The use of a reversible proteasome inhibitor in a model of Reduced-Size Orthotopic Liver transplantation in rats

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A B S T R A C T
Ischemia/reperfusion injury (IRI), inherent in liver transplantation (LT), is the main cause of initial deficiencies and primary non-function of liver allografts. Living-related LT was developed to alleviate the mortality resulting from the scarcity of suitable deceased grafts. The main problem in using living-related LT for adults is graft size disparity. In this study we propose for the first time that the use of a proteasome inhibitor (Bortezomib) treatment could improve liver regeneration and reduce IRI after Reduced-Size Orthotopic Liver transplantation (ROLT). Rat liver grafts were reduced by removing the left lateral lobe and the two caudate lobes and preserved in UW or IGL-1 preservation solution for 1 h liver and then subjected to ROLT with or without Bortezomib treatment. Our results show that Bortezomib reduces IRI after LT and is correlated with a reduction in mitochondrial damage, oxidative stress and endoplasmic reticulum stress. Furthermore, Bortezomib also increased liver regeneration after reduced-size LT and increased the expression of well-known ischemia/reperfusion protective proteins such as nitric oxide synthase, heme oxygenase 1 (HO-1) and Heat Shock Protein 70. Our results open new possibilities for the study of alternative therapeutic strategies aimed at reducing IRI and increasing liver regeneration after LT. It is hoped that the results of our study will contribute towards improving the understanding of the molecular processes involved in IRI and liver regeneration, and therefore help to improve the outcome of this type of LT in the future.

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Introduction

Ischemia/reperfusion injury (IRI) is inherent in liver transplantation (LT) and is the main cause of initial deficiencies and primary non-function of liver allograft (Busuttil and Tanaka, 2003). Therefore, minimizing the adverse effects of IRI could increase both the number of suitable transplantation grafts and patients who successfully recover from LT. The first step towards this objective is to fully understand the mechanisms involved in IRI.

Living-related LT was developed to address the high mortality rate caused by the lack of suitable deceased grafts (Malago et al., 1995; Samstein and Emond, 2001). The main problem in using living-related LT for adults is graft size disparity (Kawasaki et al., 1998; Schiano et al., 2001). In addition I/R, which is inevitable in LT, impairs liver regeneration after hepatectomy (Foschi et al., 1993; Selzner et al., 1999).

Different strategies have been proposed and used to reduce IRI and improve liver regeneration after reduced-size LT (ROLT), which include the pharmacological modulation of several factors (Padrissa-Altés et al., 2009), ischemic preconditioning (Padrissa-Altés et al., 2010) and the use of preservation solutions (Zaouali et al., 2011). A new preservation solution named the Institute Georges Lopez-1 (IGL-1)® solution has been proposed as an effective alternative to the University of Wisconsin solution (UW solution) in clinical kidney transplantation (Badet et al., 2005a, 2005b), and in experimental orthotopic LT models (Ben Abdennebi et al., 2006; Franco-Gou et al., 2007). IGL-1® solution is characterized by inversion of K+ and Na+ concentrations in the UW solution and contains polyethylene glycol as an osmotic support rather than HES. Its benefits are associated in part, with the prevention of oxidative stress and its capacity to generate nitric oxide (NO) (Ben Mosbah et al., 2006).
The proteasomes are complex proteinases responsible for the degradation of intracellular proteins (Ciechanover, 1994). The 26S proteasome is part of the ubiquitin proteasome pathway (UPP) and is responsible for the degradation of ubiquitinated protein substrate. Ubiquitination is the process by which ubiquitin is covalently conjugated to a substrate protein via an isopeptide linkage to the substrate's lysine residue, or by attaching to its N-terminal amino acid. Substrates that have chains of ubiquitin can be identified by the 26S proteasome which degrades the substrate into peptides and recycles the ubiquitin (Pickart, 2001). There are several natural and synthetic compounds that act as proteasome inhibitors and their potential to be used in the treatment of human diseases other than hepatic IRI has been considered in previous reviews (Lee and Goldberg, 1998; Murray and Norbury, 2000; Myung et al., 2001). One of these compounds – Bortezomib (BRZ) – has been approved for clinical trials. BRZ has been shown to block the UPP pathway in cancer (Velcade; Millennium Pharmaceuticals), and its use was approved by the US Food and Drug Administration in 2003 (Bedford et al., 2011).

Several studies have shown UPS inhibition to be protective against IRI in different organs. Majetschak et al. (2008) reported that proteasome activity was reduced during cold ischemia in a model of murine heart transplantation. Other studies have demonstrated that proteasome inhibition can reduce injury in models of isolated perfused rat heart through the decrease in the adherence of polymorphonuclear leukocytes to the endothelium (Campbell et al., 1999). Other studies have shown that proteasome inhibitors reduce inflammation (reviewed in Elliott and Ross, 2001). Regarding BRZ, there are previous reports that support our findings. Bardag-Gorce et al. (2011) showed that BRZ decreases oxidative stress in a model of rat alcoholic liver disease. They also reported that BRZ increased the expression of antioxidative enzymes and decreased ROS burst.

In the present study our aim was to investigate the effects of UPS inhibition on IRI and liver regeneration in a model of ROLT. We found that BRZ reduced IRI after LT and was correlated with a reduction in mitochondrial damage, oxidative stress and endoplasmic reticulum (ER) stress.

Our results may lead to the study of alternative therapeutic methods aimed at reducing IRI and increasing liver regeneration after LT (the main limiting factors of ROLT), which would therefore improve the success rate of this kind of LT.

Material and methods

Experimental animals

Male Sprague–Dawley rats (200–250 g) were used as donors and recipients. All animals were anesthetized with isoflurane. Research procedures complied with the European Union regulations for animal experiments (EU guideline 86/609/EEC) and the “Principles of Laboratory animal care” by NIH publication Vol 25, No. 28 revised 1996.

Experimental design

The following experimental procedures were carried out:

1) Sham (n = 6): the abdomen will be opened and the liver lobes will be briefly pulled out the abdominal cavity.

2) Reduced-size orthotopic LT (ROLT + UW) (n = 6): liver was reduced by removing the left lateral lobe and the two caudate lobes and preserved with cold University of Wisconsin (UW) solution for 1 h. ROLT was performed according to Kamada’s cuff technique without arterialization (Franco-Gou et al., 2004). Animals were killed after 6 h of reperfusion for sample collection.

3) ROLT + IGL-1 ( n = 6): Same as group 2 but using IGL-1 solution instead of UW solution.

4) ROLT + UW + BRZ ( n = 6): Same as group 2 but donor livers were treated with a UPS inhibitor. Donor and recipient animals were treated with BRZ (Selleck Chemicals, TX, USA) (0.1 mg/kg body weight, i.v.) (Anan et al., 2006) 25 min before ischemia and just after surgery, respectively.

5) ROLT + IGL-1 + BRZ ( n = 6): Same as group 3 but donor livers were treated with a UPS inhibitor. Donor and recipient animals were treated with BRZ (Selleck Chemicals, TX, USA) (0.1 mg/kg body weight, i.v.) (Anan et al., 2006) 25 min before ischemia and just after surgery, respectively.

Proteasome chymotryptic-like activity assay


Transaminase assay

Hepatic injury was assessed in terms of transaminase levels with commercial kits from RAL (Barcelona, Spain) (Zaouali, 2010). Brieﬂy, 200 μL of plasma was added to the substrate provided by the commercial kit and then alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels were measured at 365 nm with a UV spectrometer and calculated following the supplier’s instructions.

Western bloting of 20S and 19S proteasome, ATF4, CHOP and β-actin

Liver tissue was homogenized as previously described and proteins were separated by SDS-PAGE and transferred to PVDF membranes (Alfany-Fernandez et al., 2009; Massip-Salcedo et al., 2008). Membranes were immunoblotted using the following antibodies: 20S and 19S proteasome subunit (ENZO life sciences), ATF4, C/EBP-homologous protein (CHOP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Sigma Chemical, St. Louis, MO, USA). Signals were detected by the enhanced chemiluminescence kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by scanning densitometry as previously described (Massip-Salcedo et al., 2008).

Lipid peroxidation assay

Lipid peroxidation in liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction (Ben Mosbah et al., 2006).

Glutamate dehydrogenase activity

Glutamate dehydrogenase (GLDH) was used as an indirect measure of mitochondrial damage. GLDH was measured in plasma as described elsewhere (Ben Mosbah et al., 2010).

TNF-α determination

Plasma TNF-α levels were measured using a commercial immunoassay kit for rat TNF-α from Biosource (Camarillo, CA, USA) (Fernandez et al., 2004).
Histology and proliferating cell nuclear antigen (PCNA) labeling index

To quantify the severity of hepatic injury, hematoxylin and eosin-stained sections were evaluated by a point-counting method on an ordinal scale as described elsewhere (Franco-Gou et al., 2004; Padrissa-Altes et al., 2010). Hepatic proliferation was assessed in liver biopsies by immunohistochemistry as previously described (Franco-Gou et al., 2004; Padrissa-Altes et al., 2010).

Constitutive nitric oxide synthase (eNOS), heme oxygenase (HO-1) and HSP-70 immunohistochemistry

Immunohistochemistry was performed as described elsewhere (Zaouali et al., 2010a, 2010b) using the following primary antibodies: anti-NOS-3, Polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz CA, USA; anti-HO-1, Polyclonal IgG, Sigma Chemical, St Louis, MO; HSP-70 monoclonal antibody, BD Science. Images were obtained with a Nikon Eclipse E1000 fluorescence microscope.

Statistics

Data are expressed as mean ± standard error. Statistical comparison was performed by variance analysis, followed by the Student–Newman–Keuls test. p < 0.05 was considered significant.

Results

Proteasome inhibitor BRZ reduces liver injury and improves regeneration after ROLT

As compared with sham liver 20S proteasome contents and chymotryptic-like proteasome activities were reduced in all animals after ROLT and cold preservation (Figs. 1A and B). Livers submitted to ROLT and preserved in UW solution (ROLT+UW group) showed higher chymotryptic-like proteasome activities and 20S proteasome protein levels than livers submitted to ROLT and preserved in IGL-1 solution (Figs. 1A and B). The combined administration of BRZ and the use of IGL-1 caused the highest inhibition of chymotryptic-like
proteasome activities and 20S proteasome protein levels. In contrast, the 19S subset protein levels were unchanged between all experimental groups (Fig. 1B). The reduction of chymotryptic-like proteasome activities and 20S proteasome protein levels positively correlated with a significant lower AST and ALT levels determined in rats submitted to ROLT using the IGL-1 solution than ones submitted to ROLT using UW (Fig. 2A). BRZ treatment in animals submitted to ROLT resulted in a reduction of ALT and AST levels after 6 h of reperfusion (Fig. 2A). The combined use of IGL-1 solution and BRZ treatment in animals submitted to ROLT further decreased levels of transaminase in animals, much more so than the combined use of BRZ treatment and UW solution (Fig. 2A). This was corroborated with liver histological studies, which showed that the occurrence of grade 3 necrosis was lower in animals submitted to ROLT using IGL-1 solution than those submitted to ROLT using UW solution (Fig. 3B). Furthermore, BRZ treatment in animals submitted to ROLT using IGL-1 solution further reduced necrotic damage; the occurrence of grade 3 necrosis was reduced in animals treated with BRZ compared to those that were not. In addition, GLDH, which is a sensitive and specific marker of liver disease in all animals (Zaouali et al., 2010c), was analyzed and the results revealed that animals submitted to ROLT using IGL-1 solution suffered less liver damage than those submitted to ROLT using UW solution. Additionally, BRZ treatment further reduced GLDH activity levels in both groups. Animals submitted to ROLT using IGL-1 solution and treated with BRZ showed a synergic effect in liver damage, resulting in a significant decrease in the level of GLDH activity (Fig. 2B).

The lipid peroxidation assay revealed that the MDA levels that were lower in animals submitted to ROLT using IGL-1 compared to those submitted to ROLT using UW solution, were significantly lower when animals were treated with BRZ (Fig. 3A).

With regard to liver regeneration, no significant differences were observed between sham-operated animals and animals submitted to ROLT using either one of the two solutions. However, when animals were treated with BRZ there was a significant increase in PCNA-positive hepatocytes in the group in which UW solution was used, and an increase in the liver regeneration rate compared to their control groups, ROLT + UW and ROLT + IGL-1, respectively (Fig. 4).

**Mechanisms involved in the reduction of injury and improvement of regeneration by proteasome inhibition in ROLT**

Animal livers submitted to ROLT using IGL-1 solution had higher levels of ATF4 protein than those submitted to ROLT using UW solution (Fig. 5A). Livers submitted to ROLT and treated with BRZ showed even higher levels of ATF4 protein expression when compared to

![Image](image.png)

**Fig. 2.** Effect of Bortezomib treatment on hepatic injury after reduced-size LT. A) Transaminase levels (ALT and AST) and B) GLDH levels were measured in the plasma from the experimental groups shown in the graph (n = 6 for each group). *p < 0.05 versus Sham; +p < 0.05 versus ROLT + UW; °p < 0.05 versus ROLT + IGL-1.
Fig. 3. Effect of Bortezomib treatment on oxidative stress and necrosis after reduced-size LT. A) Lipid peroxidation assay: MDA levels were measured in all experimental groups shown in graph (n = 6 for each group). B) Histological analysis of the liver; a: Sham: No lesion; b: ROLT + UW and c: ROLT + IGL-1: Extensive and multifocal areas of necrosis of hepatocytes with hemorrhage and neutrophil infiltration; d: ROLT + UW + BRZ and e: ROLT + IGL-1 + BRZ: focal and small areas of hepatocyte necrosis with neutrophil infiltration. Original magnification 500. Black bar indicates 50 μm. *p < 0.05 versus Sham; +p < 0.05 versus ROLT + UW; °p < 0.05 versus ROLT + IGL-1.
their control groups. ROLT + IGL-1 + BRZ was the group with the highest levels of ATF4 protein expression (Fig. 5A). A contrasting pattern was observed with CHOP protein, whose expression was lower in groups in which IGL-1 solution was used and even lower in livers treated with BRZ (Fig. 5B).

Our results also show that the use of the preservation solution IGL-1 in ROLT can reduce TNFα in the plasma more than when UW solution is used (Fig. 6). Additionally, BRZ treatment further reduced the levels of TNFα in the plasma of animals submitted to ROLT compared to the control groups (Fig. 6). As before, animals submitted to ROLT using IGL-1 and treated with BRZ were most affected i.e. showed the lowest levels of TNFα in the plasma (Fig. 6).

In addition, by studying eNOS and HO-1 by immunofluorescence, we observed that sham livers showed weak staining for endothelial eNOS, which was localized typically in a few sinusoidal endothelial cells and in very few hepatocytes. The positivity for eNOS slightly increased (mainly in endothelial cells) when livers were submitted to ROLT using UW solution. The positivity for eNOS was even more pronounced when livers were submitted to ROLT using the IGL-1 solution, than with the UW solution. BRZ treatment increased the livers’ positivity for eNOS in both hepatocytes and endothelial cells, especially when applied before ROLT using IGL-1, rather than UW solution (Fig. 7). In sham livers, small numbers of cells expressing HO-1 were found and were distributed mainly in periportal areas. The positivity for HO-1 increased (mainly in periportal sinusoidal lining cells) when livers were submitted to ROLT using both the IGL solution and the UW solution. BRZ treatment further increased positivity for HO-1 (both in hepatocytes and in sinusoidal lining cells) compared to groups in which BRZ was not used (Fig. 8). Finally, we investigated the presence of HSP70 in livers submitted to ROLT. In sham livers

Fig. 4. Effect of Bortezomib treatment on liver regeneration after reduced-size LT. A) PCNA positive hepatocytes were analyzed in livers from all experimental groups shown in the graph (n = 6 for each group). B) Immunological staining for PCNA; a: Sham: Lower positive cells number than b: ROLT + UW and c: ROLT + IGL-1, which resulted in lower positive cell numbers than d: ROLT + UW + BRZ and e: ROLT + IGL-1 + BRZ. Original magnification 500. Black bar indicates 50 μm. *p < 0.05 versus Sham; +p < 0.05 versus ROLT + UW; °p < 0.05 versus ROLT + IGL-1.
preservation solutions were used in animals submitted to ROLT was increased following BRZ treatment when both UW and IGL-1 of HSP70 protein (both in hepatocytes and in sinusoidal lining cells) HSP70 was clearly found also in sinusoidal lining cells. The expression for protein levels of CHOP.*p of each group). A) Densitometric analysis for protein levels of ATF4 and B) Densitometric analysis for protein levels of CHOP. -p<0.05 versus Sham; +p<0.05 versus ROLT+UW; *p<0.05 versus ROLT+IGL-1.

Discussion

The two main limiting factors of living donor LT are IRI and liver regeneration; hence to improve the clinical outcome of this kind of LT investigations need to focus on how to overcome these two weaknesses and make the use of living donors a feasible option, with the desired result being to increase the number of donors. To accomplish this, it is necessary to understand the molecular basis of IRI and liver regeneration and to identify potential pharmacological treatments that can improve both situations. In this study, we report for the first time that UPS inhibitors, such as BRZ, can reduce IRI and improve liver regeneration after ROLT in an experimental model in the rat.

Here we demonstrated for the first time that the use of IGL-1 preservation solution per se reduced more the activities of proteasome when compared to UW solution during liver preservation. In addition, BRZ treatment decreased more the proteasome activity when the liver was preserved in IGL-1, compared to UW. The decrease of proteasome activity is related with the diminutions of Transaminase and GLDH levels. Our results indicate also that BRZ reduced oxidative stress and grade 3 necrosis when administered in livers submitted to ROLT, using both UW and IGL-1 solutions. BRZ is particularly effective in livers preserved in IGL-1 solution which can explain in part the suitability of this solution. The benefits of IGL-1 solution against oxidative stress and IRI have been previously described by our group (Ben Mosbah et al., 2006). However, the use of this solution in combination with a UPS inhibitor is new, and it seems to have further beneficial effects. These results are in accordance with previous studies that report that UPS inhibition is protective against IRI in different organs. For instance, Majetschak et al. (2008) proposed that UPS inhibition may be useful in maintaining the physiological ubiquitin–protein conjugate pool during organ preservation, and may therefore prolong organ preservation. Moreover, other studies have demonstrated that proteasome inhibition can reduce injury in models of isolated perfused rat heart by decreasing the ability of polymorphonuclear leukocytes to adhere to the endothelium. Other inhibitors of the proteasome, such as MG132, lactacystin and epoxomicin, were used to prevent ischemia–reperfusion injury of the organs (Alexandrova et al., 2008; Geng et al., 2009; Jing et al., 2011; Yao et al., 2007). All of these compounds inhibit proteasome activity, and even epoxomicin can inhibit the activity of the immunoproteasome, the key complex in the inflammatory response (Fenteany and Schreiber, 1998; Meng et al., 1999). However, these compounds are not highly specific to proteasome and are irreversible inhibitor which causes more damage than cytoprotective effects obtained with BRZ treatment. There is also published data that describes UPS involvement in IRI and does not support the notion that proteasome inhibitors could be potential candidates for the treatment of IRI (Ge et al., 2007; Powell et al., 2005; Rudic et al., 2010). For instance, a study on cardiac I/R reported that under conditions that foster excessive inhibition of the proteasome, the removal of oxidized proteins by the 20S proteasome would be impaired, thus hindering recovery (Powell et al., 2005). Numerous proteins, some of which may be pro-apoptotic, would then accumulate leading to the death cells. These contradictory results can be explained by differences in the experimental models as well as in dose, type of inhibitor and time of inhibition, which reinforces the idea that the effect of UPS inhibition is somewhat dependent on the degree of proteasome activity inhibition in the different tissues (for example, leukocytes vs. heart). To account for this, we used BRZ, which has been approved for clinical trials. BRZ has proven therapeutic potential of intervention of the UPS described in cancer (Velcade; Millennium Pharmaceuticals), and was approved by the US Food and Drug Administration in 2003. It is currently approved for the treatment of multiple myeloma and mantle cell lymphoma (Kane et al., 2007; Suh and Goy, 2008; Wei and Roberts, 2008). The concentration used in this study was in the range of the recommended therapeutic concentration (Anan et al., 2006).

In terms of hepatic regeneration, the results presented in this study show for the first time that the use of BRZ as a pharmacological treatment in ROLT increases liver regeneration after surgery, especially when livers are preserved in UW solution. BRZ induced regeneration in the liver after 6 h of reperfusion in ROLT, at which stage the levels of regeneration in transplanted livers were as low as in the sham group. In the group in which livers submitted to ROLT using IGL-1 solution the increase in liver regeneration was not significant, but there was an obvious tendency towards this end.
The mechanisms by which BRZ has such protective effects can be multiple and diverse. However, BRZ inhibits reversibly UPS and this is intimately related to the ER (Vembar and Brodsky, 2008). We investigated whether UPS inhibition induced ER stress. Indeed, we observed that BRZ treatment induced ATF4 protein synthesis and reduced CHOP protein synthesis. ER stress activates the unfolded protein response (UPR), which is the specific signaling that occurs between the nucleus and the ER in response to ER stress (Schroder and Kaufman, 2005). Different stimuli are signaled through several protein kinases to upregulate the protein folding capacity of the ER. Although pathways that respond to ER stress help cope with incorrectly folded protein in the ER, destructive pathways still occur with large accumulations of incorrectly folded protein. When these adaptive responses are not sufficient to relieve ER stress, the damaged cells undergo apoptosis (Kim et al., 2006). In this sense, different proteins and factors are involved in both of these processes. For instance, ATF4 promotes the activation of the UPR to rescue the ER situation (Blais et al., 2004). Moreover, ATF4 is known to induce antioxidant genes and genes of the ER protein maturation machinery (Harding et al., 2003), which further illustrates that this protein is involved in the rescue of cell death. However, some authors have proposed that CHOP proteins have evolved to promote the death of individual cells in response to insurmountable levels of ER stress (Oyadomari et al., 2002a). Others have claimed that CHOP plays an important role in

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![Fig. 6. Effect of Bortezomib treatment on TNFα production after reduced-size LT. TNFα levels were measured in plasma of animals of all experimental groups (n=6 for each group). *p<0.05 versus Sham; +p<0.05 versus ROLT+UW; °p<0.05 versus ROLT+IGL-1.](image)

![Fig. 7. Effect of Bortezomib treatment on eNOS protein levels after reduced-size LT. eNOS immunohistochemistry was performed in livers from animals of all experimental groups (n=6 for each group). Sham livers showed only weak staining for endothelial eNOS, which was localized typically in a few sinusoidal endothelial cells and in very few hepatocytes. The positivity for eNOS slightly increased (mainly in endothelial cells) when livers were submitted to ROLT using UW solution. The positivity for eNOS was even more augmented when livers were submitted to ROLT using IGL-1 solution, rather than with UW solution. BRZ treatment increased positivity for eNOS, both in hepatocytes and in endothelial cells, especially when applied before ROLT using IGL-1, rather than with UW solution.](image)
promoting ER stress-induced apoptosis (Oyadomari and Mori, 2004) and that its deletion ameliorates tissue damage during ER stress (Oyadomari et al., 2001, 2002b; Zinszner et al., 1998). Our results show that levels of the ATF4 protein were upregulated when IGL-1 solution was used instead of UW solution and that this upregulation was even higher in those livers submitted to ROLT using IGL-1 solution and BRZ. The increase of ATF4 protein levels correlated with a reduction in liver injury. These results are correlated with the fact that stress pathways such as hypoxia activate this protein to promote stress resistance, redox homeostasis and inhibition of apoptosis (Rzymski et al., 2009). In addition, ATF4 plays a crucial role in forming resistance to the proteasome inhibitor BRZ by inducing the prosurvival pathways, such as autophagy, which relieve the protein overload in BRZ treated cells (Rzymski et al., 2009). ATF4 is also selectively degraded by the proteasome under normal growth conditions, which means that under BRZ treatment conditions, ATF4 protein levels are even higher (Lassot et al., 2001, 2005). Furthermore, ATF4 can form dimers with the redox-sensitive NRF2 and regulate response to oxidative stress following ER stress by the expression of HO-1, NAD(P)/H:quinone reductase1 (NQO1) and glutathione S transferase (GST) (Cullinan and Diehl, 2004; Cullinan et al., 2003). This correlates with our results for immunofluorescence, which show an increase in HO-1 protein levels in groups in which ATF4 is also upregulated.

Finally, several studies have demonstrated that ER stress and proteasome inhibition trigger autophagy (Ding et al., 2007; Ogata et al., 2006; Shintani and Klionsky, 2004; Yorimitsu and Klionsky, 2007). This process could help the cell recover from the protein overload in BRZ treated animals (Yorimitsu and Klionsky, 2007). It may also increase ATP availability and thus alleviate the decrease in the energetic metabolism observed after ROLT. All these observations indicate that ATF4 upregulation offers a protective strategy for the cell to survive after ROLT by decreasing ER and oxidative stress and by ameliorating the energetic state of the cell. Furthermore, the decrease in CHOP protein levels due to BRZ inhibition protects the cells against apoptosis and consequently the liver tissue is better preserved after ROLT.

BRZ treatment had an effect not only on ER stress but also on TNFα production in the liver by decreasing its synthesis. TNFα is known to participate in the early stages of liver regeneration (Akerman et al., 1992; Kahn et al., 1994; Yamada et al., 1997) but is also well known for its damaging effects in the liver after I/R (Peralta et al., 1997). Even though TNFα may be essential for regeneration (Gallucci et al., 2000) this cytokine can still cause damage in conditions of IRI. Thus, the reduction in TNFα observed in groups treated with BRZ may be beneficial in terms of preserving the liver and decreasing injury but may also mean that there is not enough TNFα to induce liver regeneration. Contrary to what one may expect, our results showed that TNFα levels did not correlate with liver regeneration rates, revealing that in this model – and possibly due to the differential effect of TNFα on IRI and liver regeneration – TNFα may not play a major role in liver

Fig. 8. Effect of Bortezomib treatment on HO-1 protein levels after reduced-size LT. HO-1 immunohistochemistry was performed in livers from animals from all experimental groups (n = 6 for each group). In sham livers, a small number of cells expressing HO-1 were found and were distributed mainly in periportal areas. The positivity for HO-1 increased (mainly in periportal sinusoidal lining cells) when livers were submitted to ROLT using IGL solution, and also using UW solution. BRZ treatment further increased positivity for HO-1 (both in hepatocytes and in sinusoidal lining cells) compared to those groups in which BRZ was not used.
regeneration, but merely a secondary role. We observed that BRZ induced hepatic regeneration regardless of the preservation solution used. Further studies will be needed to clarify the mechanisms behind this effect. Nonetheless, we cannot rule out the possibility that the induction of liver regeneration is a consequence of the reduction in liver injury brought on by the use of BRZ, as it is well known that hepatic regeneration is reduced under I/R conditions (Foschi et al., 1993; Portugal et al., 1995; Selzner et al., 1999). In addition, a previous study carried out by our group correlated HO-1 and HSP70 induction with ischemic preconditioning, a surgical strategy that reduces IRI and improves liver regeneration after ROLT. The protection conferred by HSP70 in preconditioning was mainly related to cellular proliferation processes (Franco-Gou et al., 2006). For this, we also investigated the possible effect of BRZ on HSP70 protein levels and observed that this treatment also enhanced HSP70 protein expression.

Previous studies carried out by our group found that the use of IGL-1 better preserved livers submitted to I/R and that this was associated with an increase in the production of NO by the eNOS, which facilitates the up-regulation of other well-known cytoprotective genes, such as hypoxia-inducible factor-1 alpha (HIF-1alpha) and HO-1. During normoxic reperfusion, the presence of NO allows HIF-1alpha accumulation to inhibit prolyl-hydroxylases, thus promoting an additional over-expression of HO-1 in steatotic and non-steatotic liver grafts preserved in IGL-1 (reviewed in Zaouali et al., 2010b). In our conditions, BRZ treatment increased NO and HO-1 protein levels after ROLT, particularly when IGL-1 was used.

To conclude, the results of the present study demonstrate that the improvement of liver preservation conferred by IGL-1 solution after ROLT can be further enhanced with the use of the proteasome inhibitor BRZ, which was not only able to reduce liver injury after ROLT but also ameliorate oxidative stress, ER stress and TNFalpha production. Moreover, this treatment also upregulated NO, HO-1 and HSP 70 protein levels, and was correlated with a reduction in IRI and oxidative stress. Last but not least, BRZ treatment induced liver regeneration after ROLT, thus providing a base for future research on how to promote hepatic regeneration in this kind of transplantation in which difficulties in subsequent liver regeneration are a major limiting factor. Based on the results of our study we recommend the use of BRZ either as an additive to IGL-1 preservation solution or as an intravenous drug to improve the outcome of living-donor LTs by reducing liver injury and increasing hepatic regeneration.

Author’s contribution

SPA: designed research, performed research, analyzed the data and wrote the paper.

Fig. 9. Effect of Bortezomib treatment on HSP70 protein levels after reduced-size LT. HSP70 immunohistochemistry was performed in livers from animals of all experimental groups (n = 6 for each group). In sham livers only a small number of periportal hepatocytes were positive for HSP70 expression. In livers of animals submitted to ROLT using UW solution, the positivity for HSP70 slightly increased in parenchymal cells. And in livers of animals submitted to ROLT using IGL-1 solution, HSP70 was also clearly found in sinusoidal lining cells. The expression of HSP70 protein (both in hepatocytes and in sinusoidal lining cells) was increased by BRZ treatment in both UW and IGL-1 preservation in ROLT.
MAZ: designed research, performed research and analyzed the data. EB, TC and EBR: helped perform research. AS: performed the histological studies. RB, FBG and JO: designed research, analyzed the data and helped write the paper. JRC: designed research and wrote the paper.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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