiotensin-(1-7) in steatotic grafts. Am J Transplant. 2009;9(3):439-451.
  *Ambos autores contribuyeron igualmente al artículo.

- **Casillas-Ramírez A**, Alfany-Fernández I, Massip-Salcedo M, Juan ME, Planas JM, Serafín A, Pallàs M, Rimola A, Rodés J, Peralta C. Retinol-binding protein 4 and peroxisome proliferator-activated receptor-γ in steatotic liver transplantation. J Pharmacol Exp Ther. 2011;338(1):143-153.

A continuación se adjunta también la lista de artículos relacionados estrechamente con el tema de esta Tesis:

- Jimenez-Castro MB*, **Casillas-Ramirez A***, Massip-Salcedo M, Elias-Miro M, Serafin, A, Rimola A, Rodes J, Peralta C. Cyclic adenosine 3',5'-monophosphate in rat steatotic liver transplantation. Liver Transpl. 2011;17(9):1099-1110.
  *Ambos autores contribuyeron igualmente al artículo.

- Zaouali MA, Padrissa-Altés S, Ben Mosbah I, Alfany-Fernandez I, Massip-Salcedo M, **Casillas-Ramirez A**, Bintanel-Morcillo M, Boillot O, Serafin A, Rimola A, Rodés J, Roselló-Catafau J, Peralta C. Improved rat steatotic and nonsteatotic liver preservation by the addition of epidermal growth factor and insulin-like growth factor-I to University of Wisconsin solution. Liver Transpl. 2010;16(9):1098-1111.

- **Casillas-Ramírez A**, Zaouali A, Padrissa-Altés S, Ben Mosbah I, Pertosa A, Alfany-Fernández I, Bintanel-Morcillo M, Xaus C, Rimola A, Rodés J, Roselló-Catafau J, Peralta C. Insulin-like growth factor and epidermal growth factor treatment: new approaches to protecting steatotic livers against ischemia-reperfusion injury. Endocrinology. 2009;150(7):3153-3161.

- Ramalho FS, Alfany-Fernandez I, **Casillas-Ramirez A**, Massip-Salcedo M, Serafín A, Rimola A, Arroyo V, Rodés J, Roselló-Catafau J, Peralta C. Are angiotensin II receptor antagonists useful strategies in steatotic and nonsteatotic livers in conditions of partial hepatectomy under ischemia-reperfusion? J Pharmacol Exp Ther. 2009;329(1):130-140.

- **Casillas-Ramirez A**, Amine-Zaouali M, Massip-Salcedo M, Padrissa-Altés S, Bintanel-Morcillo M, Ramalho F, Serafín A, Rimola A, Arroyo V, Rodés J, Roselló-Catafau J, Peralta C. Inhibition of angiotensin II action protects rat steatotic livers against ischemia-reperfusion injury. Crit Care Med. 2008;36(4):1256-1266.

- Massip-Salcedo M, Zaouali MA, Padrissa-Altés S, **Casillas-Ramirez A**, Rodés J, Roselló-Catafau J, Peralta C. Activation of peroxisome proliferator-activated receptor-alpha inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion. Hepatology. 2008;47(2):461-472.

- **Casillas-Ramírez A**, Mosbah IB, Franco-Gou R, Rimola A, Roselló-Catafau J, Peralta C. [Ischemia-reperfusion syndrome associated with liver transplantation: an update]. Gastroenterol Hepatol. 2006;29(5):306-313.

- Massip-Salcedo M*, **Casillas-Ramirez A***, Franco-Gou R, Bartrons R, Ben Mosbah I, Serafin A, Roselló-Catafau J, Peralta C. Heat shock proteins and mitogen-activated protein kinases in steatotic livers undergoing ischemia-reperfusion: some answers. Am J Pathol. 2006;168(5):1474-1485.
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- Ben Mosbah I, **Casillas-Ramírez A**, Xaus C, Serafín A, Roselló-Catafau J, Peralta C. Trimetazidine: is it a promising drug for use in steatotic grafts? World J Gastroenterol. 2006;12(6):908-914.

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Artículos originados directamente a partir de la presente Tesis

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Minireview

Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation

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Abstract

Ischemia-reperfusion (I/R) injury associated with liver transplantation remains a serious complication in clinical practice, in spite of several attempts to solve the problem. The present review focuses on the complexity of I/R injury, summarizing conflicting results obtained from the literature about the mechanisms responsible for it. We also review the therapeutic strategies designed in past years to reduce I/R injury, attempting to explain why most of them have not been applied clinically. These strategies include improvements in pharmacological treatments, modifications of University of Wisconsin (UW) preservation solution based on a variety of additives, and gene therapy. Finally, we will consider new potential protective strategies using trimetazidine, 5-amino-4-imidazole carboxamide riboside (AICAR), melatonin, modulators of the renin-angiotensin system (RAS) and the phosphatidylinositol-3-OH kinase (PI3K)-Akt and the p42/p44 extracellular signal-regulated kinases (Erk 1/2) pathway. These strategies have shown promising results for I/R injury but have not been tested in experimental liver transplantation to date. Moreover, we will review ischemic preconditioning, taking into account the recent clinical studies that suggest that this surgical strategy could be appropriate for liver transplantation.

Keywords: Ischemia-reperfusion; Liver transplantation; Pharmacological strategies; Preservation solutions; Gene therapy; Ischemic preconditioning

Contents

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Introduction

Liver transplantation (LT) dates back to 1963, when Thomas Starzl carried out the first transplant on a child suffering from biliary atresia. Although LT provides effective therapy for most forms of acute and chronic liver failure, ischemia-reperfusion (I/R) injury, inherent in every LT, is the main cause of both initial poor function and primary non-function of liver allograft. The latter is responsible for 81% of re-transplantations during the first week after surgery (Clavien et al., 1992; Shaw, 1995; Jaeschke, 1996).

The shortage of organs has led centers to expand their criteria for the acceptance of marginal donors (Busuttil and Tanaka, 2003). Some of these include the use of organs from aged donors, non-heart-beating donors (NHBD), and grafts such as small-for-size or steatotic livers. However, I/R injury is the underpinning of graft dysfunction that is seen in the marginal organ (Busuttil and Tanaka, 2003). Donor age of more than 70 years was found to be associated with lower patient and graft survival. Additionally these donors also have an increased incidence of steatosis, which may potentiate cold preservation injury (Busuttil and Tanaka, 2003).

The fundamental problem with NHBD organs is the prolonged warm ischemia before cold preservation (Reddy et al., 2004). Controlled NHBDs provide organs that are far less prone to ischemic damage and tend to offer superior posttransplant function (Busuttil and Tanaka, 2003). The use of uncontrolled NHBDs is associated with a very high risk of primary nonfunction (Reddy et al., 2004).

One of the benefits of reduced-size grafts from living donors is a graft of good quality with a short ischemic time, this latter being possible because live donor procurements can be electively timed with recipient procedure (Farmer et al., 2001). On the other hand, the major concern over application of living-related liver transplantation (LRLT) for adults is graft-size disparity. The small graft needs posterior regeneration to restore the liver/body ratio. It is well known that I/R significantly reduces liver regeneration after hepatectomy (Franco-Gou et al., 2004). Moreover, increased rates of primary non-function have been reported when using donor livers with moderate steatosis compared with non-steatotic livers. As such, hepatic steatosis is the major cause of rejected grafts for LT and exacerbates the organ shortage problem (Selzner and Clavien, 2001). Therefore, minimizing the adverse effects of I/R injury could increase the number of both suitable transplantation grafts and of patients who successfully recover from LT. The first step towards achieving this objective is a full understanding of the mechanisms involved in I/R injury.

Complexity of I/R injury

A large number of factors and mediators play a part in liver I/R injury (Fan et al., 1999; Lentsch et al., 2000; Serracino-Inglott et al., 2001; Jaeschke, 2003; Banga et al., 2005). The relationships between the signalling pathways involved are highly complex and it is not yet possible to describe, with absolute certainty, the events that occur between the beginning of reperfusion and the final outcome of either poor function or a non-functional liver graft. Fig. 1 shows some of the mechanisms involved in the pathophysiology of I/R injury. The present review will focus on different results from the literature about possible sources for reactive oxygen species (ROS), nitric oxide (NO) effects and mechanisms, and the role of some proinflammatory mediators such as tumour necrosis factor-α (TNFα), and transcription factors, for example, nuclear factor kappaB (NFκB). These data will provide a better understanding of why hepatic I/R injury remains an unresolved problem in clinical practice.

Mechanisms responsible for ROS production

As regards the mechanisms responsible for ROS production, experiments with xanthine/xanthine oxidase (X/XOD) inhibitors such as allopurinol suggest that this system is the main ROS generator in hepatocytes (Adkison et al., 1986; Hamer et al., 1995; Elion et al., 1966) and it has also been implicated in LT-related lung damage (Fernandez et al., 2002). However, results obtained in experimental models of the isolated perfused liver have underestimated the importance of the X/XOD system, and suggest that mitochondria could be the main source of ROS (Jaeschke and Mitchell, 1989). On the other hand, some data challenge the pathophysiological relevance of intracellular oxidant stress during reperfusion (Jaeschke et al., 1988; Metzger et al., 1988; Jaeschke, 1991a; Grattagliano et al., 1999). Grattagliano et al. (1999) demonstrated that mitochondria do not seem to actively participate in the reperfusion-induced oxidative stress. In addition, studies by Jaeschke et al. and Metzger et al. showed that the increased vascular oxidant stress after 30 and 60 min of ischemia was attenuated by inactivation of Kupffer cells (KC) but not by high dose of allopurinol (Metzger et al., 1988; Metzger and Lauterburg, 1988; Jaeschke, 1991b; Jaeschke and Farhood, 1991). Similarly, different studies have shown that either neutrophils or KC are the predominant source of ROS (Jaeschke and Farhood, 1991; Jaeschke et al., 1992). This latter hypothesis has also been an object of controversy. The elimination of KC did not modify the deleterious effects of I/R and the activation of neutrophils is

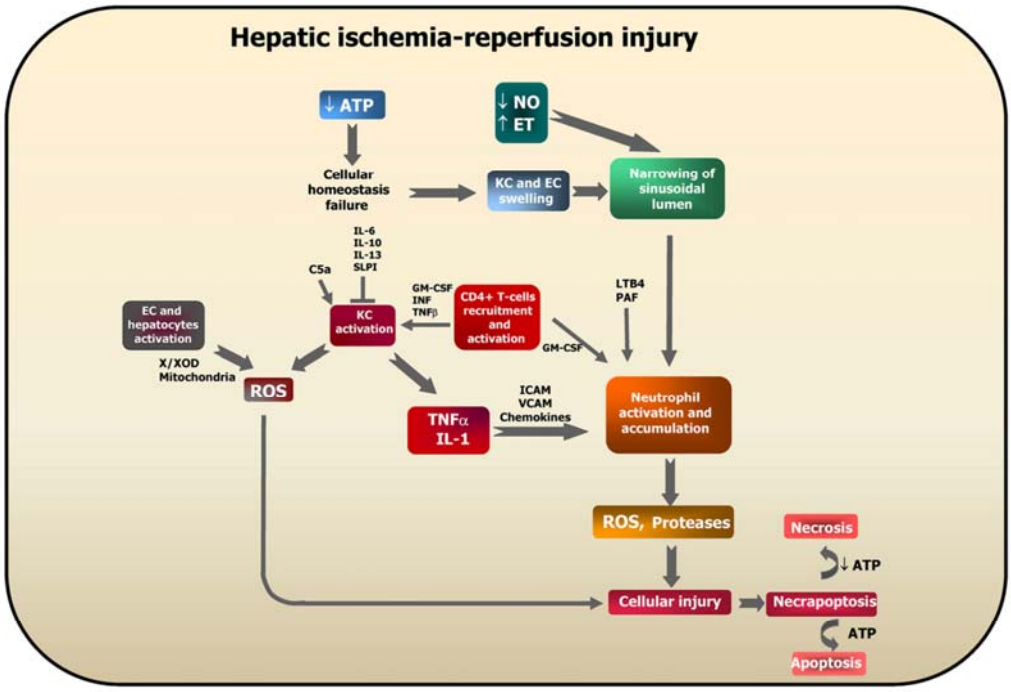

Fig. 1. Some of the mechanisms involved in the pathophysiology of ischemia-reperfusion injury. Ischemia-induced energy deficiency results in the failure of active transmembrane transport and consequently in endothelial cell (EC) and Kupffer cell (KC) swelling. This fact together with the imbalance between NO and endothelin (ET) production contributes to the narrowing of sinusoidal lumen. This leads to both accumulation of neutrophils and decreased perfusion. KC activation results in ROS, TNFα and interleukin 1 (IL-1) release. In addition ROS may derive from xanthine/xanthine oxidase (X/XOD) and mitochondria. Cytokine release throughout the induction of adhesion molecules (ICAM and vascular cell adhesion molecule [VCAM]) and CXC chemoxine leads to neutrophil activation and accumulation. These neutrophils then extravasate, causing parenchymal injury by ROS and production of proteases. In the same way, activation of complement factor C5a primes and activates KC; resident and newly accumulated CD^{4+} T-lymphocytes in the liver can produce granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (INF) and tumor necrosis factor beta (TNF-β), which amplify KC activation and promote neutrophil recruitment into the liver; platelet activating factor (PAF) can prime neutrophils for generation of superoxide whereas leukotriene B4 (LTB4) may contribute to the amplification of the neutrophil response. Additionally, anti-inflammatory factors such as interleukin-6 (IL-6), interleukin-10 (IL-10), IL-13 and secretory leukocyte protease inhibitor (SLPI) attenuate reperfusion injury. Finally, the necroapoptosis theory postulates that a process begins with a common death signal that culminates in either necrotic cell death or apoptosis, depending on the decline of cellular ATP.

not essential for reoxygenation injury (Imamura et al., 1995; Teoh and Farell, 2003).

Clearly, then, there is a range of potentially conflicting results with regard to the mechanisms responsible for ROS generation in liver I/R injury. For instance, in our opinion, in order to clarify the importance of X/XOD vs mitochondria it should be taken into account that there are differences in the experimental models evaluated, including the times of ischemia. In this line, X/XOD play a crucial role in hepatic I/R injury only in conditions in which significant conversion of XDH to XOD occurs (80–90% of XOD) such as 16 h of cold ischemia. However, this ROS generation system does not appear to be crucial at shorter ischemic periods such as 6 h of cold ischemia (Fernandez et al., 2002; Jaeschke, 2002). Thus, even after prolonged periods of ischemia, where a significant conversion of XDH to the XOD occurs, this enzyme may only play a minor role compared with mitochondria (Jaeschke and Mitchell, 1989; Jaeschke, 2002).

In addition, the drugs used for inhibiting X/XOD should be considered, since, for example allopurinol, seems to have more than one mechanisms of action. It is not only a potent inhibitor of XOD, but it may also improve ischemia-induced mitochondrial dysfunction (Elion et al., 1966; Jeon et al., 2001). In fact, evidence for reduced mitochondrial dysfunction after high doses of allopurinol was shown in a warm hepatic I/R model (Jeon et al., 2001).

Similarly, in assessing the relative contribution of intracellular vs vascular oxidant stress to hepatic I/R injury, it should also be noted that oxidative stress in hepatocytes and the stimulatory state of KC after I/R depend on the duration of ischemia, and may also differ between ischemia at 4 °C and that at 37 °C (Mochida et al., 1994), which probably leads to different developmental mechanisms of liver damage.

Mediators and transcription factors in I/R injury

Nitric oxide

It is difficult to distinguish between beneficial and harmful mediators in I/R injury. Some authors have found that NO exerts a beneficial effect on I/R injury in different organs, tissues and cells,

whereas other studies report no effect or even a deleterious action of NO (Peralta et al., 2001). In our opinion, in addition to the differences in animal species, experimental models of hepatic I/R tested, and the dose and timing of administration of the different pharmacological modulators of NO, these differential effects of NO could be explained, at least partially, by the different source of NO.

In this context, some studies suggest that although endothelial NO synthase (eNOS)-derived NO production is protective in I/R, inducible NO synthase (iNOS)-derived NO production may contribute to I/R injury. This may be a function of the NO generation kinetics of the two isoforms in I/R. The basal, low-level NO generation by the constitutively expressed eNOS isoform may abrogate the microcirculatory stresses of engraftment and reperfusion. In contrast, iNOS-derived NO cannot be generated until several hours after stimulation because of requirements for transcriptional induction of this isoform. Excess NO production may no longer be of microcirculatory benefit at this later time (Shah and Kamath, 2003). Furthermore, the excessive levels of iNOS-derived NO production may be detrimental through the generation of NOS-derived superoxide production or the generation of peroxynitrite (Billiar, 1995). Additionally, whether NO is cytoprotective or cytotoxic in hepatic I/R injury may be determined at apoptosis. For example, NO may promote apoptosis by inducing cytochrome *c* release and caspase activation (Chung et al., 2001). However, NO may also upregulate the anti-apoptotic protein Bcl-2 (Genaro et al., 1995).

In addition, to understand the different results in relation with the action mechanisms of NO, it is important to clarify whether the NO source is endogenous or exogenous. In this regard, although the beneficial role of endogenous NO could be related to an attenuation of leukocyte accumulation, the exogenous supplementation of NO did not modify this parameter but was associated with an inhibition of endothelin release (Peralta et al., 2001).

TNF and NFκB

Differential effects of NO mentioned above have also been reported for other mediators involved in hepatic I/R injury. According to the cell type and experimental or pathologic conditions, tumour necrosis factor (TNF) is protective or injurious to the liver in the context of I/R injury. TNF may stimulate cell death or it may induce hepatoprotective effects mediated by antioxidant, anti-apoptotic, and other anti-stress mediators coupled with a pro-proliferative biologic response (Bradham et al., 1998; Aggarwal, 2000; Diehl, 2000). For example, although the deleterious effect of TNF in local and systemic damage associated with hepatic I/R is well established (Peralta et al., 1999b), this mediator is also a key factor in hepatic regeneration (Teoh et al., 2003), an important process in reduced-size LT (paediatric and living-related LT). Pretreatment with low-dose TNFα was also highly protective against hepatic I/R injury (Teoh et al., 2003).

These differential effects observed for TNF can also be extrapolated to transcription factors. It is well known that NFκB can regulate various downstream pathways and thus has the potential to be both pro- and anti-apoptotic. Currently it is not clear whether the beneficial effects of NFκB activation in protection against apoptosis or its detrimental proinflammatory role predominate in liver I/R (Fan et al., 1999). Hepatic neutrophil recruitment and hepatocellular injury are significantly reduced when NFκB activation is suppressed in mice following partial hepatic I/R (Yoshidome et al., 1999). However, NFκB activation is essential for hepatic regeneration after rat LT, and reduces apoptosis and hepatic I/R injury (Bradham et al., 1999).

To understand the role of NFκB in the context of hepatic I/R, is important to consider the differences in animal species used; for instance, mechanisms of protection from apoptosis might be different in rats and mice (Chaisson et al., 2002). In addition, the experimental design used to evaluate the role of this transcription factor may also be important. Thus, some studies using adenoviral vector containing a repressor to prevent NFκB activation may not accurately reflect the role of NFκB signalling in regenerating liver because adenoviral vectors themselves cause increased TNF levels, DNA synthesis, and apoptosis in the liver before partial hepatectomy (Lieber et al., 1997; Iimuro et al., 1998).

Neutrophil accumulation

Activation of neutrophils has been implicated in the hepatic microvascular dysfunction and parenchymal damage associated with I/R (Cutrin et al., 2002). Still, a controversial topic is the question of how neutrophils actually accumulate in the liver. The classical theory argues that the increased expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin plays a key role in neutrophil accumulation and the subsequent liver damage associated with I/R (Cutrin et al., 2002; Banga et al., 2005). In contrast, it has also been reported that neutrophil accumulation observed in the liver following I/R is not dependent on the upregulation of either ICAM-1 or P-selectin (Peralta et al., 2001a).

To explain the results that neutrophil accumulation is not dependent on adhesion molecules, we subscribe to the theory proposed by Jaeschke (2003). This argues that although P-selectin and ICAM-1 appear to be relevant for neutrophil adherence in postsinusoidal venules (Vollmar et al., 1995; Essani et al., 1998), the neutrophils relevant for the injury accumulate in sinusoids, which were identified as the dominant sites for neutrophil extravasation (Chosay et al., 1997). In these capillaries, neutrophil sequestration does not depend on B2 integrins or on ICAM-1 or selectins (Vollmar et al., 1995; Jaeschke et al., 1996; Essani et al., 1998). Thus, mechanical factors such as active vasoconstriction, vascular lining cell swelling and injury, and reduced membrane flexibility after activation of the neutrophil, appear to be involved in trapping of these leukocytes in sinusoids (Jaeschke et al., 1996; McCuskey et al., 1996; Jaeschke and Smith, 1997).

The extensive vascular injury during reperfusion eliminates, in part, the sinusoidal endothelial cell (EC) barrier and the neutrophil has direct access to hepatocytes (McKeown et al., 1988; Caldwell-Kenkel et al., 1991; Jaeschke, 2003). Nevertheless, even with damaged but still present EC, transmigration may still be required (Jaeschke, 1998). As a consequence, I/R injury is only moderately or not at all attenuated by anti-ICAM therapies (Farhood et al., 1995; Vollmar et al., 1995; Nishimura et al., 1996; Rentsch et al., 2000). In regard to the role of P-selectin, sinusoidal EC neither contain Weibel Palade bodies nor do they transcriptionally upregulate relevant levels of P-selectin (Essani et al., 1998).

However, during I/R, a number of interventions directed against selectins reduced hepatic neutrophil accumulation and cell injury (Garcia-Criado et al., 1995; Yadav et al., 1999; Martinez-Mier et al., 2000; Amersi et al., 2001). Because these findings cannot be explained by the prevention of P-selectin-dependent rolling in sinusoids, it has been suggested that most liver I/R models include some degree of intestinal ischemia (Kubes et al., 2002), which leads to neutrophil accumulation in remote organs including the liver (Horie et al., 1996). Thus the lower number of neutrophils in the liver when selectins are blocked may be a secondary effect due to the protection of antiselectin therapy against intestinal reperfusion injury (Kubes et al., 2002).

Cell death

A controversy has emerged over the past years as to whether necrotic or apoptotic cell death accounts for the severe parenchymal injury observed during hepatic reperfusion. Some investigators reported that the greatest part of parenchymal injury is caused by massive necrotic alterations. In contrast, others showed that specific inhibition of apoptosis significantly prevented parenchymal injury and improved animal survival after prolonged periods of ischemia (Selzner et al., 2003a). Although it has long been assumed that necrosis and apoptosis are different processes this may not actually be the case. First we briefly review some basic background information on cell death signalling pathways in hepatocytes in order to understand the shared pathway that leads to both necrosis and apoptosis.

Apoptosis occurs through two main pathways. The first, referred to as the intrinsic (mitochondrial) pathway, is typically activated by a variety of stressors such as DNA damage, p53 activation, growth factor deprivation, metabolic disturbances (Yin and Ding, 2003; Malhi et al., 2006). The second is the extrinsic pathway that is triggered through death receptors (Malhi et al., 2006). It is well known that one of the most important regulators of intrinsic pathway is the Bcl-2 family of proteins (Ghobrial et al., 2005) (Fig. 2). The Bcl-2 family includes pro-apoptotic members such as Bax, Bak, Bad, Bid and anti-apoptotic members such Bcl-2, Bcl-Xl and Bcl-W (Reed, 1994). Following death signal, pro-apoptotic proteins undergo posttranslational modifications resulting in their activation and translocation to the mitochondria (Scorrano and Korsmeyer, 2003). Then, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome *c*, which promotes caspase 9 activation, which then activates caspase 3 and the final stages of apoptosis (Ghobrial et al., 2005).

In the extrinsic pathway, a variety of mediators, including TNFα, Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) first bind to their respective death receptors, which cause receptor oligomerization and the association of various adapter proteins, including Fas-associated death domain, TNFα receptor-associated death domain, and TNFα receptor-associated factor. Fas-associated death domain and TNFα receptor-associated death domain promote binding of procaspase 8 and its proteolytic activation to catalytic caspase 8. If sufficient amounts of caspase 8 are generated at the receptor, caspase 8 can directly activate procaspase 3 (Scaffidi et al., 1998). In hepatocytes the caspase 8 interacts with the intrinsic pathway and cleaves Bid, a BH3 only pro-apoptotic Bcl2 family member, to a truncated form, tBid. tBid translocates to mitochondria, causing mitochondrial permeabilization and release of mitochondrial effectors of apoptosis, such cytochrome *c* (Yin, 2000; Ding and Yin, 2004).

The mechanisms that induce the release of mitochondrial intermembrane proteins such as cytochrome *c* remain controversial (Jaeschke and Lemasters, 2003). In hepatocytes TNFα-and Fas-dependent signalling induces the onset of the mitochondrial permeability transition (MPT). The MPT occurs from the opening of a pore in the inner membrane, the permeability transition pore (Bernardi, 1999). MPT leads to large-amplitude mitochondrial swelling, rupture of the outer membrane, and release of cytochrome *c* and other proteins from the intermembrane mitochondrial space (Jaeschke and Lemasters, 2003).

Other mechanisms for cytochrome *c* release also seem to exist. In some models, tBid interaction with either Bax or Bak, forms channels in the mitochondrial outer membrane that release cytochrome *c* and other proteins from the intermembrane space (Korsmeyer et al., 2000; Shimizu et al., 2000; Cande et al., 2002). If MPT onset occurs in relatively few mitochondria, the organelles become sequestered into autophagosomes for lysosomal digestion, a process that eliminates the damaged and potentially toxic mitochondria (Elmore et al., 2001; Jaeschke and Lemasters, 2003). When the MPT involves more mitochondria, mitochondrial swelling leads to outer membrane rupture and cytochrome *c* release. Provided that adenosine triphosphate (ATP) is available from glycolysis and still-intact mitochondria, cytochrome *c* activate downstream caspases and other executioner enzymes of apoptosis. When MPT onset is abrupt and involves most mitochondria, ATP becomes profoundly depleted, which blocks caspase activation. Instead, ATP depletion culminates with plasma membrane rupture and the onset of necrotic cell death (Leist et al., 1997; Jaeschke and Lemasters, 2003; Paxian et al., 2003) (see Fig. 2). Hence, the new term "necrapoptosis" has been coined to describe a process that begins with a common death signal and which culminates in either cell lysis (necrotic cell death) or programmed cellular resorption (apoptosis), depending on factors such as the decline of cellular ATP levels (Lemasters, 1999).

Strategies designed in past years to prevent hepatic I/R injury

Despite improvements in pharmacological treatments, preservation solutions and gene therapy aimed at reducing hepatic I/R injury, the results to date have not been conclusive. Fig. 3 shows some of the therapeutic strategies developed to prevent I/R injury.

Pharmacological treatment

Numerous experimental studies have focused on inhibiting the harmful effects of I/R-associated inflammatory response. In this respect, drugs such as chloroquine (Kayawake et al., 1982) and chlorpromazine (Chien et al., 1978; Churchill et al., 1995) have been administered in order to prevent mitochondrial dysfunction and loss of liver cell phospholipids during hepatic ischemia. Antioxidant therapy using either tocopherol (Soltys et

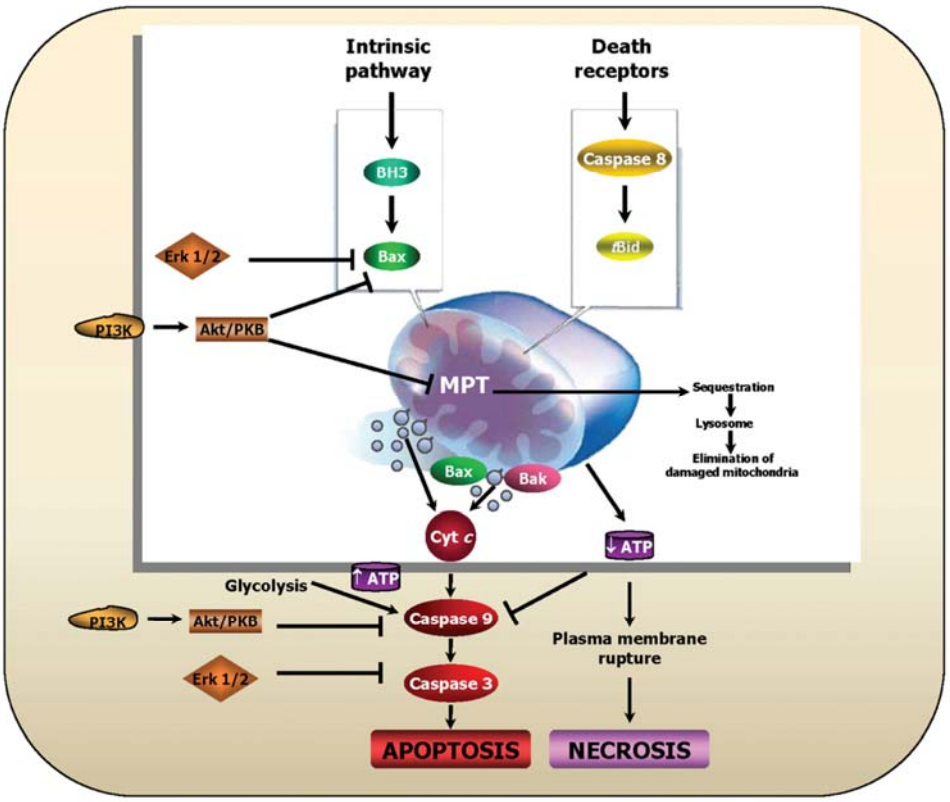

Fig. 2. Necrosis and apoptosis in hepatic ischemia-reperfusion. Activation of intrinsic and death receptor-dependent (extrinsic) pathways converge on mitochondria to induce membrane permeabilization. BH3 only Bcl-2 family members, including tBid formed by death receptor-linked caspase 8 activation, cause Bax/Bak dependent permeabilization of the outer membrane. Permeabilization may involve formation of channels in the outer membrane or induction of mitochondrial permeability transition (MPT) followed by mitochondrial swelling and outer membrane rupture. After membrane permeabilization, cytochrome *c* is released to the cytosol and activates in sequence caspase 9 and caspase 3 in a reaction requiring ATP. If MPT onset is abrupt and involves most mitochondria, ATP become profoundly depleted, which blocks caspase activation. Instead ATP depletion culminates with plasma membrane rupture and the onset of necrotic cell death. If MPT onset occurs in relatively few mitochondria, the organelles become sequestered for lysosomal digestion. The prosurvival PI3K-Akt and Erk 1/2 kinase cascades has shown anti-apoptotic mechanisms in the heart. Signalling through these pathways results in: inactivation of caspases 3 and 9, which inhibits apoptosis; inactivation of the pro-apoptotic protein Bax, one consequence of which is to prevent the release of mitochondrial cytochrome *c*; and inhibition of MPT; among other mechanisms.

al., 2001), glutathione (GSH) ester (Peralta et al., 2002), or allopurinol (Fernandez et al., 2002) has been applied in an attempt to inhibit ROS effects in reperfusion, and anti-TNF antiserum pre-treatment (Colletti et al., 1990) has also been employed to block the damaging effects of this cytokine. Therapies with dopamine (Hasselgren, 1987) or ATP-$MgCl_2$ (Chaudry, 1989) have been administered to reduce hepatic I/R injury-related microcirculatory disorders. Drugs such as adenosine (Peralta et al., 1997), NO donors (Serafin et al., 2002), L-arginine (Koti et al., 2002), and anti-ICAM-1 (Farhood et al., 1995) and anti-P-selectin (Peralta et al., 2001a) antibodies have been used to inhibit neutrophil accumulation. However, none of these treatments has managed to prevent hepatic I/R injury.

The difficulty of blocking the inflammation related to this process must be taken into account because, among other factors, many mediators and cell types are involved in this kind of inflammatory response. Pharmacological treatment-derived difficulties must also be considered. In this regard, GSH ester does not reach its site of action at the optimum concentration or at the right moment (Polyak et al., 2000). The administration of anti-TNF antibodies does not effectively protect against hepatic I/R injury, and this finding has been related to the failure of complete TNFα neutralization locally (Peralta et al., 2001a). Small changes in the dose of NO donors produce totally opposite effects (Peralta et al., 2001). In addition, possible drug side effects should not be ruled out, and, for example, pernicious systemic effects for dopamine (Shimada et al., 1999), adenosine (Casella et al., 2004) and NO donors (Lamarque and Whittle, 1995) have been described.

Although this also occurs in non-steatotic livers, modulating I/R injury in steatotic livers poses a greater problem. This latter type of liver produces ROS that are insensitive to the effects of antioxidants such as superoxide dismutase (SOD) and catalase (Yang et al., 2000). Differences in the action mechanisms between steatotic and non-steatotic livers mean that therapies which are

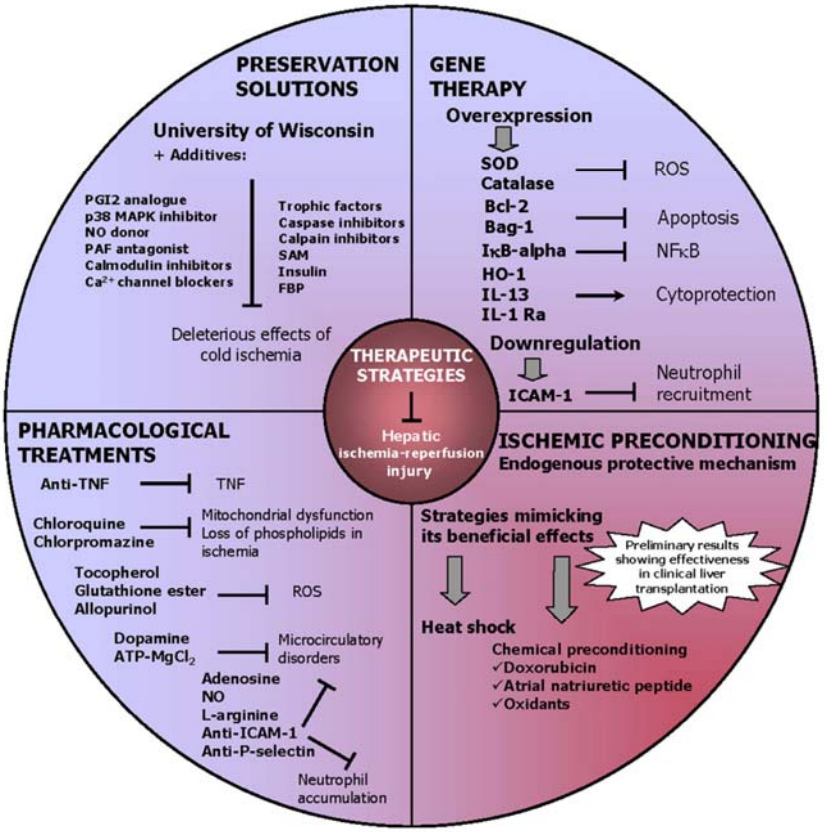

Fig. 3. Therapeutic strategies are developed in past years to prevent damage caused by hepatic ischemia-reperfusion injury. A variety of ingredients such as stable PGI2 analogue, p38 MAPK inhibitor, NO donor, PAF antagonist, calmodulin inhibitors, Ca^{2+} channel blockers, trophic factors, caspase or calpain inhibitors, SAM, insulin, or FBP were introduced into UW solution to reduce hepatic cold ischemia injury. Gene therapy based on overexpression of SOD, catalase, Bcl-2, Bag-1, a mutant inhibitor of IκBalpha, HO-1, IL-13 and IL-1Ra, or downregulation of ICAM-1 expression have also been made to ameliorate reperfusion injury. Pharmacological treatments such as anti-TNF antiserum, chloroquine, chlorpromazine, antioxidant therapy using either tocopherol, glutathione ester or allopurinol, dopamine, ATP-$MgCl_2$, adenosine, NO donors, L-arginine, anti-ICAM-1 and anti-P-selectin have been also tested. Strategies capable of mimicking benefits of ischemic preconditioning, such as heat shock preconditioning and chemical preconditioning with either doxorubicin, atrial natriuretic peptide or oxidants have been evaluated. None of these treatments have been applied in clinical practice. However, recent clinical reports suggest the effectiveness of ischemic preconditioning in liver transplantation.

effective in non-steatotic livers may prove useless in the presence of steatosis, and the effective drug dose may differ between the two liver types. Findings such as these must be taken into consideration when applying pharmacological strategies in the same way to steatotic and non-steatotic livers, because the effects may be very different. For instance, whereas in an LT experimental model a NO donor reduced oxidative stress in non-steatotic livers, the same dose increased vulnerability of steatotic grafts to I/R injury (Carrasco-Chaumel et al., 2005).

Furthermore, there may be drugs that would only be effective in steatotic livers. In the context of LT, steatotic donors have been reported to show a higher content of mitochondrial uncoupling protein-2 (UCP-2) and a reduced ability to synthesize ATP upon reperfusion, thus leading to increased mortality following I/R (Cheng et al., 2003). Hence, compounds such as cerulenin that reduce UCP-2 expression in steatotic livers, offer protection as a result of increased availability of ATP prior to I/R (Chavin et al., 2004). However, this strategy may be ineffective in non-steatotic livers because the latter do not show an overexpression of UCP-2 (Chavin et al., 1999). Similar results have been obtained with carnitine administration (Tolba et al., 2003; Yonezawa et al., 2005).

Preservation solutions

Since its introduction by Belzer et al. in the late 1980s, the University of Wisconsin (UW) solution has become the standard solution for the preservation of most organs in transplantation (Vajdova et al., 2002; Busuttil and Tanaka, 2003). The inclusion of some components in the UW solution has been both advocated and criticized. For instance, adenosine has been added to UW solution as a substrate for the regeneration of adenine nucleotides. However, simplified variants of UW solution in which adenosine was omitted were shown to have similar or even higher protective

potential during cold liver storage (Vajdova et al., 2002). The colloid hydroxyethyl starch (HES) included in UW preservation solution prevents interstitial edema (Ar'Rajab et al., 1991) but produces extended and accelerated aggregation of erythrocytes that may result in stasis of blood and incomplete washout of donor organs before transplantation (Morariu et al., 2003). Another limitation of the UW solution is that some of its constituent compounds (allopurinol, lactobionate) do not offer very good protection because they are not present at a suitable concentration and encounter problems in reaching their site of action. Indeed, studies in humans have suggested that the allopurinol in the UW preservation solution was unable to prevent the subsequent X/XOD-derived superoxide radical production during reperfusion (Pesonen et al., 1998).

A variety of ingredients such as the stable prostacyclin (PGI2) analogue OP-4183 (Goto et al., 1993), the p38 mitogen-activated protein kinase (MAPK) inhibitor FR167653 (Yoshinari et al., 2001), the NO donor sodium nitroprusside (Rodriguez et al., 1999), the platelet-activating factor (PAF) antagonist E5880 (Takada et al., 1995), calmodulin inhibitors (Anaise et al., 1990), Ca^{2+} channel blockers such as nisoldipine (Takei et al., 1990; Vajdova et al., 2002), trophic factors (Ambiru et al., 2004), caspase or calpain inhibitors, S-adenosylmethionine (SAM) (Vajdova et al., 2002), insulin (Li et al., 2003), or fructose-1,6-biphosphate (FBP) (Moresco et al., 2004) were introduced into UW preservation solution, with promising results. However, none of these modifications to UW solution composition have found their way into routine clinical practice. For instance, studies aimed at enrichment of UW solution with caspase inhibitors showed that this prevents sinusoidal endothelial cells apoptosis (Vajdova et al., 2002), but it has also been demonstrated that such inhibitors have little effect on necrosis, and this could mean no protection in the steatotic liver where the predominant form of cell death is necrosis (Selzner et al., 2003a). Along this line, addition of precursors for ATP resynthesis such as SAM only resulted in a poor initial ATP recovery during liver reperfusion (Vajdova et al., 2002). Insulin and FBP were recommended and added to UW preservation solution with the aim of stimulating gycolysis and modulating KC activity, respectively. However, further studies showed that these modifications in UW solution may exacerbate graft ischemic injury and decrease the graft survival rate in rat LT (Li et al., 2003; Moresco et al., 2004).

Gene therapy

Advances in molecular biology provide new opportunities to reduce liver I/R injury by using gene therapy. To suppress the ROS burst, SOD and catalase have been transfected by either adenovirus, liposomes or polyethyleneglycol (Fan et al., 1999; Mari et al., 2002; Selzner et al., 2003a). To inhibit apoptosis, overexpression of Bag-1 and Bcl-2, mainly by using adenovirus, has been tested (Selzner et al., 2003a). To limit neutrophil recruitment and activation, reduction in ICAM-1 expression was obtained by using liposomes (Sonnenday et al., 2004). Cytoprotective strategies based on expression of genes such as heme oxygenase-1 (HO-1), anti-inflammatory cytokine interleukin-13 (IL-13) and interleukin-1 receptor antagonist (IL-1Ra) have been developed employing adenoviral or liposome vector (Coito et al., 2002; Harada et al., 2002; Ke et al., 2003; Pachori et al., 2004). Attempts have also been made to modulate the NFκB effect through adenoviral transfection of a mutant inhibitor of kappaB-alpha (IκBalpha), which would inhibit NFκB and ameliorate the hepatic inflammatory response to I/R (Fan et al., 1999; Okaya and Lentsch, 2005). However, there are a number of problems inherent in gene therapy, for example, vector toxicity, difficulties in increasing transfection efficiencies and protein expression at the appropriate time and site, and the problem of obtaining adequate mutants (in the case of NFκB) due to controversy about NFκB activation (Somia and Verma, 2000; Wheeler et al., 2001; Chaisson et al., 2002).

New potential protective strategies

The present communication will now center on emerging protective strategies such as enrichments of UW solution and pharmacological treatments with favorable results in I/R injury but that up to now have not been evaluated in experimental LT. Moreover, we will discuss ischemic preconditioning taking into account the novel clinical reports that suggest the effectiveness of this surgical procedure in LT.

Pharmacological treatments and preservation solutions

Trimetazidine

Trimetazidine (TMZ), which has been used as an anti-ischemic drug in the heart for over 35 years (Veitch et al., 1995; Ikizler et al., 2003) reduced liver injury and improved liver regeneration and survival rate in an experimental model of partial hepatectomy under hepatic blood inflow occlusion (Kaya et al., 2003). Studies examining the underlying protective mechanisms of TMZ suggest that mitochondria, energy metabolism, oxidative stress and microcirculation might be important targets through which TMZ exerts its cytoprotective effect (Guarnieri and Muscari, 1993; Hauet et al., 1998; Elimadi et al., 2001; Ikizler et al., 2003). Interestingly, severe mitochondrial damage (Rashid et al., 1999; Caraceni et al., 2004), decreased ATP level (Selzner et al., 2003b; Caraceni et al., 2004), increased ROS production (Serafin et al., 2002, 2004b), and impaired microcirculation (Teramoto et al., 1993; Hakamada et al., 1997) have also been proposed as factors that might leave steatotic livers vulnerable to I/R injury. A recent study indicated the benefits of TMZ in orthotopic heart transplantation performed in pigs, when TMZ was added to the cardioplegic solution (Castedo et al., 2005). Taking these observations into account TMZ could be used as a pharmacological treatment in vivo as well as a new additive to UW solution for liver preservation.

AICAR

Studies carried out in past years showed that 5-amino-4-imidazole carboxamide riboside (AICAR) administered as pharmacological treatment improved the ability of the heart to recover from I/R and also added to the cardioplegic solution (Galinanes et al., 1992, 1995). Recently, evidence has shown that AICAR is a useful pharmacologic treatment for protection against hepatic

injury in both steatotic and non-steatotic liver transplantation. Benefits from AICAR were linked to an increment of NO synthesis which, in turn, reduced oxidative stress (Carrasco-Chaumel et al., 2005). If AICAR can be equally protective in LT when it is used as an additive in UW solution, this could be a promising strategy.

Modulators of the renin-angiotensin system

Recent research has suggested an important role for the renin-angiotensin system (RAS), known for its regulation of blood pressure and fluid homeostasis, in both I/R injury and liver regeneration after partial hepatectomy (Takada et al., 2001; Ramalho et al., 2002; Guo et al., 2004). Furthermore, angiotensin-converting enzyme (ACE) inhibitors (captopril and enalapril) and angiotensin II (Ang-II) type 1 receptor blockers (losartan and candesartan) were able to downregulate cellular adhesion molecules (e.g., ICAM-1), inhibit the synthesis of proinflammatory cytokines and chemokines [(e.g., TNFα, cytokine-induced neutrophil chemoattractant-1 (CINC-1)] and reduce I/R injury (Anthuber et al., 1997; Araya et al., 2002; Guo et al., 2004). In addition, ACE inhibitors (lisinopril, captopril and enalaprilat) showed a potent stimulating effect on hepatocellular proliferation during liver regeneration after experimental partial hepatectomy (Ramalho et al., 2002). It was recently reported that candesartan, a potent and long-lasting Ang-II type 1 receptor antagonist, induces upregulation of plasma concentration of the hepatocyte growth factor (HGF), the most potent mitogen for mature hepatocytes (Matsumoto and Nakamura, 1996; Araya et al., 2002). Further research is therefore needed to establish whether RAS modulation can be as advantageous in small grafts for LT which require a process of hepatic regeneration.

Modulators of PI3K-Akt and Erk 1/2 pathway

Trophic factors such as insulin-like growth factor (IGF), cardiotrophin-1 and fibroblast growth factor (FGF) have been shown to protect against I/R injury in the heart through the activation of phosphatidylinositol-3-OH kinase (PI3K)-Akt and p42/p44 extracellular signal-regulated kinases (Erk 1/2) (Brar et al., 2001; Yamashita et al., 2001; Buehler et al., 2002). This pathway has been implicated in cellular survival, through recruitment of anti-apoptotic protection pathways (Cross et al., 2000). In the heart, PI3K-Akt has been shown to increase NO, inhibit opening of the MPT pore, and activate protein kinase C (PKC) and mitochondrial Raf-1, which has been shown to phosphorylate and inactivate the pro-apoptotic factor, Bad (Hausenloy and Yellon, 2004). Activation of either the PI3K-Akt or the Erk 1/2 pathway inhibits the conformational change in Bax required for its translocation to the mitochondria (Yamaguchi and Wang, 2001; Tsuruta et al., 2002; Weston et al., 2003). Moreover Erk 1/2 kinase activation has been shown to inhibit apoptosis, by inhibiting caspase 3 activation (Terada et al., 2000) and Akt activation can suppress the mitochondrial apoptotic death pathway by inactivating caspase 9 (Cardone et al., 1998) (Fig. 2). Interestingly, PI3K-Akt is a cell signalling mechanism also involved in the benefits of liver ischemic preconditioning in isolated hepatocytes (Carini and Albano, 2003). The modulation of therapeutic targets such as the anti-apoptotic pro-survival PI3K-Akt and Erk 1/2 kinase cascades could open new perspectives for limiting I/R injury associated with LT.

Surgical strategies

Ischemic preconditioning (IP) consists of a brief period of ischemia followed by a short interval of reperfusion prior to a prolonged ischemic stress (Peralta et al., 1997). Vasoactive substances such as adenosine, NO, bradykinin, etc., have been considered the major players in triggering preconditioning (Cutrin et al., 2002). In addition to the extracellular mediators, IP involves activation of intracellular messengers such as PKC, AMP-activated protein kinase (AMPK), p38 MAPK, Ik kinase; signal transducer and activator of transcription-3 (STAT3) and transcription factors including NFκB and heat shock transcription factor 1 (HSF1) (Carini and Albano, 2003; Selzner et al., 2003a). The downstream consequences of these pathways could be cytoprotective by abrogation of cell death pathways, stimulating antioxidant and other cellular protective mechanisms including MnSOD and heat shock proteins (HSPs), and by initiating entry into the cell cycle (Cutrin et al., 2002; Selzner et al., 2003a). The benefits of IP on energy metabolism, inflammatory mediators including ROS and TNF, mitochondrial dysfunction, KC activation, and microcirculatory disorders associated with I/R injury have also been described (Glanemann et al., 2003; Kim et al., 2004; Serafin et al., 2004a; Banga et al., 2005).

Since the effectiveness of IP was first described, numerous efforts have been made to find strategies capable of mimicking its beneficial effects. One of these strategies is known as heat shock preconditioning, in which the organ or the whole body is temporarily exposed to hyperthermia prior to hepatic ischemia (Matsumoto et al., 2001). Chemical preconditioning with either doxorubicin (Ito et al., 2000), atrial natriuretic peptide (Gerbes et al., 1998) or oxidants (Peralta et al., 1999a) decreases hepatic injury in several experimental models of I/R. However, their possible clinical application seems limited owing to difficulties in implementing them in clinical practice, toxicity problems and the side-effects that have been identified (Fried et al., 1990; Olson and Mushlin, 1990; Peralta et al., 1999a).

To date, IP has been successfully applied in human liver resections in both steatotic and non-steatotic livers. The effectiveness of IP in hepatic surgery was first reported by Clavien et al. (2000, 2003), but unfortunately, in this study, it proved ineffective in elderly patients. It is well known that the impact of cold ischemia on organ function becomes even more significant as the age of the donor increases (Busuttil and Tanaka, 2003). Recent research indicates that melatonin prevents oxidative stress and inflammatory response in hepatocytes from elderly rats and this could improve the viability of liver grafts from elderly donors and increase the effectiveness of IP (Castillo et al., 2005).

A recent clinical study by Koneru and colleagues showed no effects of IP on cadaveric donor livers compared with controls. However, the study consisted of clamping the hepatic vessels for a period of 5 min, and as the authors concluded, that may be insufficient to obtain a beneficial effect from IP (Koneru et al., 2005). Another clinical study carried out by Azoulay and colleagues using the model of cadaveric whole liver transplantation

showed that IP based on 10 min of ischemia was associated with better tolerance to ischemia. However, this was at the price of decreased early function (Azoulay et al., 2005). Early this year, Jassem et al. (2006) concluded that 10 min of preconditioning was effective to protect cadaveric donor allografts from cold ischemia, reduced inflammatory response and resulted in better graft function. Further randomized clinical studies are necessary to confirm whether IP is appropriate for LT in clinical practice. Interestingly, the effectiveness of IP in clinical practice in major liver hepatectomy opens up new possibilities in LRLT, since the ischemia period is similar in both surgical procedures. Moreover, IP increases liver regeneration, the most critical aspect to be considered in LRLT (Franco-Gou et al., 2004).

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Therapeutic Targets in Liver Transplantation: Angiotensin II in Nonsteatotic Grafts and Angiotensin-(1–7) in Steatotic Grafts

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Numerous steatotic livers are discarded as unsuitable for transplantation because of their poor tolerance of ischemia-reperfusion(I/R). The injurious effects of angiotensin (Ang)-II and the benefits of Ang-(1–7) in various pathologies are well documented. We examined the generation of Ang II and Ang-(1–7) in steatotic and nonsteatotic liver grafts from Zucker rats following transplantation. We also studied in both liver grafts the effects of Ang-II receptors antagonists and Ang-(1–7) receptor antagonists on hepatic I/R damage associated with transplantation. Nonsteatotic grafts showed higher Ang II levels than steatotic grafts, whereas steatotic grafts showed higher Ang-(1–7) levels than nonsteatotic grafts. Ang II receptor antagonists protected only nonsteatotic grafts against damage, whereas Ang-(1–7) receptor antagonists were effective only in steatotic grafts. The protection conferred by Ang II receptor antagonists in nonsteatotic grafts was associated with ERK 1/2 overexpression, whereas the beneficial effects of Ang-(1–7) receptor antagonists in steatotic grafts may be mediated by NO inhibition. Our results show that Ang II receptor antagonists are effective only in nonsteatotic liver transplantation and point to a novel therapeutic target in liver transplantation based on Ang-(1–7), which is specific for steatotic liver grafts.

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Introduction

Up to 30% of all livers retrieved for organ transplantation exhibit steatotic transformations (1,2). The increasing demand for organs for transplantation has led to the acceptance of steatotic livers, which have poor tolerance to ischemia-reperfusion (I/R) injury (3,4). The use of these marginal organs for transplantation is associated with increased risk of graft dysfunction or failure after surgery (4). In addition, many steatotic livers are discarded for transplantation, exacerbating the critical shortage of donor livers (3). Therefore, minimizing the adverse effects of I/R in steatotic liver transplantation is an urgent need.

The classical view of the renin-angiotensin system (RAS) is an enzymatic cascade by which angiotensinogen is hydrolyzed to angiotensin-I, which is then converted to angiotensin (Ang)-II by the angiotensin-converting enzyme (ACE) and binds to its receptor subtypes, Ang II type-I receptor (AT1R) and Ang II type-II receptor (AT2R) (5). In addition to the systemic RAS, a local RAS has been identified in the liver, indicating that liver can synthesize Ang II independently of the circulating RAS (6,7). Indeed, both steatotic and nonsteatotic livers generate Ang II under warm I/R conditions and Ang II receptor (ATR) antagonists protect both liver types against I/R injury (7), indicating the injurious effects of Ang II under these conditions. To our knowledge,

no data on the role of ATR antagonists in steatotic liver transplantation have been reported.

However, this classical view has recently been challenged, particularly the main axis (ACE-Ang II-ATR), in which Ang II, ATR and the enzyme ACE have major roles (8). A large body of evidence suggests that Ang-(1–7) is a potent endogenous effector hormone of the RAS (9). This is reinforced by the identification of Mas as a receptor for Ang-(1–7) (10,11). In addition to Ang-(1–7) and its receptor Mas, the recently described homologue of ACE, ACE2 may form the ACE 2-Ang-(1–7)-Mas axis (12,13). This axis induces opposite effects to those elicited by the classical ACE-Ang II-ATR axis (12,14). Ang-(1–7) shows protective effects in myocardial infarction (15), diabetes-induced cardiovascular dysfunctions (16) and hepatic fibrosis (17).

Here we examined the generation of Ang II and Ang-(1–7) in steatotic and nonsteatotic liver grafts from Zucker rats following transplantation. We also studied in both liver grafts the effects of Ang II receptor antagonists and Ang-(1–7) receptor antagonists on hepatic I/R damage associated with transplantation.

Material and Methods

Experimental animals

Homozygous (obese, Ob) and heterozygous (lean, Ln) Zucker rats (Iffa-Credo, L'Abresle, France) aged 10–11 weeks were used (232 rats). Steatosis in Zucker rats is not associated with inflammation. Ob rats showed moderate macrovesicular and microvesicular fatty infiltration into hepatocytes, whereas Ln rats showed no evidence of steatosis (Figure 1). All procedures were performed under isofluorane inhalation anesthesia (18). This study respected European Union regulations (Directive 86/609 EEC) for animal experiments. Animals were randomly distributed into groups as described below.

Experimental design

1) Sham (n = 12 rats): Ln and Ob animals (6 in each group) were subjected to transversal laparotomy, and silk ligatures were applied in the right suprarenal vein, diaphragmatic vein and hepatic artery.
2) Transplantation (TR) (n = 24 rats). This group was divided into two subgroups: 2.1) (n = 12 rats, 6 donor rats and 6 recipient rats, 6 transplantations): Steatotic livers from Ob Zucker rats (n = 6 rats) were flushed and preserved with cold University of Wisconsin (UW) solution for 6 h (18). Standard orthotopic liver transplantation in Ln Zucker rats (n = 6 rats) was performed according to the Kamada cuff technique, without hepatic artery reconstruction (19); 2.2) (n = 12 rats, 6 donor rats and 6 recipient rats, 6 transplantations): Nonsteatotic livers from Ln Zucker rats (n = 6 rats) were flushed and preserved with cold UW solution for 6 h (18). Standard orthotopic liver transplantation in Ln Zucker rats (n = 6 rats) was performed according to the Kamada cuff technique, without hepatic artery reconstruction (19).
3) Transplantation + AT1R antagonist (AT1R antagonist) (n = 24 rats): Same as group 2, but treated with losartan, an AT1R antagonist (5 mg/kg orally) 24 h and 1.5 h before the surgical procedure (7).
4) Transplantation + AT2R antagonist (AT2R antagonist) (n = 24 rats): Same as group 2, but treated with PD123319, an AT2R antagonist (30 mg/kg i.v.) 5 min before the surgical procedure (7).

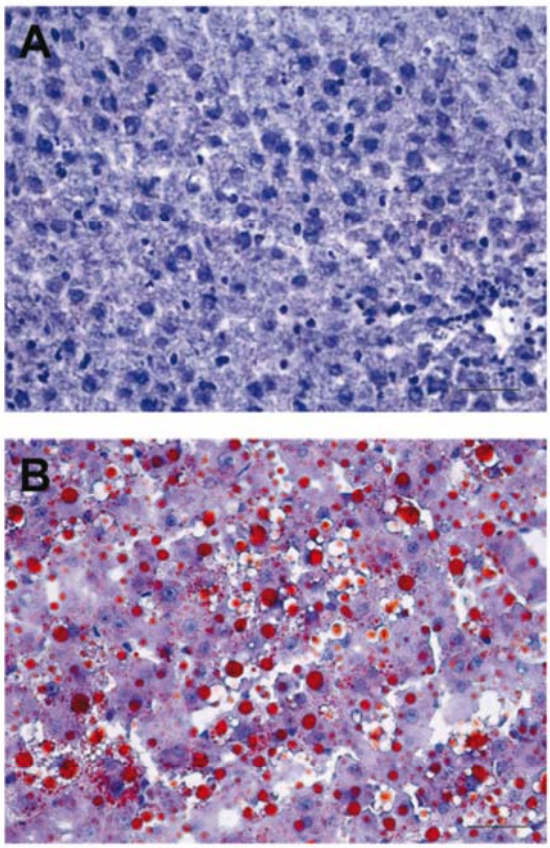

Figure 1: Difference of steatosis in Ob versus Ln Zucker rats shown by red oil staining. (A) Ln rats showed no evidence of steatosis. (B) Ob rats showed fatty infiltration in hepatocytes (bar = 50 μm).

5) Transplantation + Ang-(1–7)-Mas antagonist (Ang-(1–7)-Mas antagonist) (n = 24 rats): Same as group 2, but treated with A-779, an Ang-(1–7) Mas receptor antagonist (48 μg/kg i.v.) 5 min before the surgical procedure (20).
6) Transplantation + AT1R antagonist+ ERK 1/2 inhibitor (AT1R antagonist+ERK 1/2 inhibitor (n = 12 rats): Same as group 2.2, but treated with losartan, an AT1R antagonist (5 mg/kg orally) 24 h and 1.5 h before the surgical procedure (7) and PD98059, an ERK 1/2 inhibitor (4 mg/kg, i.v) 5 min before the surgical procedure (21).
7) Transplantation + AT2R antagonist + ERK 1/2 inhibitor (AT2R antagonist+ERK 1/2 inhibitor (n = 12 rats): Same as group 2.2, but treated with PD123319, an AT2R antagonist (30 mg/kg i.v.) 5 min before the surgical procedure (7) and PD98059, an ERK 1/2 inhibitor (4 mg/kg, i.v) 5 min before the surgical procedure (21).

Plasma and liver samples were collected 4 h after transplantation.

The conditions of this study (including the times of cold ischemia and reperfusion) were established on the basis of the results of previous studies (22,23). A cold ischemic period of 6 h is long enough to induce liver damage

after transplantation in both liver grafts and to allow high survival at 4 h after transplantation (23). Therefore, these experimental conditions were appropriate to evaluate the levels of Ang II and Ang-(1–7) in both liver grafts as well as the mechanisms responsible for the effects of Ang II receptor antagonists and Ang-(1–7) receptor antagonists on hepatic I/R injury associated with transplantation.

To evaluate the effect of Ang II receptor antagonists on survival in recipients transplanted with nonsteatotic liver grafts, the following experimental groups were carried out: (A) recipients transplanted with nonsteatotic liver grafts without any treatment, (B) recipients transplanted with nonsteatotic liver grafts treated with AT1R antagonist and (C) recipients transplanted with nonsteatotic liver grafts treated with AT2R antagonist. For each experimental group mentioned above there were 20 rats, 10 donor rats and 10 recipient rats, 10 transplantations. To evaluate the effect of Ang-(1–7)-Mas antagonist on survival in recipients transplanted with steatotic liver grafts, the following experimental groups were carried out: (D) recipients transplanted with steatotic liver grafts without any treatment and (E) recipients transplanted with steatotic liver grafts treated with Ang-(1–7)-Mas antagonist. For each experimental group mentioned above there were 20 rats, 10 donor rats and 10 recipient rats, 10 transplantations. As previously reported (23), the survival of receptors was monitored for 14 days. Preliminary studies from our group demonstrated that the doses and the pretreatment times of the different drugs used in this study were the most effective for protecting both liver types against damage associated with transplantation. The drugs used in this study did not significantly affect systolic blood pressure, which was measured by a noninvasive tail-cuff method (Pressure Meter LE5001, Panlab, Spain).

Reverse transcription and real-time PCR: Quantitative real-time PCR analysis was performed using the Assays-on-Demand TaqMan probes (Rn00593114_m1 for angiotensinogen, Rn00561094_m1 for ACE and Rn00667869_m1 for β-Actin) (Applied Biosystems, Foster City, CA). The TaqMan gene expression assay was performed according to the manufacturer's protocol (Applied Biosystems).

Western blotting of NOS, ERK 1/2 and ACE2: This was done as described elsewhere (18,24), using the following antibodies: constitutive and inducible nitric oxide synthase (cNOS, iNOS) (Transduction Laboratories, Lexington, KY), total p42/p44 extracellular signal-regulated kinases (ERK 1/2) and phosho-ERK 1/2 (Cell Signaling Technology Inc, Beverly, MA), ACE2 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-Actin (Sigma Chemical, St. Louis, MO).

Biochemical determinations: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Ang I, Ang II, Ang-(1–7), malondialdehyde (MDA), nitrite and nitrate and nitrotyrosine levels (as an index of peroxynitrite) and caspase 3 activity assay (as an index of apoptosis, since caspase 3 participates in the final execution phase of apoptosis) (25) were measured as described elsewhere (7,18,26–28).

Hepatic blood flow measurement

Hepatic tissue blood flow was measured by a laser Doppler flowmeter (LD5000, Transonic Systems, Ithaca, NY) (29). We also measured flow in the hepatic portal vein by an ultrasonic transit time volume flowmeter (T206, Transonic Systems) (30,31).

Histology

To appraise the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method on an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate-to-severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (18,29,32). Steatosis in liver was evaluated by red-oil staining on frozen specimens according to standard procedures.

TUNEL staining

DNA fragmentation was determined using a TUNEL assay in deparaffinized liver samples. We used an *in situ* cell-death detection kit according the manufacturer's instructions (Chemicon, Temecula, CA) (29). TUNEL-positive nuclei were counted (33).

Immunohistochemical location of nitrotyrosine

Immunohistochemical staining for nitrotyrosisne was performed by using the Vectastain Universal Quick kit (Vector Lab, Burlingame, CA) and polyclonal rabbit antisera against nitrotyrosine (Upstate Biotechnology, Lake Placid, NY) (18). To quantify the degree of nitrotyrosine staining, a 0–3 grading system was used: grade 0, minimal or no staining; grade 1, mild staining; grade 2, moderate staining; and grade 3, strong staining (34).

Statistics: Data are expressed as means ± standard deviation and were compared statistically via analysis of variance followed by Student's–Newman–Keuls test. A p-value of <0.05 was considered significant.

Results

Ang II and Ang-(1–7) in nonsteatotic and steatotic liver transplantation

TR significantly increased levels of Ang II precursors (Angiotensinogen mRNA, ACE mRNA and Ang I) in both liver grafts (Figure 2). Similarly, TR significantly increased Ang II levels in both liver grafts, but Ang II levels were markedly higher in nonsteatotic grafts (Figure 3; Ang II values: 24.9 ± 3.7 and 7.77 ± 3.2, in TR of nonsteatotic and steatotic livers, respectively). TR significantly increased Ang-(1–7) and ACE2 levels in both liver grafts, with Ang-(1–7) and ACE2 levels significantly higher in steatotic grafts (Figure 3; Ang-(1–7) values: 6.45 ± 0.5 and 4.29 ± 0.4, in TR of steatotic and nonsteatotic livers, respectively).

Effect of Ang II receptor antagonists and Ang-(1–7) receptor antagonist on hepatic damage in nonsteatotic and steatotic liver transplantation

In AT1R antagonist and AT2R antagonist groups of nonsteatotic grafts, plasma transaminase levels were significantly lower than in the TR group (Figure 4; ALT values: 467.7 ± 138.6, 509.5 ± 168.4 and 1540 ± 291.1, in AT1R antagonist, AT2R antagonist and TR, respectively). However, in Ang(1–7)-Mas antagonist group of nonsteatotic grafts, plasma transaminase levels were similar to those found in the TR group (ALT values: 1434 ± 323.8 and 1540 ± 291.1, in Ang(1–7)-Mas antagonist and TR, respectively). In AT1R antagonist and AT2R antagonist groups of steatotic grafts, plasma transaminase levels were similar to those found in the TR group (Figure 4; ALT values: 3732 ± 707.3, 3363 ± 508.9 and 3708 ± 526.1, in AT1R antagonist, AT2R antagonist and TR, respectively). However, in Ang-(1–7)-Mas antagonist group of steatotic graft, plasma

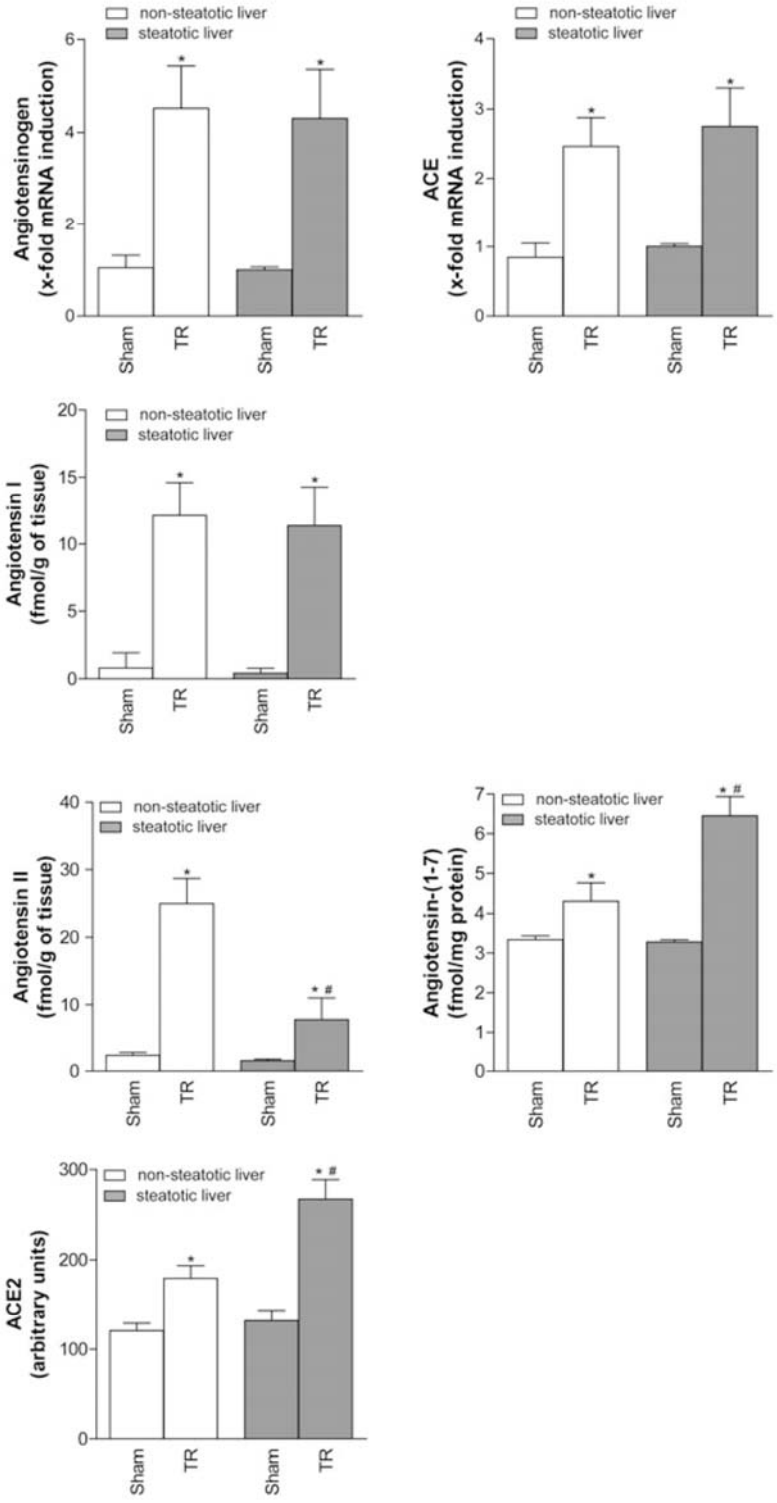

Figure 2: Precursors of Ang II in both liver grafts. Angiotensinogen mRNA expression, ACE mRNA expression and Ang I levels were analyzed in steatotic and nonsteatic grafts. PCR fluorescent signals for angiotensinogen and ACE were standardized to PCR fluorescent signals obtained from an endogenous reference (β-Actin). Comparative and relative quantifications of angiotensinogen and ACE gene products normalized to β-Actin and control Sham group were calculated by the $2^{-\Delta\Delta CT}$ method. *$p < 0.05$ versus sham. Angiotensinogen, ACE and Ang I levels were similar in both liver grafts.

Figure 3: Ang II, Ang(1–7) and Ang-(1–7) enzyme forming, ACE2 in both liver grafts. Ang II levels, Ang-(1–7) levels and ACE2 protein expression were analyzed in steatotic and nonsteatic grafts. *$p < 0.05$ versus sham. Steatotic grafts showed lower Ang II levels and higher Ang-(1–7) and ACE2 levels compared with non-steatotic grafts ($^{\#}p < 0.05$).

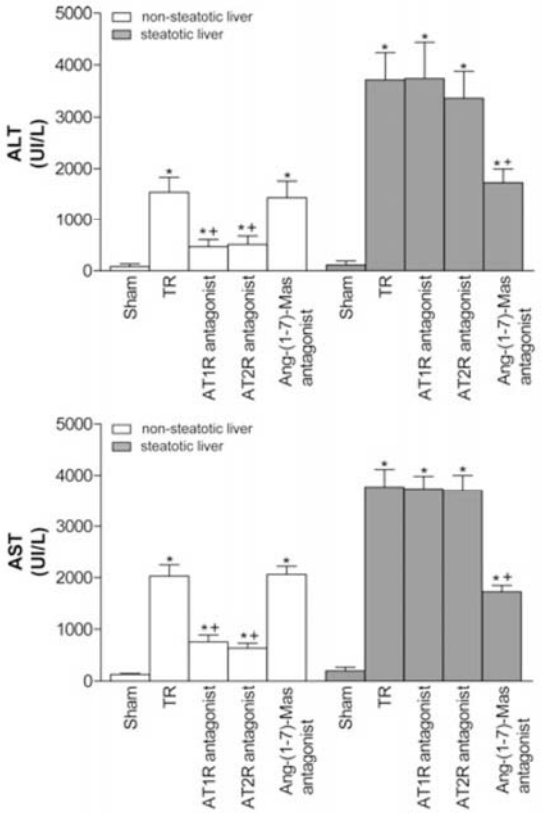

Figure 4: Effect of Ang II receptor antagonists and Ang-(1–7) receptor antagonist on transaminases in nonsteatotic and steatotic liver transplantation. ALT and AST levels were measured in plasma. *p < 0.05 versus sham, +p < 0.05 versus TR.

transaminase levels were significantly lower than in the TR group (Figure 4; ALT values: 1729 ± 262.4 and 3708 ± 526.1, in Ang(1–7)-Mas antagonist and TR, respectively). The histological study of nonsteatotic liver of TR group showed multifocal areas of moderate coagulative necrosis and neutrophil infiltration, randomly distributed throughout the parenchyma (Figure 5A). AT1R antagonist and AT2R antagonist reduced the extent and number of necrotic areas in nonsteatotic livers, since patchy areas of incipient hepatocyte necrosis and scattered areas of coagulative hepatocyte necrosis were observed (Figure 5B and C). This was consistent with damage score values (Figure 5; 0.90 ± 0.4, 0.83 ± 0.4 and 1.84 ± 0.3, in AT1R antagonist, AT2R antagonist and TR, respectively). However, Ang-(1–7)-Mas antagonist led to histological lesions in nonsteatotic livers (Figure 5D) that were similar to those of the TR group (Figure 5A). Damage score values were: 1.82 ± 0.2 and 1.84 ± 0.3, in Ang-(1–7)-Mas antagonist and TR, respectively (Figure 5). The histological study of steatotic livers of TR group showed extensive and confluent areas of severe coagulative necrosis with neutrophil infiltration (Figure 5E). AT1R antagonist and AT2R antagonist led to histological lesions in steatotic livers (Figure 5F, G) that were similar to those of the TR group (Figure 5E). Damage score values were: 2.70 ± 0.4, 2.80 ± 0.3 and 2.83 ± 0.2, in AT1R antagonist, AT2R antagonist and TR, respectively (Figure 5). However, Ang(1–7) Mas antagonist reduced the extent and number of necrotic areas in steatotic livers since multifocal areas of moderate coagulative necrosis with neutrophil infiltration were observed (Figure 5H). Damage score values were: 1.65 ± 0.4 and 2.83 ± 0.2, in Ang(1–7) Mas antagonist and TR, respectively (Figure 5).

The TUNEL assay indicated no apoptosis in steatotic or nonsteatotic grafts undergoing liver transplantation in any group (Figure 6). Caspase 3 activity was at baseline in all groups (Figure 6).

Recipients transplanted with nonsteatotic grafts without any treatment had an 80% survival rate (8 of 10) at 14 days (Figure 7). The treatment with AT1R antagonist or AT2R antagonist in recipients transplanted with nonsteatotic grafts resulted in 100% survival (10 of 10). Recipients transplanted with steatotic grafts without any treatment showed 30% survival (3 of 10) at 14 days, most of the deaths occurring within 2 days. The treatment with Ang-(1–7)-Mas antagonist reduced lethality in recipients transplanted with steatotic grafts, and resulted in a 70% survival rate (7 of 10) at 14 days (Figure 7).

Protective mechanisms of Ang II receptor antagonists in nonsteatotic liver transplantation and Ang-(1–7) receptor antagonist in steatotic liver transplantation

Ang II receptor antagonists in nonsteatotic liver transplantation: With respect to the changes in blood flow revealed using the Laser Doppler flowmeter, the microcirculation in nonsteatotic grafts after transplantation was significantly impaired when compared with the sham group (Figure 8A). Ang II receptor antagonists did not modify hepatic blood flow in nonsteatotic grafts when compared with the TR group. These results were confirmed by measurements of blood flow in the portal vein (Figure 8A). As shown in Figure 8B, phosphorylated ERK 1/2 levels in nonsteatotic grafts of the TR group were of the same order as those of the sham group. However, Ang II receptor antagonist significantly increased phosphorylated ERK 1/2 in nonsteatotic grafts when compared with the TR group (Figure 8B; phosphorylated ERK 1/2 values: 185.3 ± 17.2, 194.6 ± 26.9 and 100.7 ± 20.1, in AT1R antagonist, AT2R antagonist and TR, respectively). Protein levels of total ERK 1/2 were unchanged in all groups (data not shown). Our results indicated that the inhibition of ERK 1/2 abolished the beneficial effects of Ang II receptors antagonists on hepatic damage in nonsteatotic grafts. In fact, AT1R antagonist+ERK 1/2 inhibitor and AT2R antagonist+ERK 1/2 inhibitor resulted in transaminase levels of the same order as those of the TR group. For instance, ALT values

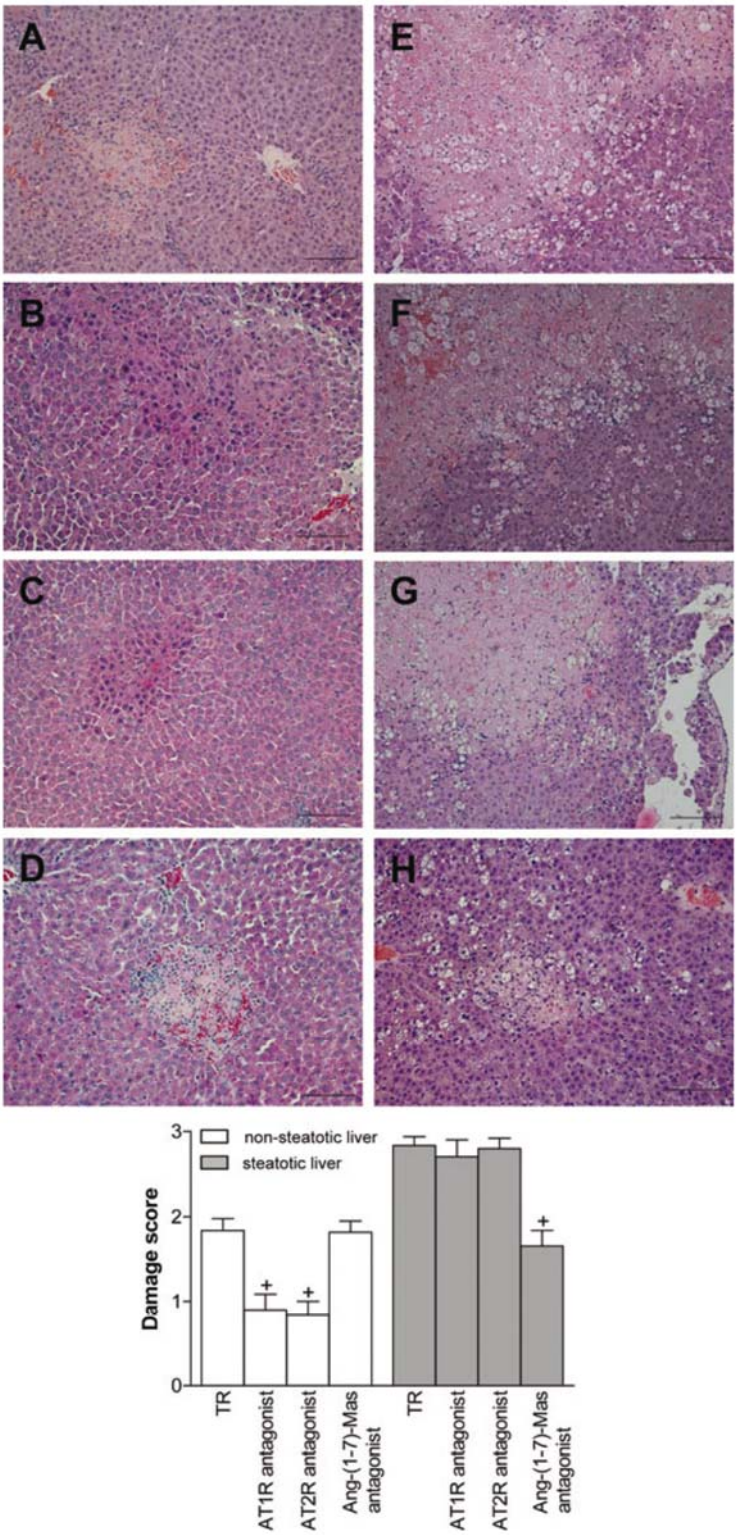

Figure 5: Histological analysis of non-steatic and steatotic liver grafts. (A to H) Representative photographs of histological changes in liver. (A) TR (nonsteatotic graft), small area of coagulative hepatic necrosis with neutrophil infiltration; (B) AT1R antagonist (nonsteatotic graft), irregular area of incipient hepatocyte necrosis; (C) AT2R antagonist (nonsteatotic graft), irregular area of incipient hepatocyte necrosis; (D) Ang(1–7)-Mas antagonist (nonsteatotic graft), hepatic lesions similar to TR; (E) TR (steatotic graft), widespread coagulative hepatic necrosis with neutrophil infiltration; (F) AT1R antagonist (steatotic graft), hepatic lesions similar to TR; (G) AT2R antagonist (steatotic graft), hepatic lesions similar to TR; (H) Ang(1–7)-Mas antagonist (steatotic graft), small area of coagulative hepatic necrosis with neutrophil infiltration (H&E staining, bar = 100 μm). Damage score was performed as described in Material and Methods. $^{+}p < 0.05$ versus TR.

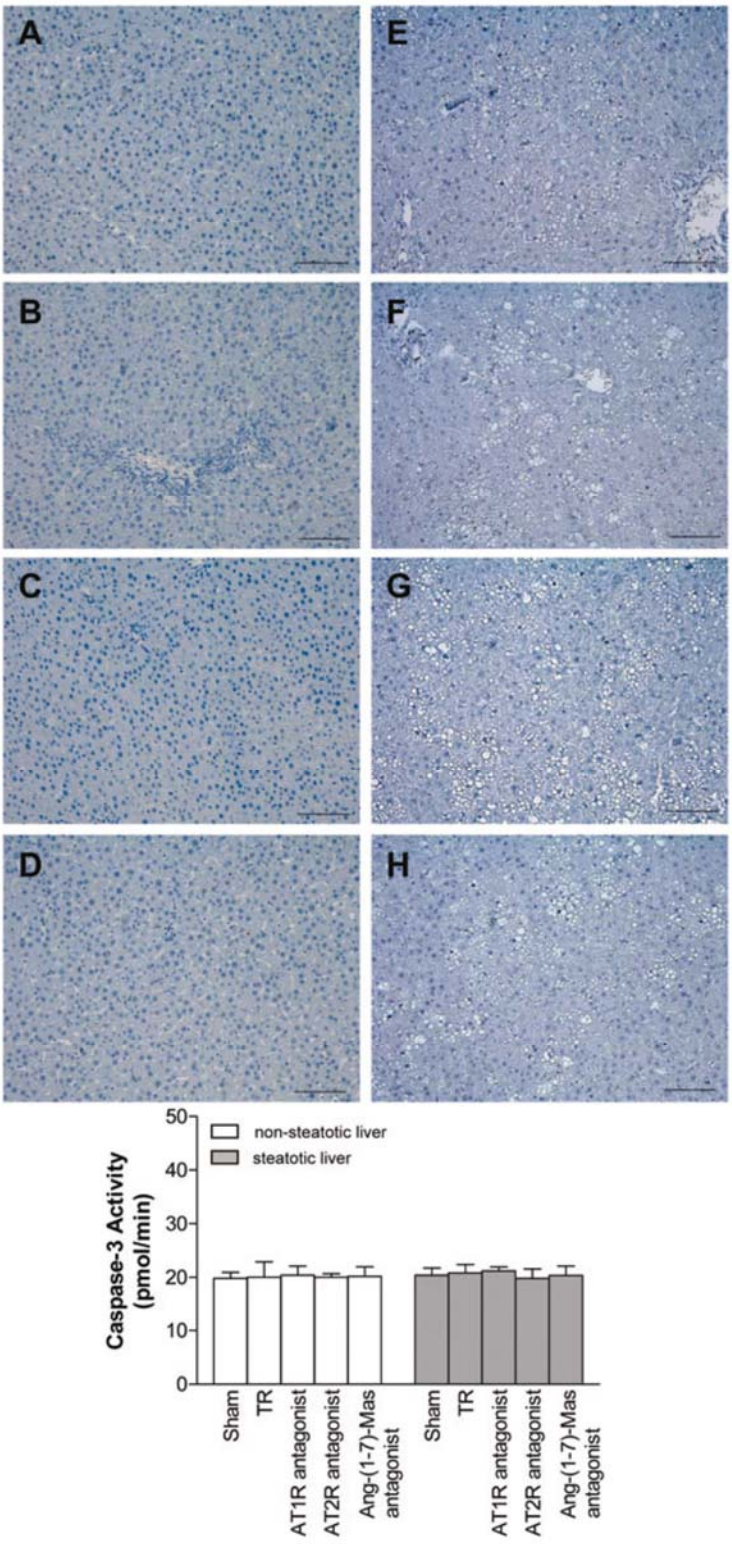

Figure 6: Effect of Ang II receptor antagonists and Ang-(1–7) receptor antagonist on apoptosis in both liver grafts. A to H) Apoptotic hepatic cells identified with the TUNEL assay. (A) TR (nonsteatotic graft), (B) AT1R antagonist (nonsteatotic graft), (C) AT2R antagonist (nonsteatotic graft), (D) Ang(1–7)-Mas antagonist (nonsteatotic graft), (E) TR (steatotic graft), (F) AT1R antagonist (steatotic graft), (G) AT2R antagonist (steatotic graft), (H) Ang(1–7)-Mas antagonist (steatotic graft). No TUNEL-positive cells were observed in any group (bar = 100 μm). Caspase 3 activity levels were analyzed in both liver grafts. No significant differences in caspase 3 activity levels were observed in any group.

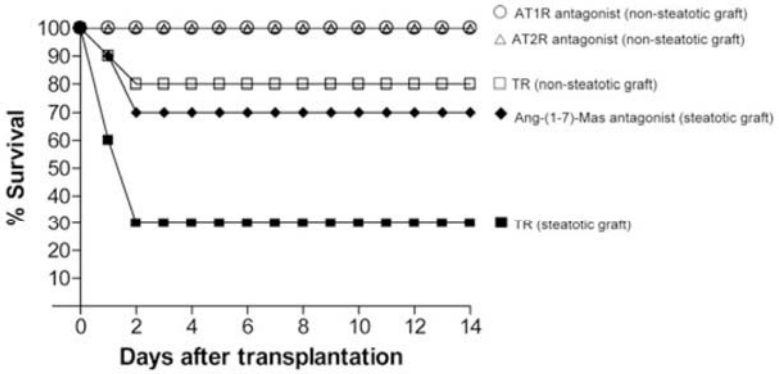

Figure 7: Survival of recipients transplanted with nonsteatotic or steatotic liver grafts at 14 days after transplantation. Recipients transplanted with nonsteatotic liver grafts without treatment (□), recipients transplanted with nonsteatotic liver grafts with AT1R antagonist treatment (○), recipients transplanted with nonsteatotic liver grafts with AT2R antagonist treatment (△), recipients transplanted with steatotic liver grafts without treatment (■), recipients transplanted with steatotic liver grafts with Ang-(1–7) Mas antagonist treatment (♦).

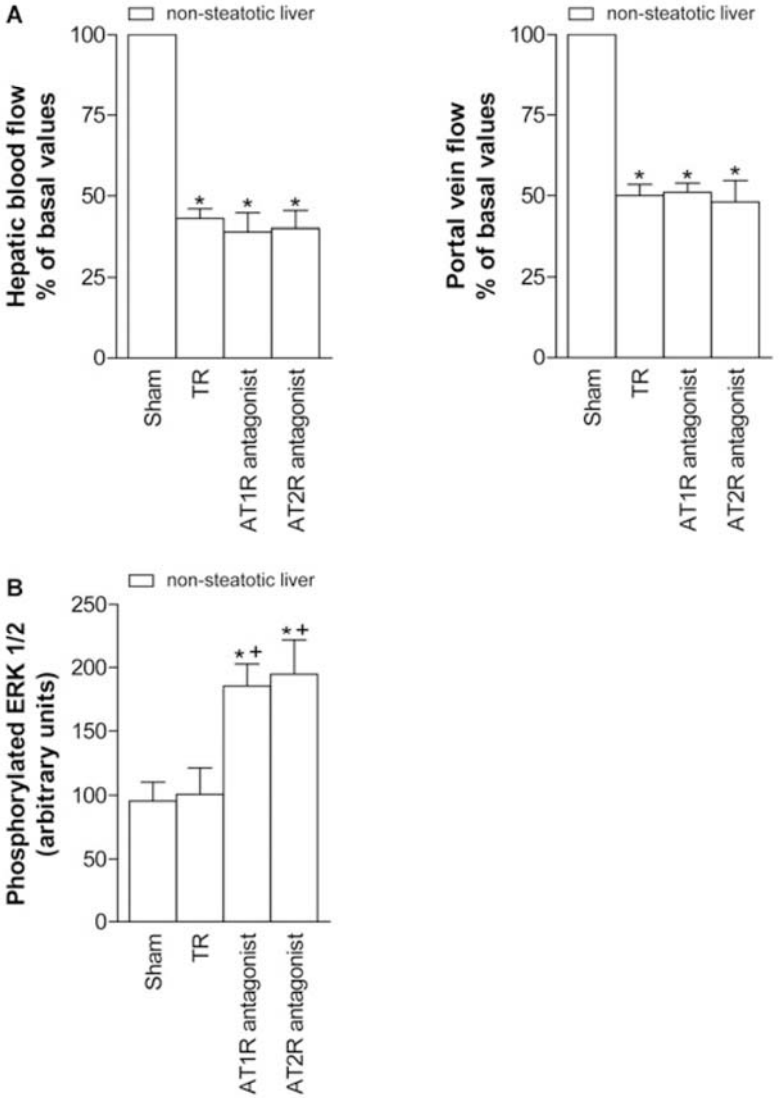

Figure 8: Protective mechanisms of Ang II receptor antagonists in nonsteatotic liver transplantation. (A) Hepatic blood flow and portal vein flow after transplantation of nonsteatotic liver grafts. Hepatic blood flow and portal vein flow are expressed as percentage of basal values. (B) Protein levels of phosphorylated ERK 1/2 were analyzed in nonsteatotic liver grafts. *$p < 0.05$ versus sham, $^{+}p < 0.05$ versus TR.

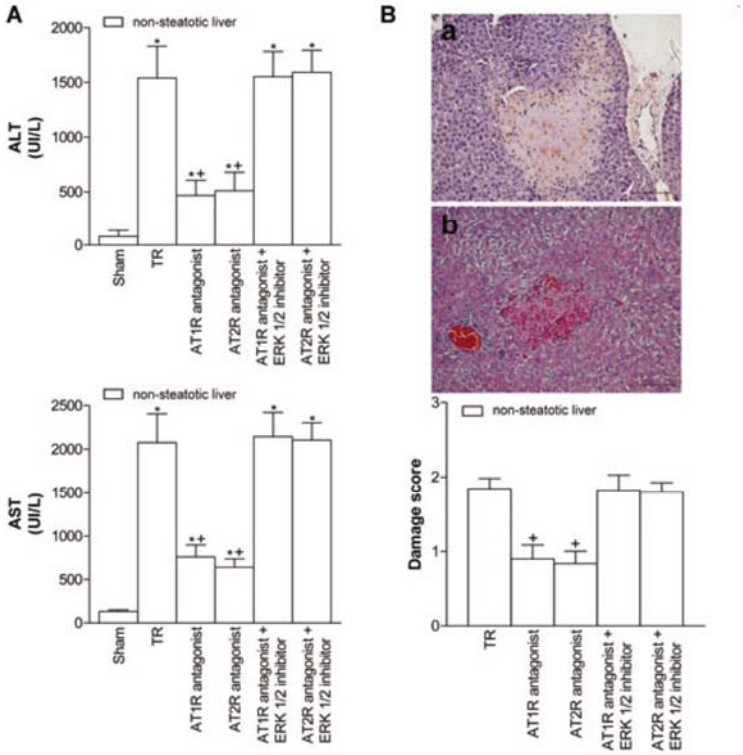

Figure 9: Involvement of ERK 1/2 in the beneficial effects of Ang II receptor antagonists on hepatic damage in nonsteatotic liver transplantation. (A) Transaminase levels. (B) Representative photographs of histological changes in liver and damage score. (a) Nonsteatotic liver grafts corresponding to AT1R antagonist+ERK 1/2 inhibitor and (b) AT2R antagonist+ERK 1/2 inhibitor groups showed small area of coagulative hepatic necrosis with neutrophil infiltration (H&E staining, bar = 100 μm). Damage score was performed as described in Material and Methods. *$p < 0.05$ versus sham, $^{+}p < 0.05$ versus TR.

were 467.7 ± 138.6, 1552 ± 229.9 and 1540 ± 291.1, in AT1R antagonist, AT1R antagonist+ ERK 1/2 inhibitor and TR, respectively (Figure 9A). The histological study on nonsteatotic liver of AT1R antagonist+ERK 1/2 inhibitor and AT2R antagonist+ERK 1/2 inhibitor groups showed moderate and multifocal areas of coagulative necrosis and neutrophil infiltration, randomly distributed throughout the parenchyma (Figure 9B.a and B.b). These histological lesions were similar to those observed in the TR group (Figure 5A). Damage score values were: 0.90 ± 0.4, 1.82 ± 0.5 and 1.84 ± 0.3, in AT1R antagonist, AT1R antagonist+ERK 1/2 inhibitor and TR, respectively (Figure 9B). As seen in nonsteatotic liver grafts corresponding to TR, AT1R antagonist and AT2R antagonist groups (Figure 6), the TUNEL assay indicated no apoptosis in nonsteatotic grafts of AT1R antagonist+ERK 1/2 inhibitor or AT2R antagonist+ERK 1/2 inhibitor groups (Figure 10A and B, respectively). In these groups, caspase 3 activity was at baseline (Figure 10).

Ang-(1–7)receptor antagonist in steatotic liver transplantation: In steatotic grafts, TR significantly increased NO generation when compared with the Sham group (Figure 11; nitrite and nitrate values: 17.68 ± 3.1 and 0.95 ± 0.3, in TR and Sham, respectively). The synthesized NO originates from cNOS (Figure 11) since Western blot did not reveal iNOS changes in any of the study groups (data not shown). As seen in NO generation, TR also significantly increased MDA and nitrotyrosine levels when compared with the sham group, which indicated the presence of peroxynitrite ($ONOO^{-}$) in steatotic livers (Figure 11; nitrotyrosine values: 6.66 ± 0.7 and 1.70 ± 0.2, in TR and Sham, respectively). Ang-(1–7)-Mas antagonist reduced NO generation in steatotic grafts when compared with the TR group (Figure 11; nitrite and nitrate values: 5.20 ± 1.6 and 17.68 ± 3.2, in Ang-(1–7)-Mas antagonist and TR, respectively). Also, Ang-(1–7)-Mas antagonist significantly reduced MDA and nitrotyrosine levels when compared with the TR group, indicating a reduction in peroxynitrite levels in steatotic grafts (Figure 11; nitrotyrosine values: 3.36 ± 0.6 and 6.66 ± 0.7, in Ang-(1–7)-Mas antagonist and TR, respectively). The results of immunohistochemical studies of nitrotyrosine (Figure 12) were consistent with those obtained by biochemical methods. In steatotic grafts, immunohistochemical analysis revealed positive staining in TR group (Figure 12B). Ang-(1–7)-Mas antagonist decreased nitrotyrosine staining in steatotic grafts (Figure 12C) when compared with the TR group (score of nitrotyrosine values: 0.43 ± 0.1, 1.90 ± 0.2 and 0.85 ± 0.1, in Sham, TR and Ang-(1–7)-Mas antagonist, respectively).

Discussion

The beneficial effects of Ang II receptor antagonists on damage in both steatotic and nonsteatotic livers have previously been reported under warm I/R conditions (6,7).

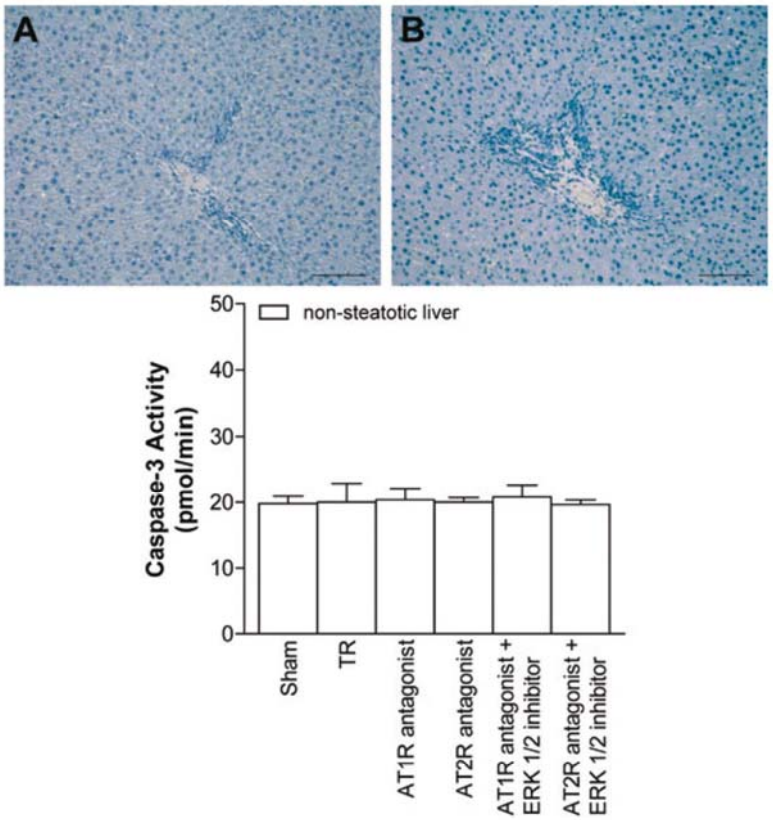

Figure 10: Apoptosis in nonsteatotic liver grafts treated with Ang II receptor antagonists and ERK 1/2 inhibitor. (A and B) Apoptotic hepatic cells identified with the TUNEL assay. (A) AT1R antagonist+ERK 1/2 (nonsteatotic graft), (B) AT2R antagonist +ERK inhibitor (nonsteatotic graft). No TUNEL-positive cells were observed in any group. (bar = 100 μm). Caspase 3 activity levels were analysed in nonsteatotic grafts. No significant differences in caspase 3 activity levels were observed in any group.

However, the utility of these strategies in the setting of liver transplantation in both liver grafts has not previously been determined. In this study, we report that Ang II receptor antagonists are effective as therapeutic strategies only in nonsteatotic liver transplantation.

The underlying protective mechanisms of Ang II receptor antagonists in nonsteatotic liver grafts were evaluated. Ang II causes vasoconstriction via G-protein-coupled AT1R receptor stimulation (35,36). Toshimitsu So and colleagues reported that AT1R antagonist ameliorated vasoconstriction in myocardial I/R (37). As the disturbances of hepatic microcirculation seems to be involved in the mechanism of injury in nonsteatotic livers (38), we evaluated whether the beneficial effects of Ang II receptor antagonists observed in nonsteatotic liver grafts could be due to improved liver blood flow. A prior study by Hiroyuki Masuko and collogues indicated that AT1R antagonist, CV-11974, ameliorated hepatic tissue blood flow in nonsteatotic livers subjected to 2 h of warm ischemia (38). However, this study indicates that the benefits of Ang II receptor antagonists on hepatic damage in nonsteatotic liver grafts subjected to 6 h of cold ischemia were not reflected by changes in hepatic blood flow. These differential effects of Ang II receptor antagonists on blood flow in liver could be explained, at least partially, by the different experimental models of I/R evaluated.

A previous report in hepatic fibrosis indicated that Ang II receptor antagonist could modulate ERK 1/2 activation (39). Our results suggest that the protection conferred by Ang II receptor antagonists in nonsteatotic liver grafts may be mediated through upregulation of ERK 1/2, which is [37] L.S. Zisman, R.S. Keller, B. Weaver, Q. Lin, R. Speth and M.R. Bristow et al., Increased angiotensin-(1–7)-forming activity in failing human heart ventricles: evidence for up regulation of the angiotensin-converting enzyme homologue ACE2, *Circulation* 108 (2003) (14), pp. 1707–1712. Full Text via CrossRef | View Record in Scopus | Cited By in Scopus (79) a pro-survival kinase that limits cell death (40). Once activated, ERK 1/2 is translocated from cell cytoplasm to the nucleus, and then activates downstream transcription factors that trigger survival signaling, mainly antiapoptotic pathways, which protect liver graft against I/R injury associated with transplantation (41,42). Next we evaluated whether the activation of ERK induced by Ang II receptor antagonist in nonsteatotic grafts decreased the cell death by necrosis or apoptosis. Apoptosis is a key feature of reperfusion injury following liver transplantation in nonsteatotic livers (25). Shiho Natori and colleagues reported

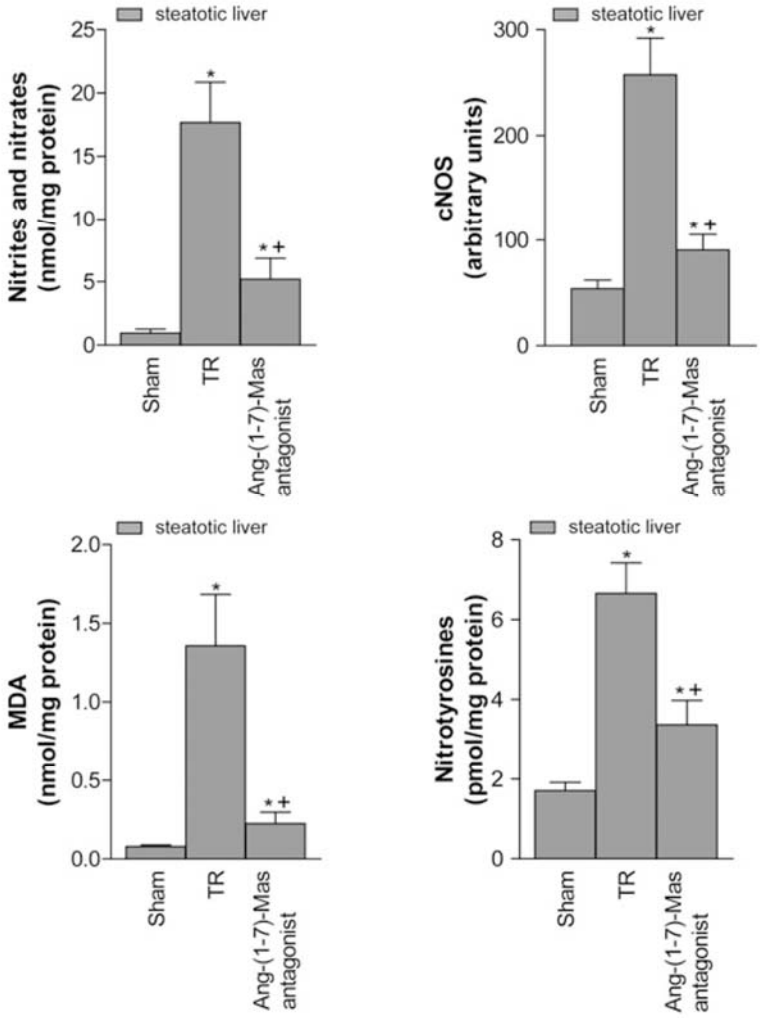

Figure 11: Protective mechanisms of Ang-(1–7) receptor antagonist in steatotic liver transplantation. Nitrites and nitrates, cNOS protein levels, MDA and nitrotyrosine levels were evaluated in steatotic liver grafts. $^{*}p < 0.05$ versus sham, $^{+}p < 0.05$ versus TR.

that the treatment with caspase inhibitors prevented the increase in caspase 3 activity, attenuated cell apoptosis and improved survival in recipients transplanted with nonsteatotic liver grafts stored for 30 h in UW solution (25). In addition, Raffaele Cursio and colleagues. suggested that ERK 1/2 may decrease apoptosis in nonsteatotic livers undergoing 2 h of warm ischemia (43). However, in our conditions, 6 h of cold ischemia, the increase in ERK 1/2 phosphorylation in nonsteatotic liver grafts pretreated with Ang II receptor antagonists contributes to the decrease in liver necrosis rather than apoptosis. Previous studies from our group (29) and the results presented herein indicate that in our conditions, 6 h of cold ischemia, apoptosis is not the predominant form of hepatocyte death postreperfusion in either type of liver.

ACE2 degrades Ang II to Ang-(1–7) (13,44). We observed upregulation of ACE2 in steatotic liver grafts, which was associated with decreased Ang II and high Ang-(1–7) levels. Many studies have described Ang-(1–7) as a promising candidate for the treatment of diseases, including myocardial infarction, diabetes and hepatic fibrosis (15–17). Here we report evidence of the injurious effects of Ang-(1–7) in steatotic liver transplantation. Ang-(1–7) receptor antagonist reduced necrotic cell death and increased survival in recipients transplanted with steatotic liver grafts. Our results indicate the clinical potential of Ang-(1–7) blocking-based therapies in steatotic liver transplantation, which could be specific for this type of liver. Our results on NO and oxidative stress obtained after Ang-(1–7) antagonist treatment could explain why Ang-(1–7) had an adverse role in steatotic liver transplantation. The intracellular signaling mechanisms of Ang-(1–7) involve release of NO, which is beneficial in other pathologies (20). However, NO generation from Ang-(1–7) in steatotic liver grafts could be detrimental if it combines with superoxide to form

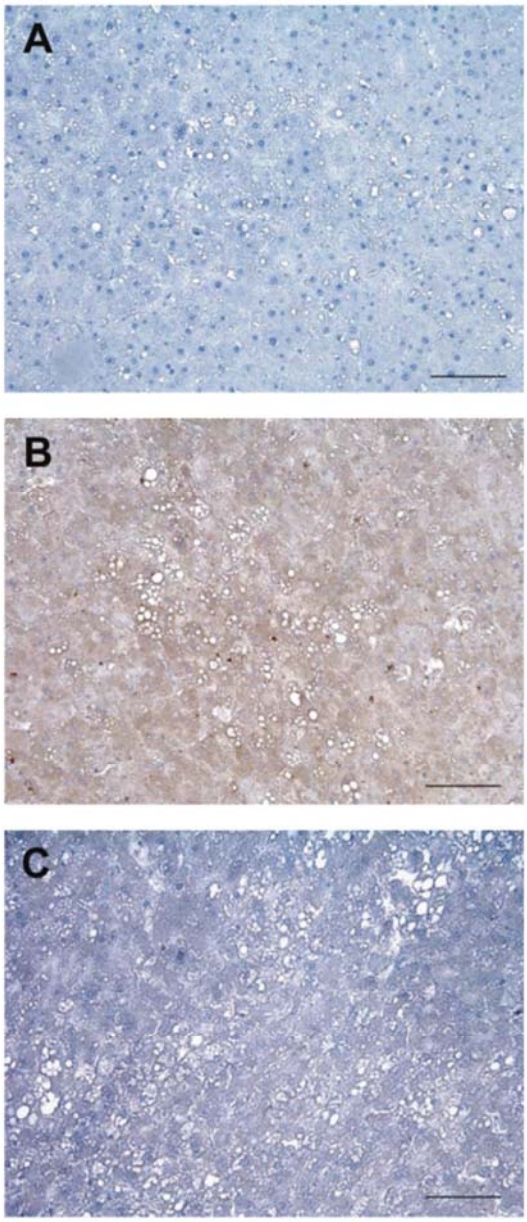

Figure 12: Immunohistochemical detection of nitrotyrosine in steatotic liver grafts. (A) Sham, absence; (B) TR, moderate staining; (C) Ang-(1–7)-Mas antagonist, slight staining (bar = 100 μm).

$ONOO^-$ (45). This could occur in steatotic liver grafts, since all these factors (high NO, high ROS and presence of $ONOO^-$) were observed in this type of liver. The oxidative and cytotoxic effects of $ONOO^-$ are well documented (45). Oxidative stress has been implicated in the vulnerability of steatotic liver grafts to I/R injury associated with liver transplantation (29). Therefore, Ang-(1–7) receptor antagonists, through NO inhibition, could protect steatotic liver grafts against oxidative stress and hepatic injury.

In the light of all previously exposed, in the context of I/R injury associated with liver transplantation, the axis ACE-Ang II-ATR and ACE2-Ang-(1–7)-Mas play a major role in nonsteatotic and steatotic grafts, respectively. From the point of view of clinical application, our findings may open up new possibilities for therapeutic intervention. Ang II is an appropriate therapeutic target only in nonsteatotic liver transplantation. For steatotic liver grafts, our results indicate a novel target for therapeutic interventions in liver transplantation within the RAS cascade, based on Ang-(1–7). Thus, Ang II receptor antagonists could be effective in nonsteatotic liver transplantation whereas Ang-(1–7) receptor antagonists could be suitable in steatotic liver transplantation.

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Retinol-Binding Protein 4 and Peroxisome Proliferator-Activated Receptor-γ in Steatotic Liver Transplantation

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ABSTRACT

Numerous steatotic livers are discarded for transplantation because of their poor tolerance of ischemia-reperfusion (I/R). The injurious effects of retinol-binding protein 4 (RBP4) in various pathologies are well documented. RBP4 levels are reduced by peroxisome proliferator-activated receptor-γ (PPARγ) agonists. Strategies aimed at increasing PPARγ protect steatotic livers under warm ischemia. Ischemic preconditioning (PC) based on brief periods of I/R protects steatotic liver grafts against I/R injury, but the responsible mechanism is poorly understood. We examined the roles of RBP4 and PPARγ in I/R injury associated with steatotic liver transplantation and the benefits of PC in such situations. We report that RBP4 and PPARγ expression levels in nonsteatotic livers were similar to those found in the sham group. However, reduced RBP4 and increased PPARγ levels were observed in steatotic livers. Treatment with either RBP4 or a PPARγ antagonist was effective only in steatotic livers. PC, which increased RBP4 levels, and RBP4 treatment both reduced PPARγ levels and hepatic injury in steatotic livers. When PPARγ was activated, neither RBP4 treatment nor PC (despite RBP4 induction) protected steatotic livers. In conclusion, steatotic liver grafts are more predisposed to down-regulate RBP4 and overexpress PPARγ. RBP4 treatment and PC, through RBP4 induction, reduced PPARγ levels in steatotic liver grafts, thus protecting them from the PPARγ detrimental effects.

Introduction

Numerous steatotic livers are discarded for transplantation because of their poor tolerance to ischemia-reperfusion (I/R), exacerbating the critical shortage of donor livers (D'Alessandro et al., 1991; Ploeg et al., 1993). Therefore, minimizing the adverse effects of I/R in steatotic liver transplantation is an urgent need. New insights into the mechanisms of steatotic liver graft failure could result in new strategies to protect steatotic liver grafts against I/R injury associated with transplantation.

Retinol-binding protein 4 (RBP4) is an adipokine synthesized by the liver, whose known function is to transport retinol in the circulation. However, the role of RBP4 in the liver is largely unknown (Blaner, 1989; Graham et al., 2006; Wagnerberger et al., 2006). Since the discovery of RBP4 in 1992, diverse studies have demonstrated that RBP4 levels are elevated in diabetes, obesity, cardiovascular diseases, and inflammation (Yang et al., 2005; Cho et al., 2006; Gra-

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A.C.-R. and I.A.-F. contributed equally to this study.

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ABBREVIATIONS: I/R, ischemia-reperfusion; RBP4, retinol-binding protein 4; PPARγ, peroxisome proliferator-activated receptor-γ; PC, ischemic preconditioning; AMPK, AMP-activated protein kinase; Ob, obese; Ln, lean; UW, University of Wisconsin; GW9662, 2-chloro-5-nitro-*N*-phenylbenzamide; rosiglitazone, (*RS*)-5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione; AICAR, aminoimidazole-4-carboxamide ribonucleoside; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TR, transplantation; PCR, polymerase chain reaction; ED_{50}, median effective dose.

ham et al., 2006; Lee et al., 2007; Yao-Borengasser et al., 2007). The possibility that lowering RBP4 levels might protect steatotic liver grafts against I/R injury associated with transplantation has not been evaluated to date.

Peroxisome proliferator-activated receptor-γ (PPARγ) agonists reduce I/R injury in steatotic livers under warm hepatic ischemia (Casillas-Ramírez et al., 2008). The role of PPARγ in steatotic liver transplantation has yet to be identified. There is a relationship between PPARγ and RBP4, whereby PPARγ agonists reduce RBP4 mRNA in adipose tissue of mice and serum RBP4 in diabetic subjects (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008). This study evaluates the role of RBP4 and PPARγ in steatotic liver transplantation. In addition, we compare the effects of pharmacological treatments that modulate RBP4 and PPARγ in steatotic liver transplantation with those obtained after applying ischemic preconditioning (PC). PC is an endogenous protective mechanism by which brief periods of vascular occlusion confer protection against subsequent sustained hepatic I/R (Casillas-Ramírez et al., 2006; Massip-Salcedo et al., 2007). To date, despite intense research efforts, PC is the only surgical strategy that has been successfully applied in patients with steatotic livers undergoing warm ischemia (Clavien et al., 2003). Previous experimental studies of our group have shown the effectiveness of PC in reducing I/R injury in steatotic liver transplantation (Carrasco-Chaumel et al., 2005), but whether PC is appropriate for steatotic liver transplantation in clinical practice remains to be clarified. Although the mechanisms by which PC protects steatotic liver grafts are unknown, liver protection depends on AMP-activated protein kinase (AMPK) activation (Carrasco-Chaumel et al., 2005). Until now, no data had been reported on the capacity of PC to modify RBP4 levels in livers undergoing I/R. However, this possibility should not be ruled out. We have suggested that PC exerts its effect on PPARγ in steatotic livers under warm hepatic ischemia (Casillas-Ramírez et al., 2008) and PPARγ agonists reduce RBP4 levels in various pathologies (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008). Only a full appraisal of the underlying protective mechanisms of PC can lead to both new pharmacological strategies to effectively protect steatotic liver grafts and new applications of PC in clinical practice of steatotic liver transplantation.

Materials and Methods

Experimental Animals

The present study was performed using homozygous (obese, Ob) and heterozygous (lean, Ln) Zucker rats (Iffa-Credo, L'Abresle, France) aged 10 to 11 weeks. Ob rats showed moderate macrovesicular and microvesicular fatty infiltration in hepatocytes, whereas Ln rats showed no evidence of steatosis (Carrasco-Chaumel et al., 2005). Analysis of triglyceride content and fatty droplet accumulation in steatotic livers confirmed that the drugs used in this study did not modify liver steatosis (data not shown). All procedures were performed under isofluorane inhalation anesthesia (Carrasco-Chaumel et al., 2005). This study conformed to European Union regulations (Directive 86/609 EEC) for animal experiments. Animals were randomly distributed into groups as described below.

Drugs

Recombinant RBP4 and anti-RBP4 antibodies were purchased from AdipoGen Inc. (Seoul, Korea). GW9662 (2-chloro-5-nitro-*N*-phenylbenzamide) and rosiglitazone (*RS*)-5-[4-(2-[methyl(pyridin-2-yl)amino]ethoxy)benzyl]thiazolidine-2,4-dione were purchased from Alexis Biochemicals (Lausen, Switzerland). Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and adenine 9-β-D-arabinofuranoside was purchased from Sigma-Aldrich (St. Louis, MO).

Experimental Design

The experimental design of the present study is summarized in Table 1. The experimental protocols are the following:

Protocol 1. RBP4 and PPARγ levels in nonsteatotic and steatotic liver transplantation were examined. In group 1 (sham; $n = 12$ rats), Ln and Ob animals (six in each group) were subjected to transverse laparotomy, and silk ligatures were applied in the right suprarenal vein, diaphragmatic vein, and hepatic artery (Carrasco-Chaumel et al., 2005).

Group 2 (TR, 12 transplantations, $n = 24$ rats) was divided into two subgroups. In subgroup 2.1 (six transplantations, $n = 12$ rats), steatotic livers from donor rats (Ob Zucker rats, $n = 6$ rats) were flushed with University of Wisconsin (UW) solution and removed from donor rats. Steatotic livers were them preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, $n = 6$ rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979). In subgroup 2.2 (six transplantations, $n = 12$ rats), nonsteatotic livers from donor rats (Ln Zucker rats, $n = 6$ rats) were flushed with UW solution and removed from donor rats. Nonsteatotic livers were then preserved in ice-cold UW solution for 6 h (ice-cold ischemia) (Carrasco-Chaumel et al., 2005) and implanted into recipient rats (Ln Zucker rats, $n = 6$ rats) according to the Kamada cuff technique without hepatic artery reconstruction (Kamada and Calne, 1979).

Group 3 (PC+TR; 12 transplantations, $n = 24$ rats) was the same as group 2 but with PC (induced by 5 min of ischemia followed by 10 min of reperfusion) before livers were flushed and preserved in UW solution for 6 h (Carrasco-Chaumel et al., 2005).

Protocol 2. This protocol involved dose-response studies of RBP4 and PPARγ antagonist on hepatic injury in steatotic liver transplantation (groups 4 and 5).

Protocol 3. Hepatic injury and RBP4 and PPARγ levels after the pharmacological modulation of RBP4 and PPARγ (groups 6–12) were evaluated.

Protocol 4. AMPK involvement in PC-induced effects on RBP4 and PPARγ in steatotic liver transplantation (groups 13 and 14) was investigated.

Protocol 5. The changes in RBP4 and PPARγ levels in steatotic liver grafts during cold ischemia before the implantation of steatotic liver grafts in the recipient (groups 15–18) were investigated.

The conditions of this study (including the times of cold ischemia, reperfusion, and PC period) were established on the basis of the results of previous studies (Carrasco-Chaumel et al., 2005). A cold ischemic period of 6 h is long enough to induce liver damage after transplantation in liver grafts and allow high survival 4 h after transplantation. In addition, the PC period used in the present study (5 min of ischemia followed by 10 min of reperfusion) is effective against hepatic I/R injury in both liver types (Carrasco-Chaumel et al., 2005). Thus, these experimental conditions were appropriate for evaluating RBP4 and PPARγ levels in both liver types and their effects on hepatic I/R injury associated with transplantation.

All of the drugs used were administered in the donor rats. The doses and pretreatment times used for the different drugs are shown in Table 2. Control experiments were performed using the vehicles of the drugs used in this study.

For analytical determinations, plasma and liver samples from protocols 1 to 4 were collected 4 h after transplantation from the recipient. Liver samples from donor rats were collected after 6 h of

TABLE 1
Experimental design of the current study

Protocol	
Protocol 1. RBP4 and PPARγ levels in nonsteatotic and steatotic liver transplantation	
1. Sham (n = 12, 6 Ln and 6 Ob)	Dissection of hepatic ilium vessels
2. TR (12 transplantations), divided into two groups	Livers preserved in UW solution and subsequently transplanted
2.1 TR (6 transplantations), graft Ob, 6 h ice-cold ischemia	Recipient Ln, 4-h reperfusion
2.2 TR (6 transplantations), graft Ln, 6 h ice-cold ischemia	Recipient Ln, 4-h reperfusion
3. PC + TR (12 transplantations)	Same as group, but with PC
Protocol 2. Dose-reponse studies of RBP4 and PPAR γ antagonist on hepatic injury in steatotic liver transplantation	
4. TR + RBP4 (6 transplantations for each dose evaluated)	Same as group 2.1, but treated with different doses of RBP4
5. TR + PPARγ antagonist (6 transplantations for each dose evaluated)	Same as group 2.1, but treated with different doses of PPARγ antagonist
Protocol 3. Hepatic injury and RBP4 and PPARγ levels after the pharmacological modulation of RBP4 and PPARγ in steatotic liver transplantation	
6. TR + RBP4 (12 transplantations)	Same as group 2, but treated with RBP4
7. PC + TR + anti-RBP4 (6 transplantations)	Same as group 2.1, but with PC and treated with anti-RBP4
8. TR + PPARγ antagonist (12 transplantations)	Same as group 2, but treated with PPARγ antagonist
9. PC + TR + PPARγ agonist (6 transplantations)	Same as group 2.1, but with PC and treated with PPARγ agonist
10. TR + RBP4 + PPARγ agonist (6 transplantations)	Same as group 2.1, but treated with RBP4 and PPARγ agonist
11. PC + TR + anti-RBP4 + PPARγ antagonist (6 transplantations)	Same as group 2.1, but with PC and treated with anti-RBP4 and PPARγ antagonist
12. TR + RBP4 + PPARγ antagonist (6 transplantations)	Same as group 2.1, but treated with RBP4 and PPARγ antagonist
Protocol 4. Effect of AMPK on RBP4 and PPARγ in steatotic liver transplantation	
13. TR + AMPK activator (6 transplantations)	Same as group 2.1, but treated with AMPK activator
14. PC + TR + AMPK inhibitor (6 transplantations)	Same as group 2.1, but with PC and treated with AMPK inhibitor
Protocol 5. RBP4 and PPARγ levels in steatotic liver grafts after ice-cold ischemia	
15. Ischemia, graft Ob, 6 h ice-cold ischemia (n = 6)	Steatotic livers from Ob animals, preserved in UW solution
16. PC + Ischemia (n = 6)	Same as group 14, but with PC
17. Ischemia + AMPK activator (n = 6)	Same as group 14, but treated with AMPK activator
18. PC + Ischemia + AMPK inhibitor (n = 6)	Same as group 14, but with PC and treated with AMPK inhibitor

TABLE 2
Drug administration protocol of the current study

Protocol		Drug	Dose and Pretreatment Time
2	RBP4	Recombinant RBP4	25, 50, 75, 100, 125, 150, 175, or 200 μg/kg i.v., in donor rat 30 min before surgical procedure
	PPARγ antagonist	GW9662	250, 500, 750, 1000, 1250, or 1500 μg/kg i.p., in donor rat 1 h before surgical procedure
3	RBP4	Recombinant RBP4	150 μg/kg i.v., in donor rat 30 min before surgical procedure
	Anti-RBP4	Anti-RBP4 antibody	3000 μg/kg i.v., in donor rat 30 min before surgical procedure
	PPARγ antagonist	GW9662	1000 μg/kg i.p., in donor rat 1 h before surgical procedure
	PPARγ agonist	Rosiglitazone	3000 μg/kg i.p., in donor rat 1 h before surgical procedure
4 and 5	AMPK activator	AICAR	100 mg/kg i.v., in donor rat 5 min before surgical procedure
	AMPK inhibitor	Adenine 9-β-D-arabinofuranoside.	100 μg/kg/min i.v. in donor rat for 10 min before surgical procedure

ice-cold ischemia (protocol 5); thus, in these rats steatotic liver grafts were not subjected to transplantation.

Reverse Transcription and Real-Time Polymerase Chain Reaction

Quantitative real-time PCR analysis was performed using the Assays-on-Demand TaqMan probes (Rn01451317_g1 for RBP4, Rn00440945_m1 for PPARγ, and Rn00667869_m1 for β-actin; Applied Biosystems, Foster City, CA) following the manufacturer's protocol.

Biochemical Determinations

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and triglyceride levels were measured following previously described protocols (Carrasco-Chaumel et al., 2005; Man et al., 2006). RBP4 and PPARγ were measured using enzyme-linked immunosorbent assay kits (Adipogen Inc., Seoul, Korea, and Antibodies-online GmbH, Aache, Germany, respectively) according to the manufacturer's instructions.

Histology

To appraise the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method on an ordinal scale as follows: grade 0, minimal or no evidence of injury; grade 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (Serafín et al., 2004). To appraise the hepatic fatty droplet content, red-oil staining on frozen specimens was evaluated according to standard procedures (Carrasco-Chaumel et al., 2005).

Statistics

Data are expressed as means ± S.E. and were compared statistically via one-way analysis of variance followed by a post hoc Student-Newman-Keuls test. A P value <0.05 was considered significant. The dose responses of RBP4 and PPARγ antagonist were analyzed using Prism version 4 (GraphPad Software Inc., San Diego, CA). To derive median effective dose (ED_{50}), a nonlinear approximation model of the least-square method based on a competition curve using one component was calculated. Data were evaluated by one-way analysis of variance and Bonferroni's post-test.

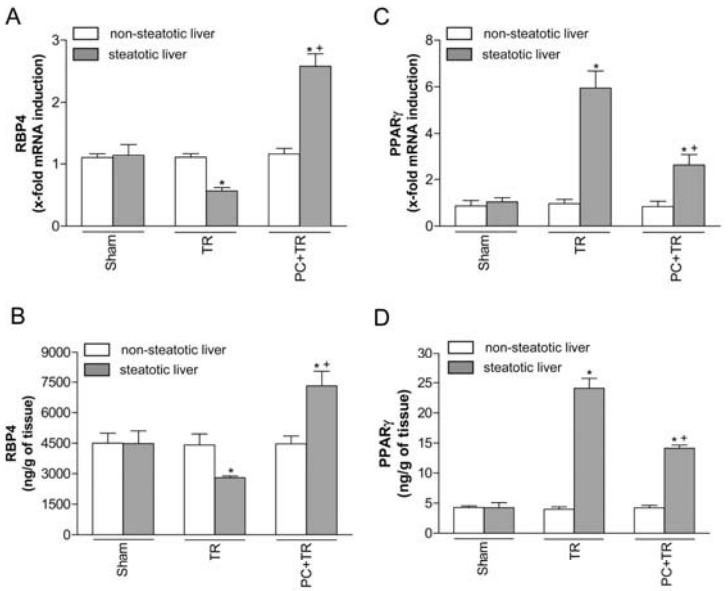

Fig. 1. RBP4 and PPARγ levels in nonsteatotic and steatotic liver transplantation. Expression of mRNA and protein levels of RBP4 (A and B) and PPARγ (C and D) were measured in both liver types 4 h after transplantation. PCR fluorescent signals for RBP4 and PPARγ were normalized to PCR fluorescent signals obtained from an endogenous reference (β-actin). Comparative and relative quantification of RBP4 or PPARγ gene products normalized to β-actin and the control sham group were calculated by the $2^{-\Delta\Delta CT}$ method. All subjects of all groups were included in each measurement. *, $P < 0.05$ versus sham; +, $P < 0.05$ versus TR.

Results

RBP4 and PPARγ Levels in Nonsteatotic and Steatotic Liver Transplantation. RBP4 mRNA and protein levels in nonsteatotic liver grafts of the TR and PC+TR groups were similar to those found in the sham group (Fig. 1, A and B). In steatotic liver grafts, a reduction in RBP4 mRNA and protein levels was observed in the TR group compared with the results found in the sham group (Fig. 1, A and B). In the PC+TR group, RBP4 mRNA and protein levels were increased in steatotic liver grafts compared with the TR group.

PPARγ mRNA and protein levels in nonsteatotic liver grafts of the TR and PC+TR groups were similar to those found in the sham group (Fig. 1, C and D). In the presence of steatosis, PPARγ mRNA and protein levels in the TR group were markedly higher than in the sham group. In the PC+TR group, PPARγ mRNA and protein levels were reduced in steatotic liver grafts compared with the TR group (Fig. 1, C and D).

Dose-Response Effect of RBP4 and PPARγ Antagonist on Hepatic Injury in Steatotic Liver Transplantation. We evaluated the relevance of changes in RBP4 and PPARγ levels observed in steatotic liver grafts undergoing transplantation on hepatic injury. For this, we administered RBP4 at doses of 25, 50, 75, 100, 125, 150, and 200 μg/kg in donor rats 30 min before the surgical procedure, and the effects on hepatic injury were determined 4 h after transplantation in recipients (Fig. 2, A–C). Our results indicated that RBP4 protected steatotic liver grafts against damage in a dose-dependent manner. The ED_{50} values for AST, ALT, and damage score were 50.29 ± 1.05, 91.12 ± 1.06, and 43.81 ± 1.44 μg/kg, respectively (Fig. 2, A–C). The most effective dose of RBP4 in reducing the parameters of hepatic injury in steatotic liver grafts was 150 μg/kg, because higher doses were not associated with lower hepatic damage. The pretreatment time of RBP4 used in the present study (30 min before the surgical procedure) was selected on the basis of previous studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed using longer pretreatment times (60 or 120 min before the surgical procedure; data not shown).

We administered a PPARγ antagonist at doses of 250, 500, 750, 1000, 1250, 1500, and 2000 μg/kg in donor rats 1 h before the surgical procedure, and the effects on hepatic injury were determined 4 h after transplantation in recipients (Fig. 2, D–F). The PPARγ antagonist protected steatotic liver grafts against damage in a dose-dependent manner. The ED_{50} values for AST, ALT, and damage score were 503.0 ± 5.53, 315.0 ± 1.03, and 224.9 ± 2.07 μg/kg, respectively (Fig. 2, D–F). The most effective dose of PPARγ antagonist in protecting steatotic livers against damage was 1000 μg/kg. Higher doses were unnecessary because they were not associated with lower hepatic damage (Fig. 2, D–F). The pretreatment time of the PPARγ antagonist (1 h before the surgical procedure) was selected on the basis of previous studies (Sivarajah et al., 2005; Casillas-Ramírez et al., 2008) and preliminary studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (2 or 3 h before the surgical procedure; data not shown).

Hepatic Injury and RBP4 and PPARγ Levels after the Pharmacological Modulation of RBP4 and PPARγ. As shown in Fig. 3, A and B, RBP4 treatment at the selected dose, 150 μg/kg (TR+RBP4 group), reduced transaminase levels in steatotic liver grafts with respect to those recorded

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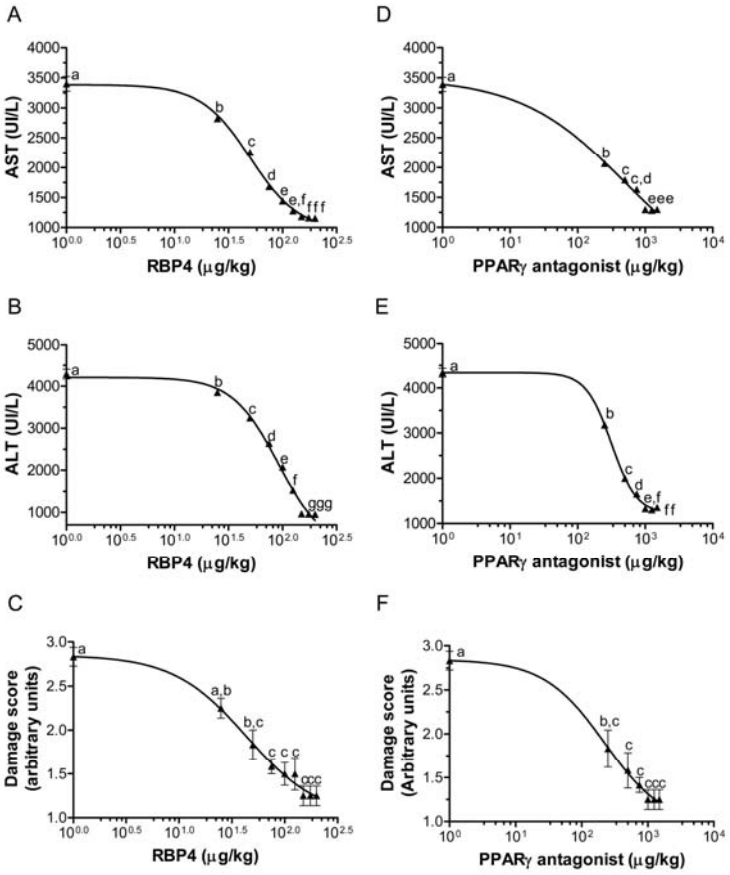

Fig. 2. Dose-response studies of RBP4 and PPARγ antagonist on hepatic injury in steatotic liver transplantation. A to C, the effects of RBP4 treatment on transaminase levels (A and B) and damage score (C) in steatotic liver transplantation were assessed. D to F, the effects of PPARγ antagonist treatment on transaminase levels (D and E) and damage score (F) in steatotic liver transplantation were assessed. Donor rats were treated with RBP4 (0, 25, 50, 75, 100, 125, 150, 175, and 200 μg/kg) or PPARγ antagonist (0, 250, 500, 750, 1000, 1250, and 1500 μg/kg). Transaminase levels and damage scores were measured in plasma and steatotic livers from recipient rats, respectively, 4 h after transplantation. Means without a common letter are different, $P < 0.05$.

in the TR group. Nonsteatotic liver grafts were not protected against hepatic injury in the TR+RBP4 group. Indeed, transaminase levels and damage score values in nonsteatotic liver grafts of the TR+RBP4 group were similar to those of the TR group (AST: 2135 ± 232 and 2075 ± 329 U/l for the TR+RBP4 and TR groups, respectively; ALT: 1549 ± 129 and 1540 ± 205 U/l for the TR+RBP4 and TR groups, respectively; damage score values: 1.80 ± 0.20 and 1.80 ± 0.12 for the TR+RBP4 and TR groups, respectively). We evaluated whether the increase in RBP4 induced by PC+TR protects steatotic liver grafts. We attempted to identify the dose of anti-RBP4 antibody that reduced RBP4 levels to those of the TR group. Among the doses evaluated (1000, 3000, and 5000 μg/kg), the appropriate dose of anti-RBP4 antibody was 3000 μg/kg (data not shown). At this dose, the administration of anti-RBP4 antibody in the PC+TR group (PC+TR+anti-RBP4 group) resulted in RBP4 protein levels similar to those of the TR group (2804 ± 80, 7316 ± 709, and 2863 ± 212 ng/g tissue for the TR, PC+TR, and PC+TR+anti-RBP4 groups, respectively). This effect was associated with transaminase levels similar to those detected in the TR group (Fig. 3, A and B). The damage score values showed a similar pattern to that described for transaminase (Fig. 3C). Thus, the administration of anti-RBP4 antibody to the PC+TR group (PC+TR+anti-RBP4 group) abolished the benefits of PC+TR on hepatic damage. The pretreatment time of anti-RBP4 antibody used in the present study (30 min before the surgical procedure) was selected on the basis of previous studies from our group. Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (60 or 120 min before the surgical procedure; data not shown).

PPARγ antagonist treatment at the selected dose, 1000 μg/kg (TR+PPARγ antagonist group), resulted in lower biochemical and histological parameters of hepatic injury in steatotic liver grafts than in the TR group (Fig. 3). In nonsteatotic liver grafts, the TR+PPARγ antagonist did not induce changes in hepatic injury. Indeed, transaminase levels and damage score values in nonsteatotic liver grafts of the TR+PPARγ antagonist group were similar to those of the TR group (AST: 2143 ± 192 and 2075 ± 329 U/l for the TR+PPARγ antagonist and TR groups, respectively; ALT:

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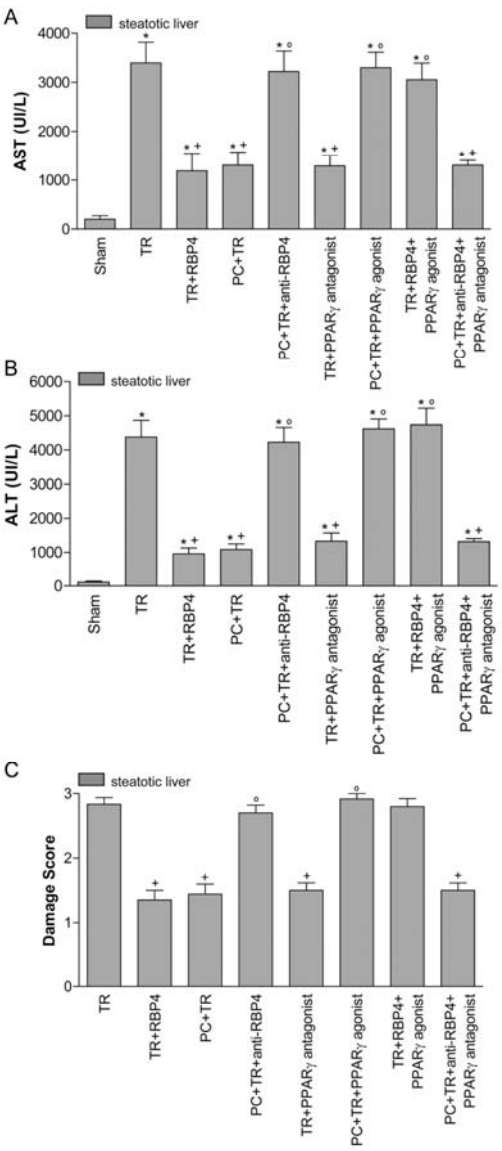

Fig. 3. Hepatic injury after pharmacological modulation of RBP4 and PPARγ in steatotic liver transplantation. Transaminase levels (A and B) and damage score (C) were analyzed in plasma and steatotic liver, respectively, 4 h after transplantation. All subjects of all groups were included in each measurement. *, $P < 0.05$ versus sham; +, $P < 0.05$ versus TR; ○, $P < 0.05$ versus PC+TR.

1630 ± 124 and 1540 ± 205 U/l for the TR+PPARγ antagonist and TR groups, respectively; damage score values: 1.70 ± 0.26 and 1.80 ± 0.12 for the TR+PPARγ antagonist and TR groups, respectively). We then evaluated whether reduction of PPARγ levels induced by PC+TR protects steatotic liver grafts. We required a dose of PPARγ agonist that would raise PPARγ to the levels of the TR group. Among the evaluated doses (1000, 3000, and 5000 μg/kg), the appropriate dose of PPARγ agonist was 3000 μg/kg (data not shown). PPARγ agonist administration at 3000 μg/kg (PC+TR+PPARγ agonist group) abolished the benefits of PC+TR, resulting in transaminase and damage score values in steatotic liver grafts similar to those observed in the TR group (Fig. 3). The pretreatment time of PPARγ agonist used in the present study (1 h before the surgical procedure) was selected on the basis of previous studies from our group and others (Yue et al., 2001; Casillas-Ramírez et al., 2008). Our results indicated that this pretreatment time resulted in parameters of hepatic injury similar to those observed at longer pretreatment times (2 or 3 h before the surgical procedure; data not shown).

The histological findings revealed that steatotic liver grafts of the TR group showed extensive and confluent areas of coagulative necrosis with neutrophil infiltration (Fig. 4A) that were reduced in number and extension in the TR+RBP4 group (Fig. 4B). The hepatic lesions observed in steatotic liver grafts of the PC+TR (Fig. 4C) and PC+TR+anti-RBP4 (Fig. 4D) groups were comparable with those observed in the TR+RBP4 (Fig. 4B) and TR (Fig. 4A) groups, respectively. In the TR+PPARγ antagonist group (Fig. 4E), the extent and the number of necrosis areas in steatotic liver grafts were reduced compared with the TR group (Fig. 4A). The hepatic lesions observed in the PC+TR+PPARγ agonist group (Fig. 4F) were similar to those observed in the TR group (Fig. 4A).

RBP4 and PPARγ levels were investigated after the pharmacological modulation of PPARγ and RBP4. In the TR+PPARγ antagonist group, RBP4 levels in steatotic liver grafts were similar to those of the TR group (Fig. 5, A and B), indicating that PPARγ antagonist treatment did not modify RBP4 levels in steatotic liver grafts. In addition, in the PC+TR+PPARγ agonist group hepatic RBP4 levels were similar to those of the PC+TR group (Fig. 5, A and B). We evaluated the effects of different doses of RBP4 on PPARγ levels in steatotic livers 4 h after transplantation, and the calculated ED_{50} was 111.5 ± 1.33 μg/kg. The most effective dose of RBP4 in reducing PPARγ levels was 150 μg/kg. Higher doses of RBP4 were not associated with lower PPARγ levels in steatotic liver grafts (Fig. 5C). On the other hand, it should be noted that at the selected dose of RBP4 (150 μg/kg) PPARγ levels and hepatic injury were reduced in steatotic livers in the TR+RBP4 group, but not to levels observed in the sham group (Figs. 3 and 5, D and E). Then, we assessed whether the administration of a PPARγ antagonist in the TR+RBP4 group would inhibit PPARγ levels and hepatic injury in steatotic livers. Our results indicated that in the TR+RBP4+PPARγ antagonist group PPARγ levels were reduced to those seen in the sham group (4.12 ± 0.59 and 4.24 ± 0.86 ng/g tissue in the TR+RBP4+PPARγ antagonist and sham groups, respectively) but resulted in parameters of hepatic injury similar to those of the TR+RBP4 group (AST: 1287 ± 432 and 1188 ± 360 U/l for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively; ALT: 1005 ± 179 and 964 ± 170 U/l for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively; damage score: 1.38 ± 0.24 and 1.35 ± 0.15 for the TR+RBP4+PPARγ and TR+RBP4 groups, respectively).

Our results indicate that RBP4 treatment (at the selected dose, 150 μg/kg) and PC mediation by RBP4 reduced PPARγ in steatotic liver grafts and ameliorated hepatic injury. In the

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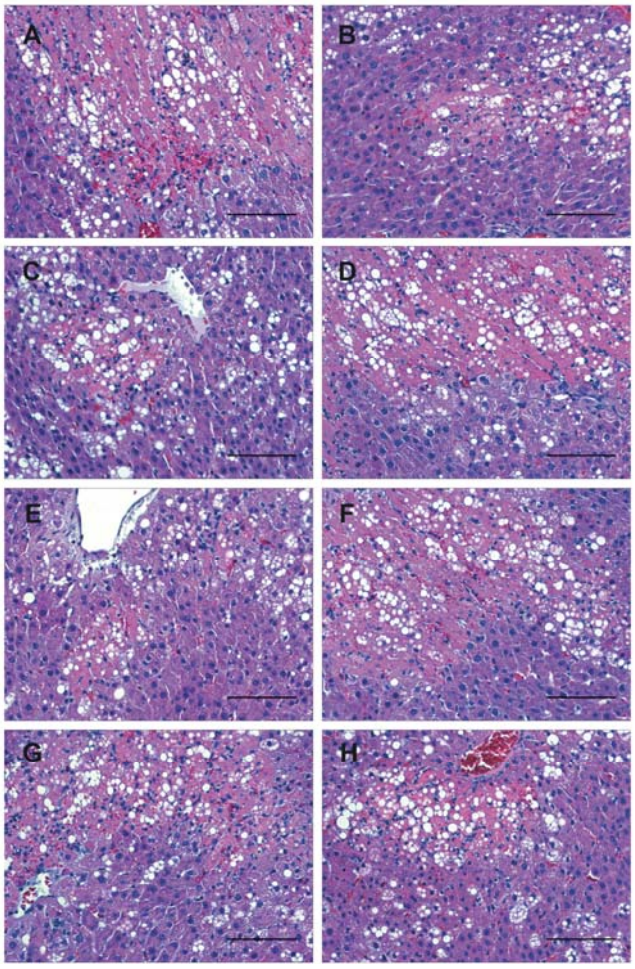

Fig. 4. Histological lesions in steatotic liver transplantation. Representative photographs of histological changes in steatotic livers are shown. A, TR, widespread coagulative hepatic necrosis with neutrophil infiltration. B, TR+RBP4, small area of coagulative hepatic necrosis with neutrophil infiltration. C, E, and H, PC+TR (C), TR+PPARγ antagonist (E), and PC+TR+anti-RBP4+PPARγ antagonist (H), hepatic lesions similar to the TR+RBP4 group. D, F, and G, PC+TR+anti-RBP4 (D), PC+TR+PPARγ agonist (F), and TR+RBP4+PPARγ agonist (G), hepatic lesions similar to the TR group. Hematoxylin and eosin staining was used. Bars, 100 μm.

TR+RBP4 group, PPARγ levels (Fig. 5, D and E) and hepatic injury (Fig. 3) were reduced in steatotic liver grafts compared with the TR group. In the TR+RBP4+PPARγ agonist group, transaminase and damage score values were similar to the TR group (Fig. 3), indicating that the PPARγ agonist abolished the benefits of RBP4 treatment in steatotic liver grafts. During RBP4 treatment, PPARγ levels (Fig. 5, D and E) and hepatic injury (Fig. 3) were reduced in steatotic liver grafts in the PC+TR group compared with the TR group. In the PC+TR+anti-RBP4 group, PPARγ levels and hepatic injury in steatotic liver grafts were similar to those in the TR group, indicating the injurious effects of anti-RBP4 antibody in steatotic liver grafts. In the PC+TR+anti-RBP4+PPARγ antagonist group, hepatic injury was similar to the PC+TR group (Fig. 3), indicating that the PPARγ antagonist prevented the injurious effects of anti-RBP4 antibody in steatotic liver grafts. In the TR+RBP4+PPARγ agonist group, histological lesions were observed in steatotic liver grafts (Fig. 4G) that were similar to those of the TR group (Fig. 4A). In the PC+TR+anti-RBP4+PPARγ antagonist group, histological lesions were observed in steatotic liver grafts (Fig. 4H) that were similar to those of the PC+TR group (Fig. 4C).

Effect of AMPK on RBP4 and PPARγ Levels in Steatotic Liver Transplantation. The beneficial effects of AMPK activators such as AICAR and the involvement of AMPK in the benefits of PC in steatotic liver grafts were previously reported by our group using the same experimental model of liver transplantation described here (Carrasco-Chaumel et al., 2005). From such studies, we selected the doses and pretreatment times for AMPK activator (100 mg/kg, 5 min before the surgical procedure) and AMPK inhibitor (100 μl/kg/min for 10 min, 10 min before the surgical procedure). This dose of AMPK activator protected steatotic liver grafts. The dose of AMPK inhibitor selected inhibited AMPK

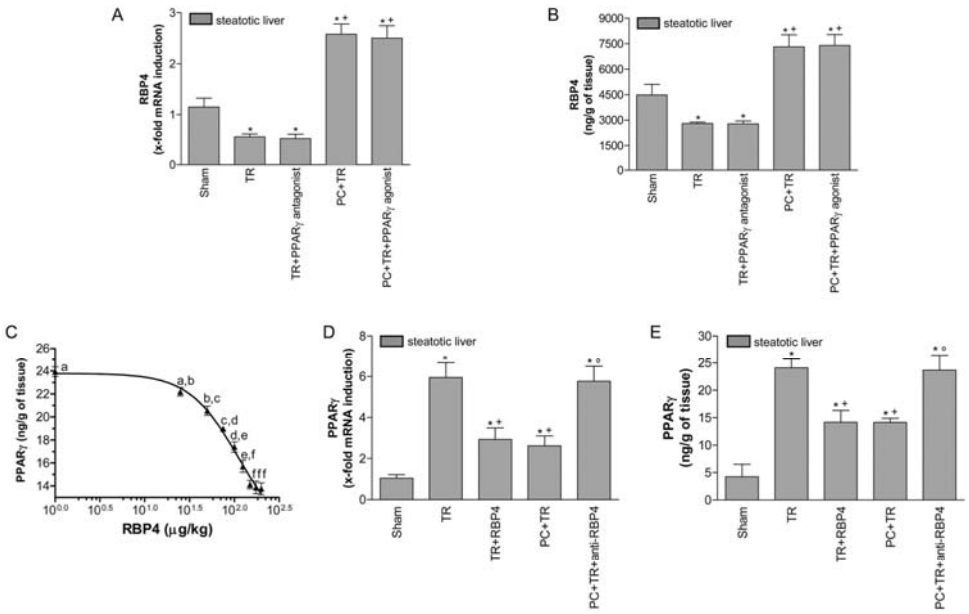

Fig. 5. RBP4 and PPARγ levels after pharmacological modulation of RBP4 and PPARγ in steatotic liver transplantation. A and B, RBP4 mRNA expression (A) and RBP4 protein (B) levels in steatotic livers 4 h after transplantation. C, dose-response study to evaluate the effect of different doses of RBP4 on PPARγ levels in steatotic livers 4 h after transplantation. Means without a common letter differ, $P < 0.05$. D and E, PPARγ mRNA (D) and PPARγ protein (E) levels in steatotic livers 4 h after transplantation. RBP4 and PPARγ mRNA expression levels in steatotic livers are as described in Fig. 1. All subjects of all groups were included in each measurement. ∗, $P < 0.05$ versus sham; +, $P < 0.05$ versus TR; ○, $P < 0.05$ versus PC+TR.

in preconditioned steatotic livers, leading to AMPK and transaminase levels similar to those of the TR group (Carrasco-Chaumel et al., 2005). The results of the present study indicated that in the TR+AMPK activator and PC+TR groups RBP4 mRNA and protein levels increased in steatotic liver grafts compared with the TR group (Fig. 6, A.1 and A.2). This finding was associated with reduced PPARγ mRNA and protein levels (Fig. 6, A.3 and A.4). Conversely, in the PC+TR+AMPK inhibitor group RBP4 mRNA and protein levels in steatotic liver grafts were similar to those of the TR group (Fig. 6, A.1 and A.2). This effect was associated with similar PPARγ mRNA and protein levels in steatotic liver grafts to those of the TR group (Fig. 6, A.3 and A.4). Thus, AMPK activators and PC, through AMPK, increased RBP4 levels in steatotic liver grafts after transplantation. This result was associated with a reduction in PPARγ levels.

RBP4 and PPARγ in Steatotic Liver Grafts after Ice-Cold Ischemia. As shown above, our results reveal a close relationship between RBP4 mRNA and RBP4 protein levels in steatotic liver grafts after transplantation, suggesting that this type of graft by itself generates RBP4 after either AMPK activator treatment or PC induction (TR+AMPK activator and PC+TR groups). In addition to the liver, adipose tissue is able to generate RBP4 that may be taken up by liver from the circulation (Gjøen et al., 1987; Tsutsumi et al., 1992). To confirm that the steatotic liver graft by itself, without the influence of other tissues or plasma constituents, can generate RBP4 after either AMPK activator treatment or PC induction, we measured RBP4 mRNA levels in steatotic liver grafts during ice-cold ischemia (before the implantation of liver grafts in the recipient). Under these conditions, the liver is isolated from the influence of other tissues and plasma constituents. Our results indicated that during ice-cold ischemia increased RBP4 mRNA levels in steatotic liver grafts were observed after either AMPK activator treatment or PC induction (Fig. 6B.1). This finding was also associated with reduced PPARγ mRNA levels (Fig. 6B.2). Conversely, in the PC+ischemia+AMPK inhibitor group, RBP4 mRNA levels in steatotic liver grafts were similar to those of the ischemia group (Fig. 6B.1). This result was associated with similar PPARγ mRNA levels in steatotic liver grafts to those of the ischemia group (Fig. 6B.2).

Discussion

In line with previous data in high-fat diet-induced obese rats (Wu et al., 2009) and Zucker rats (Lanne et al., 2006), our results indicated that the presence of fatty infiltration by itself in the liver (without any surgical intervention) does not induce changes in either RBP4 or PPARγ levels, because no differences in RBP4 or PPARγ levels were observed in steatotic or nonsteatotic livers of the sham group of Zucker rats. These results contrast with reports from the literature indicating reduced RBP4 levels (Mody et al., 2008) and high or low PPARγ levels (Zhao et al., 2004; Inoue et al., 2005) in steatotic compared with nonsteatotic livers. These different results for RBP4 and PPARγ levels could be explained, at least in part, by differences in the level of RBP4 and PPARγ

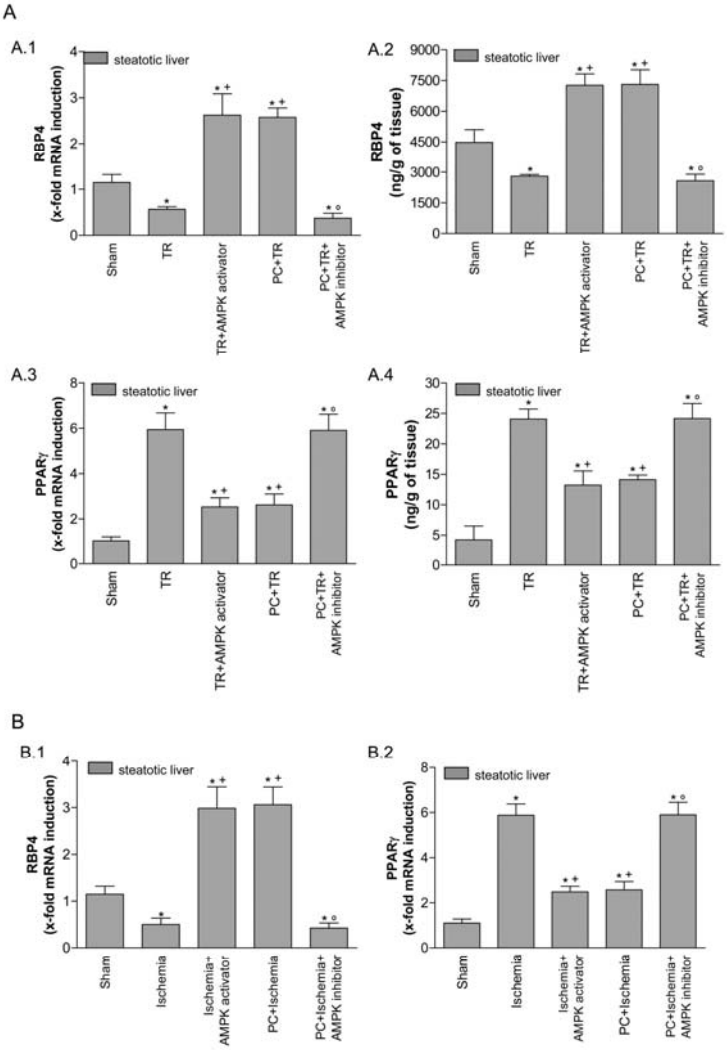

Fig. 6. A, effect of AMPK on RBP4 and PPARγ levels in steatotic liver transplantation. Expression of mRNA and protein levels of RBP4 (A.1 and A.2) and PPARγ (A.3 and A.4) were measured in steatotic livers 4 h after transplantation. *, $P < 0.05$ versus sham; +, $P < 0.05$ versus TR; ○, $P < 0.05$ versus PC+TR. B, effects of AMPK on RBP4 and PPARγ levels in steatotic livers grafts after ice-cold ischemia. RBP4 (B.1) and PPARγ (B.2) mRNA expression levels were measured in steatotic liver grafts after 6 h of ice-cold ischemia (steatotic liver grafts were not subjected to transplantation). *, $P < 0.05$ versus sham; +, $P < 0.05$ versus ischemia; ○, $P < 0.05$ versus PC+ischemia. RBP4 and PPARγ mRNA expression levels in steatotic livers were as described in Fig. 1. All subjects of all groups were included in each measurement.

regulation between rats and mice (Lanne et al., 2006), the different obesity experimental models evaluated, and the degree of steatosis.

The present study provides evidence for the generation of RBP4 in steatotic and nonsteatotic liver grafts after transplantation. In contrast with nonsteatotic liver grafts, steatotic liver grafts clearly had lower RBP4 mRNA and protein levels after transplantation. This finding is in line with previous data showing decreased RBP4 levels in liver diseases, including cirrhosis, acute hepatitis, and malnutrition (Smith and Goodman, 1971; Smith et al., 1975; McClain et al., 1979) as well as different types of inflammation, induced by either lipopolysaccharide or interleukin-6 (Rosales et al., 1996; Rosales and Ross, 1998; Gieng et al., 2005). To date, RBP4 has been described as an adipokine that exerts injurious effects in several pathologies, including diabetes and cardiovascular diseases (Yang et al., 2005; Cho et al., 2006; Graham et al., 2006; Lee et al., 2007; Yao-Borengasser et al., 2007). Here, we report evidence of the beneficial effects of RBP4 treatment in steatotic liver transplantation.

The protective pathway of PC based on AMPK activation has been demonstrated elsewhere (Carrasco-Chaumel et al., 2005). AMPK mediates some of the effects of hormones such as adiponectin and resistin (Kahn et al., 2005; Kola et al., 2006; Lage et al., 2008). Here, we report, for the first time, that RBP4 could be a downstream effector of AMPK in stea-

totic liver transplantation, and we suggest a new mechanism, namely, the induction of RBP4 generation, that might explain why AMPK activation induced by PC protects steatotic liver grafts against I/R injury.

To date, the liver has been considered the major site of endogenous RBP4 production (Blaner, 1989; Tsutsumi et al., 1992). Although adipose tissue could be another source of RBP4, its contribution seems to be minimal, according to previously reported data indicating that increases in RBP4 levels are not associated with the amount of subcutaneous abdominal fat (Gjøen et al., 1987; Tsutsumi et al., 1992; Janke et al., 2006; Stefan et al., 2007). In the present study, increased hepatic RBP4 levels were detected in steatotic liver grafts after transplantation in the groups treated with AMPK activators and PC. This RBP4 accumulation in the liver is generated by steatotic liver grafts by themselves, without the influence of other tissues or plasma constituents. In line with this result, a relationship between mRNA and protein RBP4 levels was seen in steatotic liver grafts after transplantation. In addition, the increases in RBP4 levels observed in steatotic liver grafts after transplantation after AMPK activator treatment or PC induction were observed during ice-cold ischemia (before the implantation of liver grafts in the recipient). Taking into account the fact that the liver was isolated from the influence of other organs and plasma constituents under these conditions, it is suggested that steatotic liver grafts alone were responsible for the PC- and AMPK-induced generation of RBP4.

The results presented here indicate that both RBP4 treatment and PC, via RBP4 induction, reduced PPARγ overexpression in steatotic liver grafts, thus protecting against the worsening effects of PPARγ on hepatic injury. In steatotic liver grafts, the pharmacological modulation of RBP4 activity induced changes in PPARγ levels, and the benefits of RBP4 induction by either PC or RBP4 pretreatment on hepatic injury were abolished when PPARγ was activated. Moreover, the injurious effects of anti-RBP4 antibody on steatotic liver grafts were prevented by treatment with PPARγ antagonists. In contrast with previous studies in steatotic livers under warm ischemic conditions, indicating the benefits of PPARγ (Casillas-Ramírez et al., 2008), we report that in the setting of steatotic liver transplantation reduction in PPARγ levels protects steatotic liver grafts. Moreover, in contrast with previous studies indicating that PPARγ agonists reduce RBP4 levels (Yang et al., 2005; Teranishi et al., 2007; Lin et al., 2008), we report that in the setting of steatotic liver transplantation strategies aimed at increasing RBP4 levels (RBP4 treatment and PC induction) reduce PPARγ levels and hepatic injury. On the other hand, our results do not elucidate why levels of PPARγ and RBP4 are increased and reduced, respectively, in steatotic liver grafts, or whether RBP4 is responsible for the reduced PPARγ levels observed in steatotic liver grafts. The data presented herein are insufficient to establish a regulatory relationship between RBP4 and PPARγ in steatotic liver transplantation because other genes may be involved. Further studies (beyond the scope of this work) will be required to answer this question.

It should also be considered that RBP4 treatment, at the most effective dose (150 μg/kg), reduced hepatic injury and PPARγ levels but did not completely inhibit either hepatic injury or PPARγ in steatotic liver grafts. That RBP4 did not prevent hepatic injury cannot be explained by the fact that PPARγ levels were not completely reduced by RBP4. Treatment with a PPARγ antagonist reduced but did not prevent hepatic injury in steatotic liver grafts. In addition, the administration of a PPARγ antagonist in the TR+RBP4 group, at the most effective dose, reduced PPARγ to sham levels but resulted in hepatic injury parameters similar to those of the TR+RBP4 group. Thus, these results suggest that, in addition to RBP4 and PPARγ, other mechanisms are involved in the hepatic I/R injury associated with transplantation in steatotic liver grafts. This idea is not surprising given that numerous mechanisms and mediators are involved in hepatic I/R injury associated with transplantation (Casillas-Ramírez et al., 2006; Massip-Salcedo et al., 2007), which makes it difficult to find a strategy to prevent the hepatic I/R associated with transplantation.

In conclusion, steatotic liver grafts were found to be more vulnerable to the down-regulation of RBP4 and the overexpression of PPARγ. RBP4 treatment and PC (through AMPK induction) reduced PPARγ overexpression, thus protecting steatotic liver grafts against I/R injury associated with transplantation. In terms of clinical application, therapies based on RBP4 treatment and PPARγ antagonists might open new avenues for steatotic liver transplantation and improve the initial conditions of donor livers with low steatosis that are available for transplantation. Such therapies could also increase the use of numerous steatotic livers currently discarded for transplantation, thus reducing the risk of death of those patients on liver transplant waiting lists.

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Authorship Contributions

Participated in research design: Casillas-Ramírez, Alfany-Fenández, and Peralta.

Conducted experiments: Casillas-Ramírez, Alfany-Fernández, Massip-Salcedo, and Serafín.

Performed data analysis: Juan, Planas, Pallàs, Rimola, Rodés, and Peralta.

Wrote or contributed to the writing of the manuscript: Casillas-Ramírez, Alfany-Fernández, Massip-Salcedo, Serafín, Rimola, Rodés, and Peralta.

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Artículos relacionados estrechamente con el tema de la Tesis

LIVER TRANSPLANTATION 17:1099-1110, 2011

ORIGINAL ARTICLE

Cyclic Adenosine 3′,5′-Monophosphate in Rat Steatotic Liver Transplantation

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Numerous steatotic livers are discarded as unsuitable for transplantation (TR) because of their poor tolerance of ischemia/reperfusion (I/R). Cyclic adenosine 3′,5′-monophosphate (cAMP)–elevating agents protect against I/R injury both in nonsteatotic livers that have been removed from non–heart-beating donors and subjected to warm ischemia or cold ischemia (CIS) and in perfused, isolated livers. Ischemic preconditioning (PC), which is based on brief periods of I/R, protects steatotic liver grafts, but the mechanism that is responsible is poorly understood. This study examines the role of cAMP in the vulnerability shown by steatotic livers to TR-associated I/R injury and the benefits of PC in this situation. Steatotic livers with or without PC were transplanted into Zucker rats. The hepatic levels of cAMP were measured and altered pharmacologically. Our results indicate that the cAMP levels in the nonsteatotic liver grafts were similar to those found in a sham group. However, high cAMP levels were observed in steatotic liver grafts. The blockage of cAMP generation by adenylate cyclase inhibitor pre-treatment or PC had the following results: reduced hepatic injury and increased survival of steatotic graft recipients; greater preservation of adenosine triphosphate (ATP) and reduced lactate accumulation throughout CI. This blockade of cAMP by a nitric oxide–dependent mechanism protected steatotic liver grafts against oxidative stress and microvascular disorders after reperfusion. In conclusion, cAMP blocking–based strategies could protect patients against the inherent risk of steatotic liver failure after TR. ***Liver Transpl 17:1099-1110, 2011.*** © **2011 AASLD.**

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The increasing demand for organs for transplantation (TR) has led to the acceptance of steatotic livers despite their poor tolerance of ischemia/reperfusion (I/R) injury.[1,2] The use of these marginal organs is associated with an increased risk of graft dysfunction or failure after TR.[2] In addition, many steatotic livers are discarded, and this exacerbates the critical shortage of donor livers.[1] Therefore, minimization of the adverse effects of I/R on steatotic liver TR is urgently needed.

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; CIS, cold ischemia; cNOS, constitutive nitric oxide synthase; DBcAMP, dibutyryl cyclic adenosine 3′,5′-monophosphate; GSH, glutathione; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; Ln, lean; L-NAME, *N*(G)-nitro-L-arginine methyl ester; MDA, malondialdehyde; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; Ob, obese; $ONOO^-$, peroxynitrite; PC, ischemic preconditioning; ROS, reactive oxygen species; SOD, superoxide dismutase; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; TR, transplantation; XDH, xanthine dehydrogenase; XOD, xanthine oxidase.

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LIVER TRANSPLANTATION 16:1098-1111, 2010

ORIGINAL ARTICLES

Improved Rat Steatotic and Nonsteatotic Liver Preservation by the Addition of Epidermal Growth Factor and Insulin-Like Growth Factor-I to University of Wisconsin Solution

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This study examined the effects of epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) supplementation to University of Wisconsin solution (UW) in steatotic and nonsteatotic livers during cold storage. Hepatic injury and function were evaluated in livers preserved for 24 hours at 4°C in UW and in UW with EGF and IGF-I (separately or in combination) and then perfused ex vivo for 2 hours at 37°C. AKT was inhibited pharmacologically. In addition, hepatic injury and survival were evaluated in recipients who underwent transplantation with steatotic and nonsteatotic livers preserved for 6 hours in UW and UW with EGF and IGF-I (separately or in combination). The results, based on isolated perfused liver, indicated that the addition of EGF and IGF-I (separately or in combination) to UW reduced hepatic injury and improved function in both liver types. A combination of EGF and IGF-I resulted in hepatic injury and function parameters in both liver types similar to those obtained by EGF and IGF-I separately. EGF increased IGF-I, and both additives up-regulated AKT in both liver types. This was associated with glycogen synthase kinase-3β ($GSK3_\beta$) inhibition in nonsteatotic livers and $PPAR\gamma$ overexpression in steatotic livers. When AKT was inhibited, the effects of EGF and IGF-I on $GSK3_\beta$, $PPAR\gamma$, hepatic injury and function disappeared. The benefits of EGF and IGF-I as additives in UW solution were also clearly seen in the liver transplantation model, because the presence of EGF and IGF-I (separately or in combination) in UW solution reduced hepatic injury and improved survival in recipients who underwent transplantation with steatotic and nonsteatotic liver grafts. In conclusion, EGF and IGF-I may constitute new additives to UW solution in steatotic and nonsteatotic liver preservation, whereas a combination of both seems unnecessary. ***Liver Transpl 16:1098-1111, 2010.*** © **2010 AASLD.**

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Abbreviations: AKTinh, AKT inhibitor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSP, sulfobromophthalein; EGF, epidermal growth factor; $GSK3_\beta$, glycogen synthase kinase-3β; IGF-I, insulin-like growth factor-I; I/R, ischemia/reperfusion; Ln, lean; mRNA, messenger RNA; Ob, obese; pAKT, phosphorylated AKT; PCR, polymerase chain reaction; $pGSK3_\beta$, phosphorylated $GSK3_\beta$; $PPAR\gamma$, peroxisome proliferator-activated receptor-γ; TR, orthotopic liver transplantation; UW, University of Wisconsin.

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Insulin-Like Growth Factor and Epidermal Growth Factor Treatment: New Approaches to Protecting Steatotic Livers against Ischemia-Reperfusion Injury

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Hepatic steatosis is a major risk factor in ischemia-reperfusion (I/R). IGF-binding proteins (IGFBPs) modulate IGF-I action by transporting circulating IGF-I to its sites of action. Epidermal growth factor (EGF) stimulates IGF-I synthesis *in vitro*. We examined the effect of IGF-I and EGF treatment, separately or in combination, on the vulnerability of steatotic livers to I/R. Our results indicated that I/R impaired IGF-I synthesis only in steatotic livers. Only when a high dose of IGF-I (400 μg/kg) was given to obese animals did they show high circulating IGF-I:IGFBP levels, increased hepatic IGF-I levels, and protection against damage. In lean animals, a dose of 100 μg/kg IGF-I protected nonsteatotic livers. Our results indicated that the combined administration of IGF-I and EGF resulted in hepatic injury parameters in both liver types similar to that obtained by IGF-I and EGF separately. IGF-I increased *egf* expression in both liver types. The beneficial role of EGF on hepatic I/R injury may be attributable to p38 inhibition in nonsteatotic livers and to PPARγ overexpression in steatotic livers. In conclusion, IGF-I and EGF may constitute new pharmacological strategies to reduce the inherent susceptibility of steatotic livers to I/R injury. **(*Endocrinology* 150: 3153–3161, 2009)**

Hepatic steatosis is a major risk factor after liver surgery because steatotic livers show poor tolerance to ischemia-reperfusion (I/R) (1). Operative mortality associated with steatosis exceeds 14% after major resection compared with 2% for healthy livers (2, 3). In the case of transplantation, steatotic grafts are associated with a primary nonfunction rate of 60% compared with less than 5% for nonsteatotic grafts (4, 5). Therefore, developing protective strategies to minimize the adverse effects of I/R injury in steatotic livers is an urgent need.

IGF-I is member of the IGF superfamily and participates in numerous pathophysiological processes (6–9). The IGF-binding proteins (IGFBPs), which are abundant in the bloodstream, organs, and tissues act as major modulators of IGF-I action by transporting IGF-I to its sites of action (7, 10, 11). Interaction between growth factor families is a field of growing interest. Various *in vitro* studies indicate that epidermal growth factor (EGF) stimulates IGF-I synthesis in renal cells and isolated hepatocytes (12–14).

To our knowledge, the beneficial effects of IGF-I on hepatic I/R injury have been reported only in nonsteatotic livers (15), whereas the effects of EGF on hepatic I/R injury remain unknown. The first purpose of this study was to evaluate whether treatment with IGF-I and EGF could improve the poor tolerance of steatotic livers to I/R. Given that EGF enhances *igf1* expression in several cell types (12–14), the second purpose of this study was to evaluate whether EGF provides a stimulus for *igf1* expression in hepatic I/R. Finally, the third purpose of the present study was to evaluate the involvement of p38 MAPK (p38) and peroxisome proliferator-activated receptor-γ (PPARγ) in the effects of IGF-I and EGF on hepatic I/R injury in steatotic and nonsteatotic livers.

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Abbreviations: EGF, Epidermal growth factor; IGFBP, IGF-binding protein; I/R, ischemia-reperfusion; Ln, lean; Ob, obese; PPARγ, peroxisome proliferator-activated receptor-γ.

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Are Angiotensin II Receptor Antagonists Useful Strategies in Steatotic and Nonsteatotic Livers in Conditions of Partial Hepatectomy under Ischemia-Reperfusion?

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ABSTRACT

We examined whether angiotensin (Ang) II receptor antagonists could be considered a therapeutic strategy in steatotic and nonsteatotic livers in conditions of partial hepatectomy under ischemia-reperfusion (I/R), which is commonly applied in clinical practice to reduce blood loss. We report that Ang II type I receptor (AT1R) antagonist, but not Ang II type II receptor (AT2R) antagonist, increased regeneration in nonsteatotic livers. In the presence of steatosis, both AT1R and AT2R antagonists increased liver regeneration. This effect was stronger when the two were combined. Neither of the Ang II receptor antagonists protected nonsteatotic livers against damage. Only the AT1R antagonist, through nitric oxide inhibition, reduced damage in steatotic livers. The combination of the AT1R and AT2R antagonists in steatotic livers conferred a similar degree of protection to AT1R antagonist alone. Herein, we show that p38 mitogen-activated protein kinase (p38) was a key mechanism in the regeneration induced by the Ang II receptor antagonists in both liver types because when this signaling pathway was inhibited, the beneficial effects of the Ang II receptor antagonists on liver regeneration disappeared, regardless of hepatocyte growth factor or transforming growth factor β-hepatic levels. In conclusion, in conditions of partial hepatectomy under I/R, the AT1R antagonist for nonsteatotic livers and the AT1R and AT2R antagonists for steatotic livers improved regeneration in the remnant liver through p38 activation. In addition, the combination of the AT1R and AT2R antagonists in steatotic livers led to stronger liver regeneration than either antagonists used separately and also provided the same protection against damage as that afforded by AT1R antagonist alone.

In clinical situations, partial hepatectomy under ischemia-reperfusion (I/R) is usually performed to control bleeding during parenchymal dissection (Dixon et al., 2005). Hepatic steatosis, a major risk factor for liver surgery, has been associated with increased complication index and postoperative mortality after major liver resection (McCormack et al., 2007). Steatotic livers show impaired regenerative response and reduced tolerance to hepatic injury compared with nonsteatotic ones in conditions of partial hepatectomy under I/R (Behrns et al., 1998; Veteläinen et al., 2007). A further increase in the prevalence of steatosis in hepatic surgery is to

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ABBREVIATIONS: I/R, ischemia-reperfusion; Ang, angiotensin; p38, p38 mitogen-activated protein kinase; NO, nitric oxide; Ob, obese; Ln, lean; PH, partial hepatectomy; AT1R, Ang II type I receptor; losartan, 2 butyl-4-chloro-1-[*p*-(*o*-1*H*-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol monopotassium salt; AT2R, Ang II type II receptor; PD123319, *S*-(+)-1-([4-(dimethylamino)-3-methylphenyl]methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo(4,5-c)pyridine-6-carboxylic acid; spermine NONOate, *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; PCR, polymerase chain reaction; ACE, angiotensin-converting enzyme; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; ALT, alanine aminotransferase; HGF, hepatocyte growth factor; TGF, transforming growth factor; MDA, malondialdehyde; H&E, hematoxylin and eosin; PCNA, proliferating cell nuclear antigen.

Inhibition of angiotensin II action protects rat steatotic livers against ischemia-reperfusion injury

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Objective: **We examined whether pharmacologic strategies blocking angiotensin II actions protect steatotic livers against ischemia-reperfusion (I/R) injury. The effects of ischemic preconditioning (PC) on angiotensin II were also evaluated.**

Design: **Randomized and controlled animal study.**

Setting: **Experimental laboratory.**

Subjects: **Zucker rats.**

Interventions: **The following experimental groups were studied: I/R, ischemia-reperfusion + angiotensin-converting enzyme inhibitor (I/R+ACE inhibitor), ischemia-reperfusion + angiotensin II type I receptor antagonist (I/R+AT1R antagonist), ischemia-reperfusion + angiotensin II type II receptor antagonist (I/R+AT2R antagonist), and PC (5 mins of ischemia + 10 mins of reperfusion before I/R). In some of these groups, the action of bradykinin (BK) and/or peroxisome-proliferator-activated receptor-γ (PPARγ) was altered pharmacologically.**

Measurements and Main Results: **I/R+ACE inhibitor, I/R+AT1R antagonist, and I/R+AT2R antagonist reduced hepatic injury in steatotic livers compared with the I/R group. PC reduced angiotensin II generation and hepatic injury in steatotic livers in comparison to I/R group. Our results revealed that I/R+ACE inhibitor, I/R+AT1R antagonist, I/R+AT2R antagonist, and PC increased BK compared with the I/R group. In addition, the effects of PC on BK and hepatic injury were abolished when angiotensin II was administered. Furthermore, administration of BK receptor antagonists to the I/R+ACE inhibitor, I/R+AT1R antagonist, I/R+AT2R antagonist, and PC groups resulted in hepatic injury similar to the I/R group, indicating that the benefits of ACE inhibitor, AT1R antagonist, AT2R antagonist, and PC were abolished when the action of BK was inhibited. Experiments aimed at investigating why BK was protective in steatotic livers indicated that BK acts as a positive regulator of PPARγ. If PPARγ action was inhibited, BK did not protect steatotic livers against hepatic injury.**

Conclusions: **Pharmacologic blockers of angiotensin II action (ACE inhibitors, AT1R antagonists, and AT2R antagonists) and PC, which reduced angiotensin II generation, increased BK generation in steatotic livers after I/R. This in turn increased PPARγ and protected this type of liver against I/R injury. (Crit Care Med 2008; 36:1256–1266)**

KEY WORDS: steatotic liver; angiotensin II; bradykinin; interleukin; ischemia; ischemic preconditioning

Steatosis is assessed according to the pattern and amount of fatty infiltration in hepatic tissue sections. Macrovesicular steatosis is characterized by a single, bulky fat vacuole in the hepatocyte that displaces the nucleus to the edge of the cell. Microvesicular steatosis is characterized by accumulation of tiny lipid vesicles in the cytoplasm of hepatocytes without nuclear dislocation. Steatosis was classified as mild, moderate, or severe depending on whether <30%, 30–60%, or >60% of hepatocytes, respectively, displayed fatty infiltration (1). Hepatic steatosis is a major risk factor after liver surgery because steatotic liver tolerates ischemia-reperfusion (I/R) poorly. Operative mortality associated with steatosis exceeds 14%, compared with 2% for healthy livers, and the risks of primary nonfunction and dysfunction after surgery are also higher (2). Thus, developing protective strategies to minimize the adverse effects of I/R injury in steatotic livers is an urgent need.

Angiotensin (Ang) II is a classic endocrine hormone that regulates blood pressure and sodium homeostasis. Ang II also exerts several blood pressure-independent actions, including proinflammatory effects (3). When the renin-angiotensin system activates, angiotensinogen is hydrolyzed by renin to form Ang I. Then Ang I is converted by the angiotensin-converting en-

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Activation of Peroxisome Proliferator-Activated Receptor-α Inhibits the Injurious Effects of Adiponectin in Rat Steatotic Liver Undergoing Ischemia–Reperfusion

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Hepatic steatosis is a major risk factor in ischemia–reperfusion (I/R). Adiponectin acts as an antiobesity and anti-inflammatory hormone. Adiponectin activates peroxisome proliferator-activated receptor-α (PPAR-α), a transcription factor that regulates inflammation in liver disease. Ischemic preconditioning (PC) based on brief periods of I/R protects steatotic livers against subsequent sustained I/R injury, but just how this is achieved is poorly understood. This study explains the role of PPAR-α and adiponectin in the vulnerability shown by steatotic livers to I/R and the benefits of PC in this situation. PPAR-α and adiponectin levels in nonsteatotic livers undergoing I/R were similar to those found in the sham group. However, reduced PPAR-α and increased adiponectin levels, particularly the high molecular weight isoform, were observed in steatotic livers as a consequence of I/R. Our results suggest that mitogen-activated protein kinases (MAPKs) may be positive regulators of adiponectin accumulation in steatotic livers. The addition of adiponectin small interfering RNA (siRNA) before I/R protected steatotic livers against oxidative stress and hepatic injury. The induction of PC before I/R increased PPAR-α and reduced adiponectin levels in steatotic livers. PC, which increased PPAR-α, as well as PPAR-α agonist pretreatment reduced MAPK expression, adiponectin, oxidative stress, and hepatic injury that follows I/R. In addition, the administration of a PPAR-α antagonist in preconditioned steatotic livers eliminated the beneficial effects of PC on MAPKs, adiponectin, oxidative stress, and hepatic injury. *Conclusion*: Steatotic livers are more predisposed to down-regulate PPAR-α and overexpress adiponectin when subjected to I/R. PPAR-α agonists and adiponectin siRNA are promising candidates to protect steatotic livers. PPAR-α agonists as well as PC, through PPAR-α, inhibited MAPK expression following I/R. This in turn inhibited adiponectin accumulation in steatotic livers and adiponectin-worsening effects on oxidative stress and hepatic injury. (HEPATOLOGY 2007;46:000-000.)

Abbreviations: ALT, alanine aminotransferase; HMW, high molecular weight; IL, interleukin; I/R, ischemia–reperfusion; Ln, lean; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; mRNA, messenger RNA; Ob, obese; PC, ischemic preconditioning; PPAR-α, peroxisome proliferator-activated receptor-α; siRNA, small interfering RNA; TNF-α, tumor necrosis factor α.

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PROGRESOS EN HEPATOLOGÍA

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Síndrome de isquemia-reperfusión asociado al trasplante hepático: una visión actualizada

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RESUMEN

La lesión por isquemia reperfusión (I/R) es la causa principal tanto del mal funcionamiento inicial del injerto como del fallo primario en el trasplante hepático. La búsqueda de estrategias terapéuticas para prevenir la lesión por I/R ha conducido a la utilización de fármacos esperanzadores, aunque la gran mayoría de ellos no ha alcanzado una aplicación clínica. La terapia génica requiere mejorar las técnicas de transfección, evitar la toxicidad de vectores y una discusión ética antes de alcanzar el nivel clínico. El precondicionamiento isquémico (PC) es la primera estrategia terapéutica utilizada en la clínica para reducir la lesión por I/R en hepatectomías de tumores. Futuras investigaciones aportarán datos acerca de la efectividad del PC para reducir la lesión por I/R asociada al trasplante hepático, y aumentar la poca tolerancia de los injertos esteatósicos al síndrome de I/R para su utilización en el trasplante y aliviar, así, la carencia de órganos.

ISCHEMIA-REPERFUSION SYNDROME ASSOCIATED WITH LIVER TRANSPLANTATION: AN UPDATE

Ischemia-reperfusion (I/R) injury is the main cause of both initial graft dysfunction and primary failure in liver transplantation. The search for therapeutic strategies to prevent I/R injury has led to research into promising drugs, although most have not been used clinically. Gene therapy requires better transfection techniques, avoiding vector toxicity, and ethical debate before being used clinically. Ischemic preconditioning is the first therapeutic strategy used in clinical practice to reduce I/R injury in hepatectomies for tumors. Future research will provide data on the effectiveness of ischemic preconditioning in reducing I/R injury associated with liver transplantation, and in reducing the vulnerability of steatotic grafts to I/R syndrome so that they can be used in transplantation, thus relieving the organ shortage.

INTRODUCCIÓN

La historia del trasplante hepático (TH) se remonta al año de 1963, cuando Thomas Starzl realizó el primer TH en un niño que padecía atresia biliar, el cual sólo sobrevivió 5 h. En el mismo año, 2 meses más tarde, Starzl practicó su segundo TH, esta vez en adultos, y se considera a éste como el primer TH exitoso de la historia. El paciente falleció a los 22 días del postoperatorio a causa de una embolia pulmonar.

El TH está indicado en las enfermedades hepáticas progresivas en las que no sean posibles otras medidas terapéuticas y en las que la supervivencia esperada al año sea inferior a la que se conseguiría con el trasplante[1]. Las indicaciones más comunes para el TH son la cirrosis secundaria a infección crónica por el virus de la hepatitis C y la cirrosis alcohólica. En niños, la indicación principal suele ser la atresia biliar. Otras enfermedades hepáticas susceptibles de ser tratadas con TH comprenden cirrosis causada por el virus de la hepatitis B, hepatitis autoinmune, colangitis esclerosante primaria, carcinoma hepatocelular, enfermedades metabólicas (como la enfermedad de Wilson o la hemocromatosis hereditaria), e insuficiencia hepática aguda y grave; las causas comunes son las reacciones por hipersensibilidad o idiosincrasia a fármacos, entre otras[1,2]. Si bien el TH es la mejor opción terapéutica para determinadas entidades patológicas, como las mencionadas anteriormente, la isquemia-reperfusión (I/R), inherente a todo TH, es la causa principal tanto del mal funcionamiento inicial como del fallo primario del injerto hepático[3-5]; este último es responsable del 81% de los retrasplantes durante la primera semana tras la intervención quirúrgica[6]. Y si esto ocurre en hígados sanos, aún son mayores los casos de disfunción primaria cuando el injerto es esteatósico, de ahí que la esteatosis sea la causa del mayor número de óganos no aptos para trasplante (54%), acentuando así la problemática del banco de órganos[7]. Por tanto, minimizar

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Cell Injury, Repair, Aging and Apoptosis

Heat Shock Proteins and Mitogen-activated Protein Kinases in Steatotic Livers Undergoing Ischemia-Reperfusion: Some Answers

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Ischemic preconditioning protects steatotic livers against ischemia-reperfusion (I/R) injury, but just how this is achieved is poorly understood. Here, I/R or preconditioning plus I/R was induced in steatotic and nonsteatotic livers followed by investigating the effect of pharmacological treatments that modulate heat shock proteins (HSPs) and mitogen-activated protein kinases (MAPKs). MAPKs, HSPs, protein kinase C, and transaminase levels were measured after reperfusion. We report that preconditioning increased HSP72 and heme-oxygenase-1 (HO-1) at 6 and 24 hours of reperfusion, respectively. Unlike nonsteatotic livers, steatotic livers benefited from HSP72 activators (geranylgeranylacetone) throughout reperfusion. This protection seemed attributable to HO-1 induction. In steatotic livers, preconditioning and geranylgeranylacetone treatment (which are responsible for HO-1 induction) increased protein kinase C activity. HO-1 activators (cobalt(III) protoporphyrin IX) protected both liver types. Preconditioning reduced p38 MAPK and c-Jun N-terminal kinase (JNK), resulting in HSP72 induction though HO-1 remained unmodified. Like HSP72, both p38 and JNK appeared not to be crucial in preconditioning, and inhibitors of p38 (SB203580) and JNK (SP600125) were less effective against hepatic injury than HO-1 activators. These results provide new data regarding the mechanisms of preconditioning and may pave the way to the development of new pharmacological strategies in liver surgery. ***(Am J Pathol 2006, 168:1474–1485; DOI: 10.2353/ajpath.2006.050645)***

Steatotic livers tolerate ischemia-reperfusion (I/R) poorly.[1,2] Results obtained under normothermic conditions indicate that heat shock preconditioning (whole body hyperthermia) induces a marked expression of heat shock protein 72 (HSP72) and heme-oxygenase-1 (HO-1) in steatotic livers, which protect against hepatic injury.[3,4] The possible functions of HSPs in ischemic tissue include repair of damaged proteins, protection against oxidative stress, suppression of pro-inflammatory cytokines, and repair of the ion channel.[5,6] However, despite the benefits of heat shock preconditioning, its application in the clinical setting is limited.

In common with heat shock preconditioning, ischemic preconditioning also involves the induction of organ stress to protect steatotic liver against I/R injury.[7,8] There is some evidence in isolated hepatocytes and experimental models of perfused liver that mitogen-activated protein kinases (MAPKs) are involved in the protective mechanisms of ischemic preconditioning.[9,10] Signaling pathways involved in HSP induction include MAPKs. HSP72 induction includes p38 MAPK (p38) and c-Jun N-terminal kinase (JNK) in heart in response to hypoxia.[11] HO-1 overexpression by p38 and JNK has been reported in cultures of hepatocytes,[12] isolated endothelial cells,[13] and experimental models of lung I/R.[14] The respective roles of HSPs and MAPKs in inducing the benefits of ischemic preconditioning on I/R injury in steatotic livers remain unclear.

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BASIC RESEARCH

Trimetazidine: Is it a promising drug for use in steatotic grafts?

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Abstract

AIM: Chronic organ-donor shortage has led to the acceptance of steatotic livers for transplantation, despite the higher risk of graft dysfunction or nonfunction associated with the ischemic preservation period of these organs. The present study evaluates the effects of trimetazidine (TMZ) on an isolated perfused liver model.

METHODS: Steatotic and non-steatotic livers were preserved for 24 h in the University of Wisconsin (UW) solution with or without TMZ. Hepatic injury and function (transaminases, bile production and sulfobromophthalein (BSP) clearance) and factors potentially involved in the susceptibility of steatotic livers to ischemia–reperfusion (I/R) injury, including oxidative stress, mitochondrial damage, microcirculatory diseases, and ATP depletion were evaluated.

RESULTS: Steatotic livers preserved in UW solution showed higher transaminase levels, lower bile production and BSP clearance compared with non-steatotic livers. Alterations in perfusion flow rate and vascular resistance, mitochondrial damage, and reduced ATP content were more evident in steatotic livers. TMZ addition to UW solution reduced hepatic injury and ameliorated hepatic functionality in both types of the liver and protected against the mechanisms potentially responsible for the poor tolerance of steatotic livers to I/R.

CONCLUSION: TMZ may constitute a useful approach in fatty liver surgery, limiting the inherent risk of steatotic liver failure following transplantation.

Keywords: Steatotic liver, Ischemia–reperfusion, UW preservation solution

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INTRODUCTION

The mounting number of patients awaiting liver transplantation and the limited pool of donor organs have led to the acceptance of marginal livers such as steatotic livers, for transplantation, despite the higher risk of graft dysfunction or nonfunction which is associated with their ischemic preservation[1-3].

There is evidence indicating that the composition of preservation solutions is critical for the quality of livers kept for prolonged ischemic periods. University of Wisconsin (UW) preservation solution, considered as the gold standard of such solutions, has proved itself effective in preventing liver damage during cold ischemia and has extended storage time limits[4,5]. However, irreversible injury occurring after prolonged cold periods (between 16 and 24 h) has also been reported. The main aims of organ preservation, therefore, are striving to prolong organ tolerance[6,7].

Trimetazidine (TMZ), introduced as an anti-ischemic drug into the heart for over 35 years[8,9]. has also been used to protect kidneys exposed to the prolonged cold ischemia (48 h)[10,11] and is reported to protect liver against the deleterious effects of warm ischemia[12,13]. In addition, recently, it has been demonstrated that TMZ pre-treatment reduces liver injury and improves liver regeneration and survival rate in an experimental model of partial hepatectomy under hepatic blood inflow occlusion[14]. Studies examining the underlying protective mechanisms of TMZ suggest that this drug protects mitochondria in cardiomyocytes and isolated perfused heart by releasing the calcium accumulated in the matrix and by restoring mitochondrial membrane impermeability[8,15]. TMZ improves energy recovery *in vitro* and *ex vivo* in models of myocardial ischemia[8,15] and reduces oxidative stress

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Original Paper

How ischaemic preconditioning protects small liver grafts

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Abstract

Interleukin-1 (IL-1) and transforming growth factor-β (TGFβ) are key inhibitors of hepatocyte proliferation after hepatectomy. IL-1 inhibition by heat shock proteins (HSPs) has been reported in inflammatory processes. A recent study indicated the benefits of ischaemic preconditioning in reduced-size orthotopic liver transplantation (ROLT). The present study examined: (a) the effect of ischaemic preconditioning on IL-1 and TGFβ in ROLT; (b) whether preconditioning protects small liver grafts through HSP induction; and (c) whether the potential benefits of preconditioning on HSP is related to IL-1 inhibition. Our results, obtained with an IL-1 receptor antagonist, indicated the injurious effects of IL-1 in ischaemia-reperfusion (I/R) injury and established a relationship between IL-1 and growth factors. Thus, IL-1 reduced hepatocyte growth factor (HGF) and promoted TGFβ release, thus contributing to the impaired liver regeneration associated with ROLT. Preconditioning inhibited IL-1 through nitric oxide (NO), thereby protecting against the injurious effects of IL-1. In addition, by another pathway independent of NO, preconditioning induced HSP70 and haem-oxygenase-1 (HO-1). HO-1 protected against I/R injury and liver regeneration, whereas the benefits resulting from HSP70 were mainly related to hepatocyte proliferation. These results suggest a mechanism that explains the effectiveness of preconditioning in ROLT. They suggest, too, that other strategies, in addition to preconditioning, that modulate IL-1 and/or HSPs could be considered in clinical situations requiring liver regeneration such as small liver grafts.

Keywords: reduced-size liver transplantation; ischaemia-reperfusion; liver regeneration; IL-1; HSP; oxidative stress

Introduction

Living-related liver transplantation was developed to alleviate the mortality resulting from the scarcity of suitable cadaveric grafts [1,2]. The main problem in using living-related liver transplantation for adults is graft size disparity [3,4]. In addition, ischaemia-reperfusion (I/R), which is inevitable in liver transplantation, reduces liver regeneration after hepatectomy [5,6].

Ischaemic preconditioning, i.e. a short period of ischaemia followed by a brief period of reperfusion before a sustained ischaemic insult, improved hepatic regeneration in an experimental model of reduced-size liver transplantation [7]. This surgical strategy promoted the release of hepatocyte growth factor (HGF). However, hepatocyte growth is controlled by both growth-promoting and growth-inhibiting factors [8,9].

Transforming growth factor β (TGFβ), a potent inhibitor of hepatocyte DNA synthesis [10–12] counterbalances the stimulatory effects of mitogens such as HGF during liver regeneration [10,13]. However, TGFβ does not seem to be the sole or the most significant negative regulator of hepatocyte replication. In fact, interleukin-1 (IL-1) is the main inhibitor of hepatocyte proliferation after partial hepatectomy without ischaemia [14].

IL-1 biosynthesis is down-regulated through a mechanism related to the induction of heat shock proteins (HSPs) [15–19]. The toxicity of IL-1 for pancreatic cells can be prevented by haem-oxygenase-1 (HO-1) activators [16,17]. Over-expression of HSP70 limits lipopolysaccharide (LPS)-induced production of IL-1 [18]. *In vitro* and *in vivo* studies indicate the ability of HSPs to inhibit IL-1 release in lung cells [15,19].

Previous results indicate that ischaemic preconditioning induces HSP70 over-expression in isolated hepatocytes [20] and reduces hepatic IL-1 production under normothermic conditions [21]. However, to our knowledge, the possibility that preconditioning protects in reduced-size liver transplantation by inducing changes in HSP and/or IL-1 release has not been tested previously.

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Adenosine monophosphate-activated protein kinase and nitric oxide in rat steatotic liver transplantation

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***Background/Aims*: Hepatic steatosis is a risk factor for transplantation. We examined the role of AMP-activated protein kinase (AMPK) and nitric oxide (NO) in the benefits of preconditioning in steatotic liver transplantation.**

***Methods*: Steatotic liver transplantation with or without preconditioning was induced in Zucker rats. The activities of AMPK and NO synthase (NOS) were measured and altered pharmacologically.**

***Results*: Preconditioning or AMPK activation with aminoimidazole-4-carboxamide ribonucleoside (AICAR) increased AMPK and constitutive NOS activities and protected against lipid peroxidation, nitrotyrosine formation and hepatic injury in both grafts. Inhibition of AMPK activity removed the benefits of preconditioning. NO synthesis inhibition abolished the benefits of preconditioning or AICAR. Therefore, preconditioning or AICAR, through AMPK activation, may induce NO synthesis, thus protecting against hepatic injury in both steatotic and non-steatotic liver transplantation. In non-steatotic grafts, NO donors simulated the benefits of preconditioning. However, in steatotic grafts, NO supplementation was ineffective.**

***Conclusions*: These results indicate (a) a potential relationship between AMPK and NO in the benefits of preconditioning in steatotic liver transplantation, (b) AICAR as a new phamacological strategy in steatotic liver transplantation and (c) a differential effect of NO supplementation in both grafts.**

***Keywords*: Preconditioning; Fatty liver; Oxidative stress; Peroxynitrite; Cold ischemia; Reperfusion**

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Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; NO, nitric oxide; AICAR, aminoimidazole-4-carboxamide ribonucleoside; I/R, ischemia-reperfusion; ATP, adenosine triphosphate; AMP, adenosine monophosphate; Ob, obese; Ln, lean; TX, transplantation; UW solution, University of Wisconsin solution; PC, preconditioning; araA, adenine 9-β-D-arabinofuranoside; NAME, Nω-nitro-L-arginine methyl ester hydrochloride; AST and ALT, aspartate and alanine aminotransferase; MDA, malondialdehyde; cNOS, constitutive nitric oxide synthase; iNOS, inducible NOS; $ONOO^-$, peroxynitrite; SAMS peptide, synthetic peptide His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg; ADP, adenosine diphosphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; O_2^-, superoxide; ROS, reactive oxygen species.

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