IGL-1 solution reduces endoplasmic reticulum stress and apoptosis in rat liver transplantation

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Injury due to cold ischemia reperfusion (I/R) is a major cause of primary graft non-function following liver transplantation. We postulated that I/R-induced cellular damage during liver transplantation might affect the secretory pathway, particularly at the endoplasmic reticulum (ER). We examined the involvement of ER stress in organ preservation, and compared cold storage in University of Wisconsin (UW) solution and in Institute Georges Lopez-1 (IGL-1) solution. In one group of rats, livers were preserved in UW solution for 8 h at 4 °C, and then orthotopic liver transplantation was performed according to Kamada's cuff technique. In another group, livers were preserved in IGL-1 solution. The effect of each preservation solution on the induction of ER stress, hepatic injury, mitochondrial damage and cell death was evaluated. As expected, we found increased ER stress after liver transplantation. IGL-1 solution significantly attenuated ER damage by reducing the activation of three pathways of unfolded protein response and their effector molecules caspase-12, C/EBP homologous protein-10, X-box-binding protein 1, tumor necrosis factor-associated factor 2 and eukaryotic translation initiation factor 2. This attenuation of ER stress was associated with a reduction in hepatic injury and cell death. Our results show that IGL-1 solution may be a useful means to circumvent excessive ER stress reactions associated with liver transplantation, and may optimize graft quality.
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Cells, tissues and organs that are routinely transplanted are often exposed to environmental stress. Hypothermia is believed to protect cells by slowing their metabolism during hypoxic storage. But it also causes injury, which is aggravated by all classically used preservation solutions.[1] Injury induced by hypothermia is a major cause of primary graft non-function following liver transplantation.[2] However, the introduction of University of Wisconsin (UW) solution improved graft and patient survival.[3,4] This liquid, considered as the gold standard of preservation solution, has proved effective in preventing organ damage during cold ischemia and has extended the storage time.[3,4] However, UW solution does have certain properties that hinder organ preservation, such as its increased viscosity and the presence of hydroxyethyl starch (HES).[5,6] Recently, we have described Institute Georges Lopez-1 (IGL-1) preservation solution as an effective alternative to UW solution.[7,8] IGL-1 composition is characterized by the reversion of the ionic K/Na concentrations and the replacement of HES by polyethylene glycol 35 (PEG-35). These characteristics render IGL-1 suitable for abdominal organ preservation.[7–11] However, the mechanisms by which IGL-1 protects against ischemia reperfusion (I/R) injury and

maintains cell viability are unclear. Accumulating evidence suggested perturbations at the endoplasmic reticulum (ER) as novel subcellular effectors, possibly involved in the promotion of cell death during cold organ preservation.[12–14] Indeed, I/R stimulates a large increase in intracellular Ca^{2+} concentration resulting from a depletion of Ca^{2+} from the ER lumen.[15] In turn, this mobilization of Ca^{2+} modulates mitochondrial Ca^{2+} responses and hepatocyte susceptibility to apoptosis, which are manifested by the release of cytochrome *c* and caspase-9 activation. Moreover, once the ER homeostasis is perturbed, newly synthesized unfolded proteins accumulate in the organelle, resulting in ER stress.[16,17] To cope with accumulated unfolded proteins, mammalian cells trigger a specific adaptative response called the unfolded protein response (UPR).[12,16,17]

The UPR has three branches: inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor (ATF6). These proteins are normally held in inactive states in ER membranes by binding to intra-ER chaperones, particularly the 78-kD glucose-regulated/binding immunoglobulin protein (GRP78). In response to stimuli that divert ER chaperones to misfolded proteins, IRE1, PERK and ATF6

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initiate signal transduction processes. These events promote the expression of genes required to fold newly synthesized proteins and to degrade the unfolded proteins. Moreover, homeostasis and normal ER function are restored. However, when injury is excessive, the same ER stress signal transduction pathways can also induce cell death.[12,16,17] The first of the three branches of the UPR includes IRE1α, which, once activated, induces the unconventional splicing of the mRNA encoding X-box-binding protein 1 (XBP-1). The cytosolic domain of activated IRE1α binds the tumor necrosis factor (TNF)-associated factor 2 (TRAF2), and triggers the activation of the c-Jun N-terminal kinase (JNK), MAPK p38 and caspase-12.[16,18] The second branch is mediated by PERK, which phosphorylates Ser[21] of the α subunit of eukaryotic translation initiation factor 2 (eIF2α). eIF2α phosphorylation induces translation of a basic-region leucine zipper transcription factor ATF4 and subsequent expression of the ATF4 target genes, C/EBP homologous protein-10 (CHOP).[12,16,19] The third branch is mediated by the basic-region leucine zipper transcription factor ATF6, which is activated by regulated intramembrane proteolysis.[12,16,20]

We report the results of an experimental study that aimed to evaluate the involvement of ER stress following liver transplantation and to assess whether IGL-1 solution is more protective than UW solution against hepatic I/R injury through the attenuation of ER stress. The aim of the present study was not to distinguish between a specific and beneficial effect of Na$^+$ or PEG, or a synergistic effect, but to combine the cationic inversion and HES replacement by PEG to obtain the maximal protection of liver graft against ER stress induced by cold ischemia–reperfusion injury.

Results

Induction of UPR after liver transplantation. In order to determine whether the UPR was activated upon I/R injury associated with liver transplantation, we assessed the expression of the following three mRNAs known to be specifically induced by ER stress: GRP78, an ER chaperone, as well as *CHOP* and *GADD34*, two genes involved in growth arrest and DNA damage. We used semi-quantitative real-time PCR (Figure 1a) and western blot (Figure 1b). GRP78, CHOP and GADD34 mRNA (Figure 1a) and protein expression (Figure 1b) were induced after liver transplantation compared with the Sham group. However,
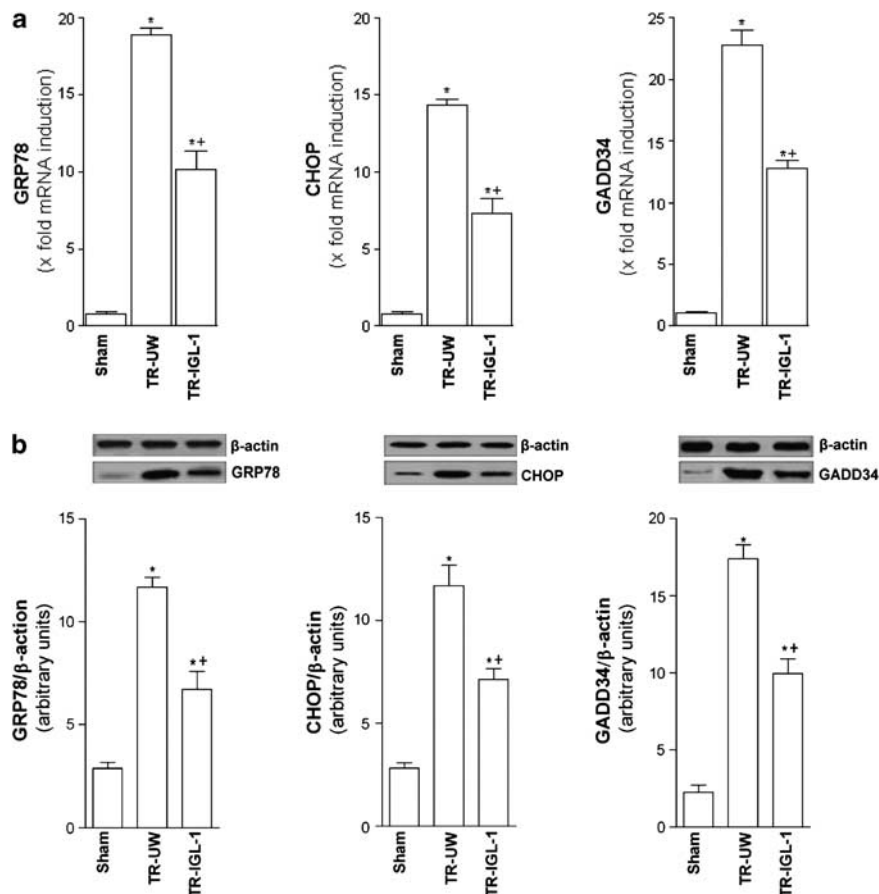
Figure 1 UPR induction after liver transplantation. (**a**) mRNA expressions and (**b**) protein level of GRP78, CHOP and GADD34 in grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). For GRP78, CHOP and GADD34 mRNA expressions in the liver, PCR fluorescent signals for GRP78, CHOP and GADD34 were standardized to PCR fluorescent signals obtained from an endogenous reference (β-actin). Comparative and relative quantifications of these gene products normalized to β-actin and the Sham group were calculated by the $2^{-\Delta\Delta CT}$ method. For GRP78, CHOP and GADD34 protein levels in the liver, the upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$ S.E.M. *$P < 0.05$ *versus* Sham; $^+P < 0.05$ *versus* TR-UW
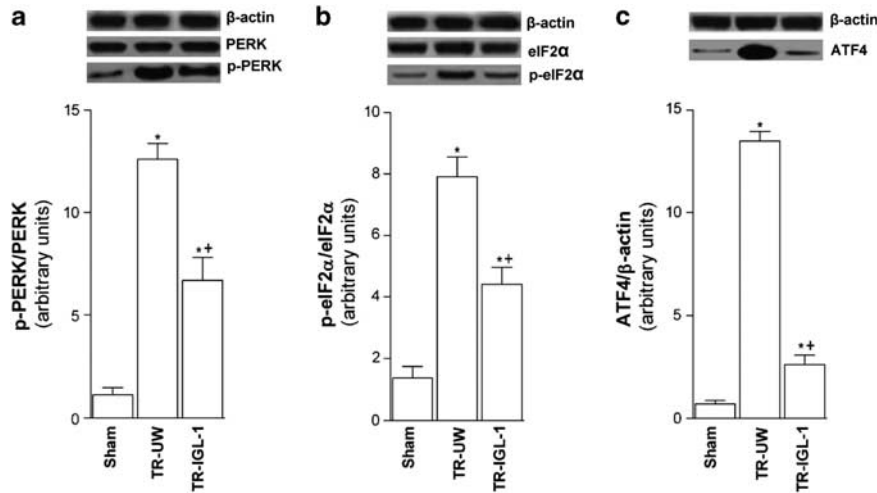
Figure 2 PERK pathway activation after liver transplantation. (**a**) Protein level of total and phospho-PERK, (**b**) total and phospho-eIF2α and (**c**) ATF4 in livers grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$ S.E.M. *$P < 0.05$ *versus* Sham; $^{+}P < 0.05$ *versus* TR-UW
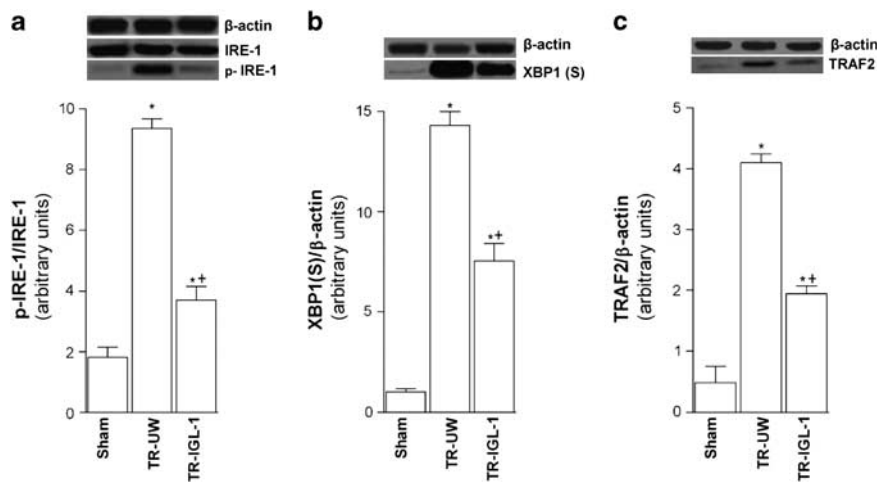
Figure 3 IRE-1 pathway activation after liver transplantation. (**a**) Protein level of total and phospho-IRE1, (**b**) XBP1 and (**c**) TRAF2 in livers grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$ S.E.M. *$P < 0.05$ *versus* Sham; $^{+}P < 0.05$ *versus* TR-UW

GRP78, CHOP and GADD34 mRNA and protein expression were lower when IGL-1 solution was used than when UW solution was used (Figures 1a and b). As the induction of GRP78, CHOP and GADD34 are indicative of the activation of the UPR, we examined which of the three branches of the UPR (PERK, IRE-1 and ATF6) were activated after liver transplantation (Figures 2, 3 and 4).

PERK To evaluate a specific activation of PERK pathway following liver transplantation, we assessed hepatic expression levels of phospho PERK, and phospho eIF2α and ATF4 activation by western blot using specific antibodies. Levels of phospho PERK increased (Figure 2a), concomitantly with eIF2α phosphorylation (Figure 2b) and ATF4 accumulation (Figure 2c). However, the activation of PERK, eIF2α and ATF4 were significantly lower for IGL-1 solution used than for UW solution (Figure 2).

IRE-1 To assess a specific activation of IRE-1 pathway, we examined hepatic phospho-IRE-1 and XBP-1s protein expression. I/R associated with liver transplantation increased phospho-IRE-1 (Figure 3a), and XBP-1s (Figure 3b), protein levels when compared with the Sham group. However, protein phospho-IRE-1 and XBP-1(S) levels were lower in the TR-IGL-1 group than in the TR-UW group (Figures 3a and b). Activated IRE-1 has been also shown to be upstream of the activation of TRAF2. Similar to the results of XBP-1, I/R increased TRAF2 protein levels (Figure 3c), but the use of IGL-1 significantly reduced TRAF2 (Figure 3c). This indicates that IGL-1 solution reduced the IRE-1 pathway activity after liver transplantation.

ATF6 To evaluate the activation of ATF6 pathway following liver transplantation, we analyzed the effect of I/R on hepatic p50ATF6α and p50ATF6β protein expression. Liver
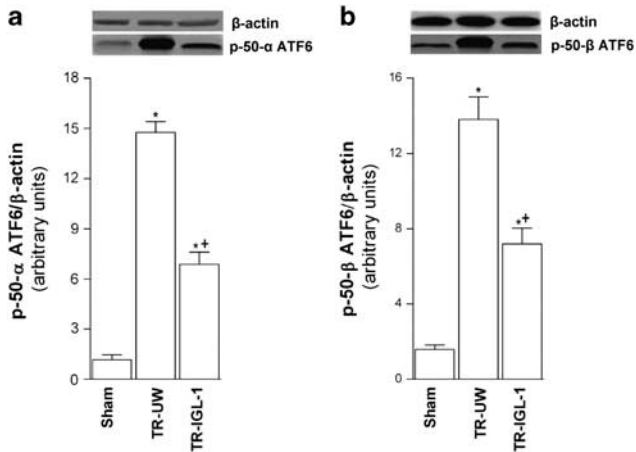
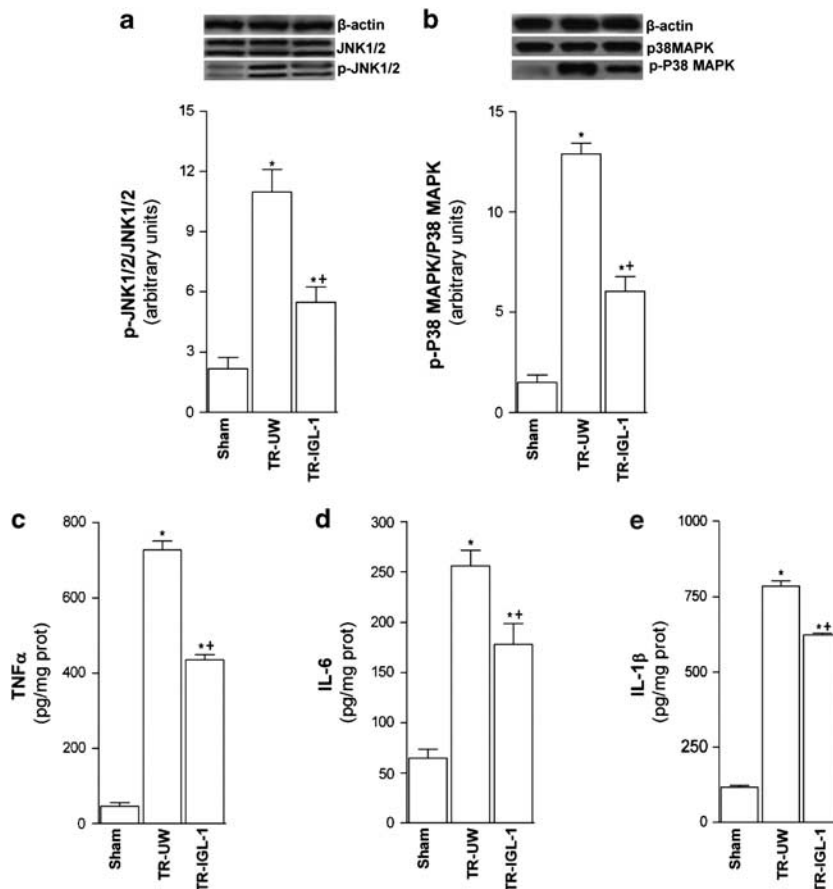**Figure 4** ATF6 pathway activation after liver transplantation. (**a**) Protein level of p50ATF6α and (**b**) p50ATF6α in livers grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$ S.E.M. *$P < 0.05$ versus Sham; $^{+}P < 0.05$ versus TR-UW

transplantation increased the expression of p50ATF6α (Figure 4a) and p50ATF6β (Figure 4b) compared with the Sham group. However, IGL-1 solution significantly reduced these expression levels compared with UW solution (Figures 4a and b).

MAPK kinase signaling To determine whether MAPK kinase signaling pathway was activated after liver transplantation, we examined the activation of JNK1/2 and p38 MAPK. Phosphorylation levels of JNK1/2 (Figure 5a) and p38 MAPK (Figure 5b) were significantly higher after liver transplantation compared with the Sham group. IGL-1 solution significantly reduced the phosphorylated levels of JNK1/2 and p38 MAPK compared with UW solution (Figures 5a and b).

Inflammatory response and hepatic injury. Owing to the association of inflammatory response and hepatocellular damage in liver transplantation with ER stress response, we examined the expression of inflammatory mediators (TNFα, IL1 β and IL6) and alanine amino transferase (ALT), aspartate amino transferase (AST) and lactate

Figure 5 JNK and P38 MAPK activation and inflammatory response after liver transplantation. (**a**) Protein levels of total and phospho p-JNK and (**b**) total and phospho-P38MAPK in liver grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$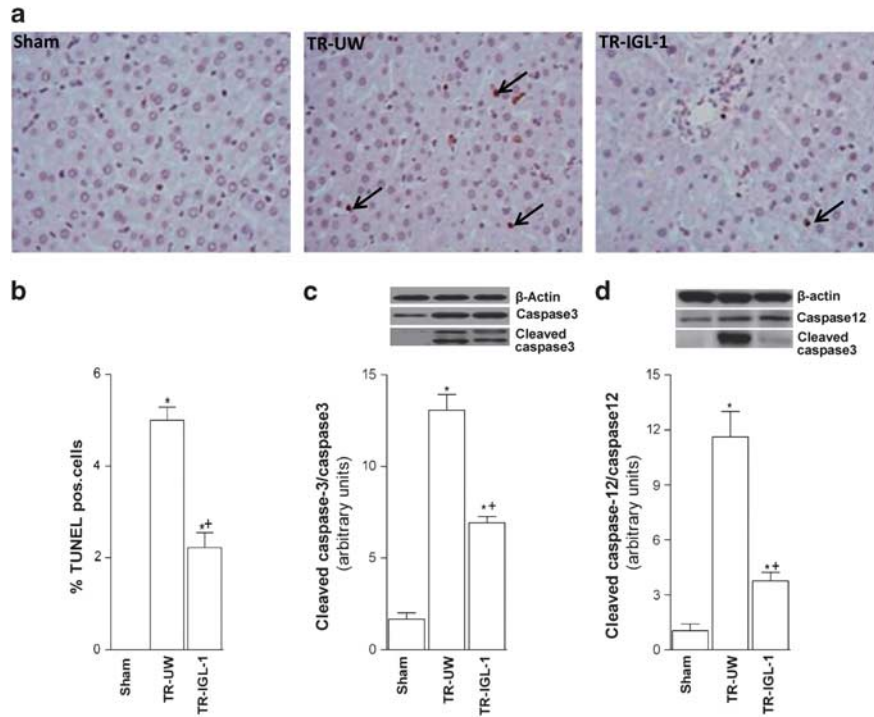 S.E.M. *$P < 0.05$ versus Sham; $^{+}P < 0.05$ versus TR-UW. (**c**) Hepatic level of TNFα, (**d**) IL6 and (**e**) IL1β in grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). $N = 6$, error bars denote $\pm$ S.E.M. *$P < 0.05$ versus Sham; $^{+}P < 0.05$ versus TR-UW

Figure 6 Cell death after liver transplantation. (**a**) TUNEL staining of the livers (original magnification × 400). Any hepatocytes stained TUNEL positive in the Sham group. Some hepatocytes stained TUNEL positive in the TR-UW group (black arrowhead). TUNEL-positive hepatocytes were significantly decreased in group TR-IGL-1. (**b**) Percentage of TUNEL-positive hepatocyte. (**c**) Protein levels of cleaved caspase-3 and (**d**) caspase-12 expression in grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote ± S.E.M. *$P < 0.05$ *versus* Sham; $^+P < 0.05$ *versus* TR-UW

Table 1 Serum levels of ALT, AST and LDH in grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1)

	Experimental groups		
	Sham	**TR-UW**	**TR-IGL-1**
ALT (U/l)	62 ± 4	602 ± 44*	280 ± 32*+
AST (U/l)	76 ± 3	1120 ± 122*	810 ± 79*+
LDH (U/l)	982 ± 74	3662 ± 104*	2062 ± 54*+

$N = 6$, *$P < 0.05$ *versus* Sham; $^+P < 0.05$ *versus* TR-UW.

dehydrogenase (LDH). TNFα (Figure 5c), IL6 (Figure 5d) and IL1β (Figure 5e) levels were higher after liver transplantation compared with the Sham group. Nevertheless, IGL-1 solution significantly decreased these levels compared with UW solution (Figures 5c and d and 6e). We report here increased hepatocellular damage after liver transplantation evidenced by increases in transaminases (ALT and AST) and LDH compared with the Sham group (Table 1). However, these increases were significantly offset by IGL-1 as preservation solution compared with the liver preserved in UW solution (Table 1).

Hepatic cell death. Excessive stress to the ER during organ preservation compromises cellular homeostasis indirectly. Here, we examined the apoptosis evaluated by percentage of TUNEL-positive hepatocytes, and by protein expression of cleaved caspase-3 and caspase-12. I/R associated with liver transplantation increased the percentage of TUNEL-positive cell (Figures 6a and b), as well as cleaved caspase-3 (Figure 6c) and caspase-12 (Figure 6d) expressions when compared with the Sham group. Decreased TUNEL-positive cells, cleaved caspase-3 and caspase-12 expression were observed when IGL-1 solution was used as the preservation solution compared with UW solution.

Mitochondrial damage. Considering the potential relationship between ER stress and mitochondrial damage, we examined mitochondrial damage by measurement of plasma glutamate dehydrogenase (GLDH) activity and cytosolic cleaved caspase-9, and mitochondrial and cytosolic cytochrome *c* activities following liver transplantation. Liver transplantation induced activation of GLDH (Figure 7a), cleaved caspase-9 (Figure 7b) and cytochrome *c* (Figure 7c) compared with values in the Sham group. However, IGL-1 reduced this activation compared with liver UW solution. This was evidenced by significant reduction in GLDH activity (Figure 7a) and protein expression of cleaved caspase-9 (Figure 7b) and cytosolic cytochrome *c* (Figure 7c) in the TR-IGL-1 group compared with the TR-UW group.

Discussion

The quality of organ preservation during ischemic storage may have a significant impact on liver allograft outcome after
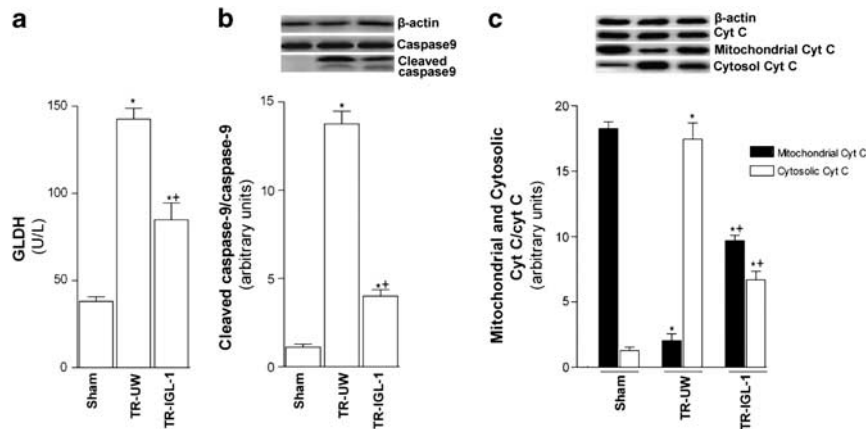
Figure 7 Mitochondrial damage after liver transplantation. (**a**) GLDH plasma level and (**b**) protein level of cleaved caspase-9 and (**c**) cytochrome c expression in grafts preserved in UW solution (TR-UW) or IGL-1 solution (TR-IGL-1). The upper panels show one representative blot of five independent experiments and the lower panels show densitometric evaluation of the independent western blot. $N = 5$, error bars denote $\pm$ S.E.M. *$P < 0.05$ versus Sham; $^+P < 0.05$ versus TR-UW

transplantation and may affect the risk of long-term complications. Thus, any improvement in the preservation of liver grafts may increase the number of viable donor livers available for transplantation.

Consistent with previous studies and that from our laboratory,[7,8,11] IGL-1 solution, based on the use of low K$^+$ high Na$^+$ concentrations and replacement of HES by PEG, while retaining all the advantages of original UW solution, improved hepatic I/R injury. The mechanism by which IGL-1 prevents microcirculatory alteration and cell swelling, and maintains cell viability remains to be determined. In the present study, we investigate the involvement of ER stress in liver I/R injury associated with liver transplantation, comparing cold storage in UW solution and IGL-1 solution. We report the accentuation of ER response following liver transplantation. The use of IGL-1 as the preservation solution significantly reduced ER stress. This reduction was associated with a decrease in inflammatory response, mitochondrial damage and hepatocellular injury. Indeed, we identified the activation of ER stress signaling pathways that are affected at transcriptional, post-transcriptional and post-translational levels. We showed induction of three mRNAs (GRP78, CHOP and GADD34) known to be specifically induced upon ER stress. A significant attenuation of the GRP78, CHOP and GADD34 mRNA and protein expression were observed when IGL-1 solution was used as the preservation solution. Additionally, the data of the present study show the activation of all three UPR arms: PERK, ATF6 and IRE1, and their downstream targets, evidenced by the induction of eIF2α, TRAF2, XBP-1(S) and ATF4 after liver transplantation compared with the Sham group. In line with these results, activation of ER stress markers has been observed in tissue biopsies from human allograft after reperfusion. Indeed, Emadali et al.[21] demonstrate an upregulation of several ER stress response chaperones in the liver during reperfusion, supporting the putative impact of alterations at the level of the ER during I/R-induced cell damage. However, here a significant reduction of activity of three UPR arms and their downstream targets was observed when livers were preserved in IGL-1 solution. These results confirm the protective

effect of IGL-1 solution on the ER stress induced after liver transplantation. It has been proposed that activation of the IRE-1 pathway regulates pro-apoptotic responses by activation of stress kinase JNK and mitogen-activated protein kinase.[22] The data of the present study reflected changes in protein phosphorylation indicating activation of JNK1/2 and P38 MAPK after liver transplantation. Our results show that the use of IGL-1 solution significantly reduces the expression of phospho JNK1/2 and P38 MAPK compared with UW solution. Taken together, these data suggest that IGL-1 protects the liver against I/R-induced apoptosis through the attenuation of ER stress, which in turn may lead to inhibition of the JNK1/2 and P38 MAPK pathways. As it has also been shown that MAPK activation can be triggered by reactive oxygen species production,[23] we cannot rule out that the inhibition of these kinases observed when IGL-1 solution was used is independent of the ER pathway attenuation. This protective role of IGL-1 solution in oxidative stress may be directly related with the presence of PEG and Na$^+$/K$^+$ inversion in the solution, and its antioxidant properties previously demonstrated in perfused rat kidney[24] and in isolated cold-stored rat hepatocytes.[25,26] Nevertheless, the mechanism by which specific PEG-35 in IGL-1 solution acts as an antioxidant molecule is unknown. On the other hand, it is well known that the UPR has been linked to several inflammatory response pathways in many cellular models and diseases.[12,27] Moreover, caspase activation links CHOP to both pro-inflammatory mediators and apoptosis, and ER stress may activate the mitochondrial apoptosis pathway.[12,28–30] Our results demonstrate that IGL-1 significantly decreases the inflammatory response, reflected by values of TNF α, IL1β and IL6 expression, compared with livers preserved in UW solution. This was associated with an important reduction of hepatic parenchymal enzyme release, evidenced here by decreased ALT, AST and LDH levels. This confirms the advantage of IGL-1 solution for protecting against hepatocellular injury associated with liver transplantation. Apoptosis is considered a hallmark of I/R-induced graft damage. The proposed pathways of this apoptosis execution after I/R include cascade-like proteolytic cleavage

activation of cysteine-like proteases, such as caspase-3 and caspase-12, a pro-apoptotic factor especially related to ER stress. Our results show slightly lower cleaved caspase-3 activity and strongly reduced caspase-12 expression, as well as reduced cell death when livers were preserved in IGL-1 solution. This attenuation of cell death and inflammatory response by IGL-1 solution was associated with an improvement of mitochondrial integrity, reflected by decreased GLDH activity, cleaved caspase-9 and cytosolic cytochrome *c* expression. This correlates with a strict relation between ER stress and mitochondrial damage as previously demonstrated.[12,28,30,31] The mechanisms by which IGL-1 solution protects from ER stress and maintains cell viability remain unclear. Based on our previous work,[8] one of the current hypotheses to explain these protective effects is related with the higher capacity of IGL-1 solution to induce constitutive nitric oxide, which is considered vital for the induction of antioxidant defense genes and those involved in ER protein folding.[32] Other potential benefits of IGL-1 solution related to PEG and lower potassium concentration, such as the anti-aggregating effect of PEG,[5] improved microcirculation, as well as an 'immuno-camouflaging' effect of PEG,[33] which protects against the initial inflammatory response,[34] should not be ruled out.

The exact relationship of ER stress-mediated cell death and cold I/R injury in liver transplantation will require further definition, but results presented here implicate ER stress on a broad scale as an important factor in this injury. We suggest that ER stress is an important potential target of IGL-1 to improve liver transplantation. However, time-course analysis of ER stress after liver transplantation might lead to a better understanding of the implication of ER stress in liver transplantation before these experimental results are confirmed in a clinical study. In conclusion, we delineate a role of ER during preservation/reperfusion of pre-damaged liver grafts, which is aggravated by the use of UW solution and attenuated by the use of IGL-1 solution. Thus, IGL-1 preservation solution may be a useful means to circumvent excessive endoplasmic stress reactions, thereby combining with other advantages of this liquid compared with standard UW solution. It may thus be a valuable alternative to UW to prevent I/R injury associated with liver transplantation and it may optimize graft quality.

Materials and Methods

Experimental animals. Male Sprague–Dawley rats weighing 250–300 g were used as donors and recipients. All animals were anesthetized with isofluorane. Research procedures complied with the European Union regulations for animal experiments (EC-guideline 86/609/CEE).

Experimental groups. All animals were randomly distributed into three groups as described below. The groups consisted of: (i) a sham ($n = 8$) group of animals subjected to anesthesia, transverse laparotomy and silk ligatures in the right suprarenal vein, diaphragmatic vein and hepatic artery; (ii) transplantation group using UW solution (TR-UW) ($n = 16$, eight transplantations), in which donor livers were flushed and preserved with cold (4 °C) UW solution (Viaspan, Madrid, Spain) for 8 h.[35,36] A standard orthotopic liver transplantation was performed according to Kamada's cuff technique, without hepatic artery construction.[37] The anhepatic phase lasted 17–20 min, as described previously.[35,36] Liver transplantation with grafts undergoing 8 h of cold ischemia is associated with high survival.[36,38] In the third group (TR-IGL-1) (iii), the same surgical procedures as described for group (ii) were carried out, but livers were flushed and preserved in

cold (4 °C) IGL-1 solution (Institute Georges Lopez-1 solution, Lyon, France). At 24 hours after transplantation, serum was obtained and aliquots of the liver were flash frozen for RNA and protein extraction.

Reverse transcription and real-time PCR. Quantitative real-time PCR analysis was performed using the Assays-on-Demand TaqMan probe ((Rn 00565250-m1 for GRP78) (Rn00492098_g1 for CHOP) and (Rn00591894_m1 for GADD34)) (Applied Biosystems, Foster City, CA, USA). The TaqMan gene expression assay was performed according to the manufacturer's protocol (Applied Biosystems).

Western blotting. Liver tissue was homogenized as previously described.[28,39,40] Total liver lysates were then used to quantify GRP78, CHOP, GADD34, ATF4, ATF6$_{\alpha}$, ATF6$_{\beta}$, XBP1(S), TRAF2, β-actin, cleaved caspase-3, caspase-12, total and phospho-PERK, total and phospho-IRE-1, total and phospho-eIF2$_{\alpha}$, total and phospho-p38 MAPK and total and phospho-JNK1/2 by western blot. Cytosolic fractions were used to quantify cleaved caspase-9 and cytochrome *c* by western blot. Mitochondrial fractions were used to quantify mitochondrial cytochrome *c* by western blot. Proteins were separated by sodium dodecyl sulfate PAGE and transferred to polyvinylidene fluoride membranes. Membranes were immunoblotted with antibodies directed against GRP78, CHOP, GADD34, ATF4, p50-ATF6$_{\alpha}$, p50-ATF6$_{\beta}$, XBP1(S), caspase-12, β-actin, total and phospho-PERK and total and phospho-eIF2$_{\alpha}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRAF2, cytochrome *c*, cleaved caspase-3 and cleaved caspase-9, total and phospho-p38 MAPK and total and phospho-JNK1/2 (Cell Signaling Technology Inc., Beverly, MA, USA) and total and phospho-IRE-1 (Thermo Fisher Scientific, Illkirch, France). The bands were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Hercules, CA, USA). The values were obtained by densitometric scanning and the Quantity One software program (Bio-Rad Laboratories) The scanning values for GRP78, CHOP, GADD34, ATF4, TRAF2, p50-ATF6$_{\alpha}$, p50-ATF6$_{\beta}$, and XBP1(S) were divided by scanning values of β-actin, and those for phosphorylated PERK, eIF2$_{\alpha}$, IRE-1, JNK1/2, p38MAPK, and cytosolic and mitochondrial cytochrome *c* were divided by the total PERK, eIF2$_{\alpha}$, IRE-1, JNK1/2, p38MAPK, and cytosolic and mitochondrial cytochrome *c*, respectively. Cleaved caspase-3, -9 and -12 were divided by the full-length fragment of each protein, respectively. All signals were normalized to the corresponding Ponceau S.[7,8]

Determination of proinflammatory cytokines. Hepatic rat cytokines (TNFα, IL-6 and IL-1β) in the liver were assayed according to the ELISA protocol given by the supplier (Invitrogen Corporation, Alcobendas, Spain).

Biochemical determinations. Plasma levels of AST, ALT and LDH were determined using commercial kits from Boehringer Mannheim (Munich, Germany).

Determination of glutamate dehydrogenase activity. GLDH is a mitochondrial enzyme, which was used as an indirect measure of mitochondrial damage. GLDH activity was measured in plasma samples, as described elsewhere.[40]

TUNEL assay. DNA fragmentation was determined using a TUNEL assay in deparaffinized liver samples, as described elsewhere.[28,41] TUNEL-positive nuclei were counted in 10 random high-power fields (× 40 objectives).

Statistics. Data are expressed as means and S.E.'s. Differences between groups were evaluated with the Student *t*-test followed by the Mann–Whitney *U*-test. $P < 0.05$ was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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