

Endoplasmic reticulum stress inhibition protects steatotic and non-steatotic livers in partial hepatectomy under ischemia–reperfusion

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During partial hepatectomy, ischemia–reperfusion (I/R) is commonly applied in clinical practice to reduce blood flow. Steatotic livers show impaired regenerative response and reduced tolerance to hepatic injury. We examined the effects of tauroursodeoxycholic acid (TUDCA) and 4-phenyl butyric acid (PBA) in steatotic and non-steatotic livers during partial hepatectomy under I/R (PH + I/R). Their effects on the induction of unfolded protein response (UPR) and endoplasmic reticulum (ER) stress were also evaluated. We report that PBA, and especially TUDCA, reduced inflammation, apoptosis and necrosis, and improved liver regeneration in both liver types. Both compounds, especially TUDCA, protected both liver types against ER damage, as they reduced the activation of two of the three pathways of UPR (namely inositol-requiring enzyme and PKR-like ER kinase) and their target molecules caspase 12, c-Jun N-terminal kinase and C/EBP homologous protein-10. Only TUDCA, possibly mediated by extracellular signal-regulated kinase upregulation, inactivated glycogen synthase kinase-3 β . This in turn, inactivated mitochondrial voltage-dependent anion channel, reduced cytochrome *c* release from the mitochondria and caspase 9 activation and protected both liver types against mitochondrial damage. These findings indicate that chemical chaperones, especially TUDCA, could protect steatotic and non-steatotic livers against injury and regeneration failure after PH + I/R.

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Therapeutic strategies to inhibit cell death in liver injury have the potential to provide a powerful tool for the treatment of liver disease characterized by cell loss, such as ischemia–reperfusion (I/R), alcoholic or nonalcoholic fatty liver disease. Indeed, with an improved understanding of the molecular pathways and the pathophysiological role of apoptosis, new drugs aimed at therapeutically modulating cell death are now available for clinical trials and/or as new therapeutic options for the treatment of several human diseases. In clinical situations, partial hepatectomy is usually performed under I/R to control bleeding during parenchymal dissection. Hepatic steatosis, a major risk factor for liver surgery, has been associated with increased complications and postoperative mortality after major liver resection. Steatotic livers show impaired regenerative response and reduced tolerance to hepatic injury compared with non-steatotic livers.¹ A further increase in the prevalence of steatosis in hepatic surgery is to

be expected. These observations highlight the need to develop protective strategies for steatotic livers in partial hepatectomy under I/R (PH + I/R). The endoplasmic reticulum (ER) regulates protein synthesis, protein folding and trafficking and intracellular calcium levels. ER stress has been associated with the development of several human chronic diseases.² In response to ER stress, a signal transduction cascade termed ‘the unfolded protein response (UPR)’^{3,4} is induced. 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-kDa glucose-regulated/binding immunoglobulin protein (GRP78). In response to stimuli that divert ER chaperones to misfolded proteins, IRE1, PERK and ATF6 initiate signal transduction processes to promote the expression of genes required for folding of newly

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Abbreviations: I/R, ischemia–reperfusion; TUDCA, tauroursodeoxycholic acid; PBA, 4-phenyl butyric acid; UPR, unfolded protein response; ER, endoplasmic reticulum; IRE1, inositol-requiring enzyme 1; GRP78, 78-kDa glucose-regulated/binding immunoglobulin protein; XBP-1, X-box-binding protein 1; TRAF2, tumor necrosis factor-associated factor 2; eIF2 α , eukaryotic translation initiation factor 2; GSK3 β , glycogen synthase kinase-3 β ; VDAC, voltage-dependent anion channel; PVDF, polyvinylidene fluoride.

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synthesized proteins and for degradation of unfolded proteins to reestablish homeostasis and normal ER function. However, when injury is excessive, these same ER stress signal transduction pathways can also induce cell death.^{3,4}

The first of the three branches of the UPR includes IRE1 α which, once activated, induces the unconventional splicing of the mRNA encoding X-box-binding protein 1 (XBP-1). The cytosolic domain of activated IRE1 α 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.^{3,5} The second branch is mediated by PERK, which phosphorylates Ser21 of the α -subunit of eukaryotic translation initiation factor 2 (eIF2 α). eIF2 α phosphorylation induces translation of a basic-region leucine zipper (bZIP) transcription factor ATF4 and subsequent expression of ATF4 target genes, C/EBP homologous protein-10 (CHOP).^{3,6} The third branch is mediated by the bZIP transcription factor ATF6, which is activated by regulated intramembrane proteolysis.^{3,7}

ER stress and mitochondrial damage are closely linked.^{8,9} ER stress induces glycogen synthase kinase-3 β (GSK3 β),^{10,11} which phosphorylates voltage-dependent anion channel (VDAC), the most abundant protein in the outer membrane of the mitochondria, and this in turn increases cell death.^{12–14} The suppression of GSK3 β activity is crucial for VDAC regulation.^{12,14} Several reports in cardiomyocytes and neuroblastoma cells have indicated that protein kinases such as extracellular signal-regulated kinase (ERK) are responsible for direct inactivation of GSK3 β .^{12,14,15}

Chemical or pharmaceutical chaperones, such as 4-phenyl butyric acid (PBA) and endogenous bile acids, and derivatives, such as tauroursodeoxycholic acid (TUDCA), modulate ER stress.^{16,17} Under conditions of I/R without hepatectomy, PBA protected non-steatotic livers against hepatic injury by inhibition of ER stress-mediated apoptosis and inflammation.¹⁷ TUDCA protected from hepatocellular necrosis under cold ischemia conditions.¹⁸ In addition, TUDCA increased hepatocellular proliferation in bile duct ligation-vagotomized rats and patients affected by chronic hepatitis.^{19,20} Given the ability of PBA and TUDCA to regulate inflammatory response, cell death and cell proliferation in various diseases,^{17–20} we examined the effects of these ER stress inhibitors (PBA and TUDCA) on hepatic injury and liver regeneration in steatotic and non-steatotic livers in PH + I/R. Under these conditions, we also analyzed the effect of PBA and TUDCA on the induction of UPR in both liver types. Our findings could contribute to the development of new pharmacological strategies based on modulating ER stress and cell death to protect steatotic and non-steatotic livers from I/R injury and to improve liver regeneration in PH + I/R.

Results

Effect of ER stress inhibitors on hepatic injury and regeneration in steatotic and non-steatotic livers in PH + I/R

Necrosis and apoptosis. Transaminase levels and damage score were higher in PH + I/R in steatotic livers than in non-steatotic livers, indicating the vulnerability of this type of liver to surgery (Figure 1a). In the PBA and TUDCA groups of

both liver types, and especially the TUDCA group, plasma transaminase levels were lower than in the PH + I/R group (Figure 1a). The histological results (Figure 1a) followed a pattern similar to that described for transaminases.

Inflammation response was also evaluated by measuring TNF α , interleukin (IL)1 β , neutrophil accumulation and oxidative stress.^{21,22} PH + I/R increased TNF α and IL1 β levels (Figure 1b), as well as neutrophil accumulation and oxidative stress (evidenced by the results of myeloperoxidase (MPO) and ROS, respectively) (Figure 1c) in both liver types compared with the sham group. In steatotic livers, all inflammation mediators tested were increased compared with non-steatotic livers. In the PBA and TUDCA groups, especially the latter, TNF α , IL1 β , MPO and malondialdehyde (MDA) levels were lower in both liver types than in the PH + I/R group (Figure 1b and c).

Apoptosis was evaluated by TUNEL and caspase 3 activation, a downstream event in the apoptotic cascade.²³ PH + I/R increased the percentage of TUNEL-positive cells and caspase 3 activation (Figure 2a) in both liver types when compared with the sham group. The results of TUNEL and caspase 3 indicated lower apoptosis in PH + I/R of steatotic livers than of non-steatotic livers, which is in accordance with previous studies indicating that apoptosis is predominant in non-steatotic livers.²³ In the PBA and TUDCA groups, and particularly in the TUDCA group, decreased cell TUNEL staining and reduced caspase 3 activity were observed in both liver types when compared with the PH + I/R group (Figure 2a). Regarding the ER apoptotic cascade, PH + I/R increased caspase 12 activity in both liver types when compared with the sham group (Figure 2b). Caspase 12 is essential for ER stress-induced apoptosis.⁵ In steatotic livers of the PH + I/R group, this mediator of ER apoptosis was lower than in non-steatotic livers. In the PBA and TUDCA groups, and particularly in the latter, caspase 12 levels were lower than in the PH + I/R group (Figure 2b). Cytochrome *c* release after mitochondrial damage induces caspase 9 release, which mediates apoptotic signals.⁶ Regarding the mitochondrial apoptotic cascade, PH + I/R increased cytosolic cytochrome *c* and caspase 9 activities in both liver types when compared with the sham group. In steatotic livers of the PH + I/R group, all mediators of mitochondrial apoptosis tested were lower than in non-steatotic livers (Figure 2b). In the PBA group of both liver types, cytosolic cytochrome *c* and caspase 9 levels were similar to those of the PH + I/R group. However, in the TUDCA group, reduced cytosolic cytochrome *c* and cleaved caspase 9 levels were observed in both liver types when compared with the PH + I/R group (Figure 2b).

ER and mitochondrial damage were evaluated in both liver types. The results of damage score indicated that the ER and mitochondria are more damaged in steatotic livers of the PH + I/R group than in non-steatotic livers. In the PBA and TUDCA groups, and particularly in the latter, ER damage was lower than in the PH + I/R group (Figure 3a). Only TUDCA protected against mitochondrial damage in both liver types. Thus, damage score levels in the mitochondria of the PBA group were similar to those of the PH + I/R group. However, reduced damage score levels were observed in the mitochondria of the TUDCA group when compared with the PH + I/R group (Figure 3a). Figure 3b shows the ultrastructural

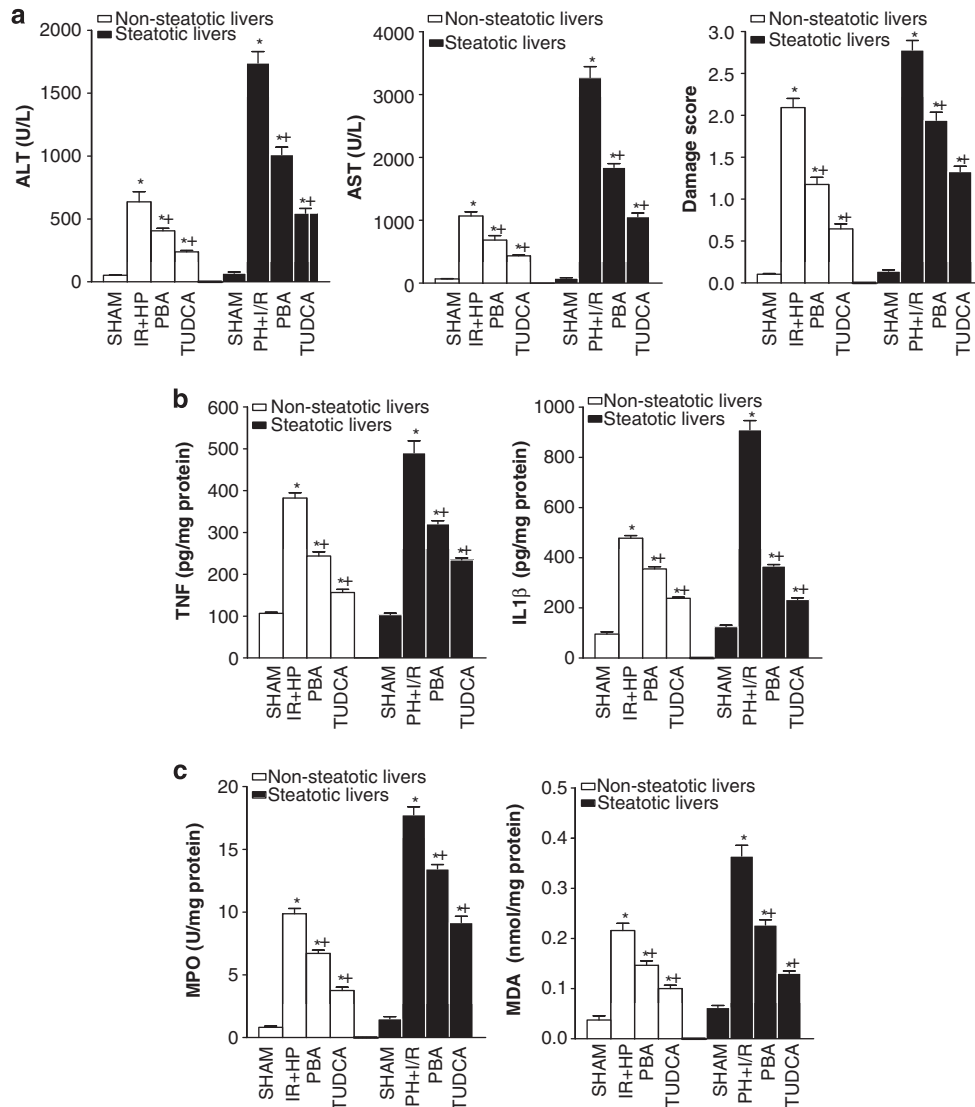


Figure 1 Hepatic damage and inflammatory response. (a) Transaminases and damage score, (b) TNF α and IL1 β levels and (c) MPO and MDA levels were measured in both liver types. * $P < 0.05$ versus sham; + $P < 0.05$ versus PH + I/R

alterations of the ER and mitochondria observed in steatotic livers of all groups. The ultrastructural alteration of ER observed in steatotic livers of the PH + I/R group were the following: ER was disordered; the membranes on either side of the vesicles show frequent in-pocketing and out-pocketing, giving profiles resembling a series of interconnecting bulbs. Increased numbers of lysosomal-related structures and cytoplasmic vesiculation and vacuolization were also observed. The PBA and TUDCA groups showed respectively, minimal and no structural alterations of ER. The ultrastructural alterations of the mitochondria observed in steatotic livers of the PH + I/R group were as follows: extensive mitochondrial cristae breakdown, mitochondrial condensation with heavy electron-dense matrix, highly swollen mitochondria that had lost all their cristae and appeared as large bags containing fine electron-dense granules and rupture of the mitochondrial membrane. In the PBA group, the ultrastructural alterations of the mitochondria were similar to those of the PH + I/R group.

However, in the TUDCA group, the mitochondrial structure was normal in steatotic livers. Electron microscopic analysis in steatotic livers showed more disruption of the plasma membrane and organelles with a relative preservation of nuclear morphology when compared with the nucleus of non-steatotic livers (electronic microscopic images not shown). This is in accordance with previous studies indicating that apoptosis and necrosis is predominant in non-steatotic livers and steatotic livers, respectively.²³

Liver regeneration. To determine the effect of PBA and TUDCA on liver regeneration, we measured PCNA and the growth factors, hepatocyte growth factor (HGF) and transforming growth factor (TGF) β . Both HGF and TGF β promote and inhibit liver regeneration, respectively.^{24,25} The rate of proliferation (assessed by PCNA) was lower in PH + I/R of steatotic livers than of non-steatotic livers (Figure 4), indicating the failure of steatotic livers to

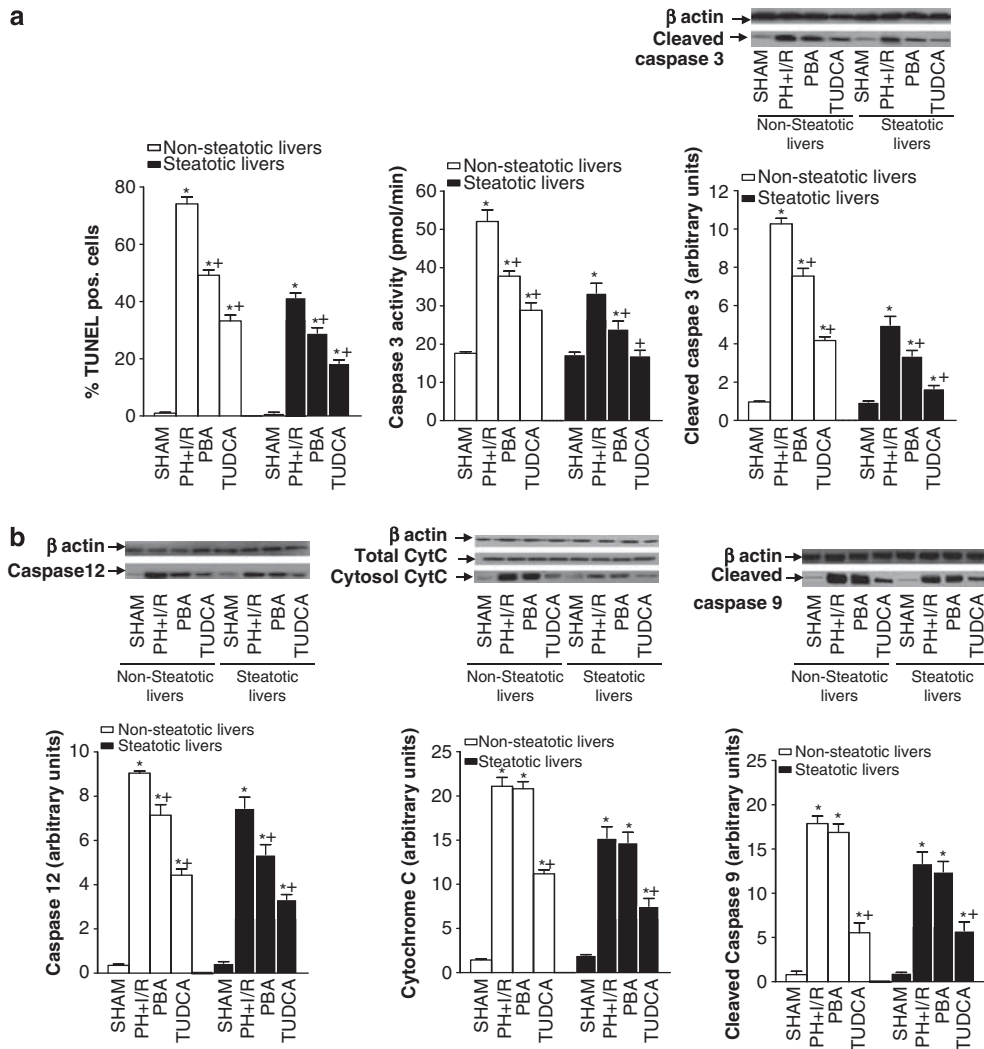


Figure 2 Apoptosis cell death, endoplasmic reticulum and mitochondrial apoptotic cascade. (a) Percentage of TUNEL-positive hepatocytes, the expression of caspase 3 activity and cleaved caspase 3 in both liver types. (b) Expression of caspase 12, cytochrome c and cleaved caspase 9 in both liver types. Representative western blots of cleaved caspase 3, caspase 12, cytochrome c and cleaved caspase 9 at the top and densitometric analysis at the bottom. * $P < 0.05$ versus sham; ⁺ $P < 0.05$ versus PH + I/R

regenerate. This was in accordance with the growth factor levels as lower HGF and higher TGF β levels were observed in steatotic livers of the PH + I/R group than in non-steatotic livers (Figure 4). In the PBA and TUDCA groups, and particularly in the TUDCA group, higher PCNA and HGF levels and lower TGF β levels were observed in both liver types when compared with the PH + I/R group (Figure 4). Total hepatic TGF β levels were similar in all groups (data not shown).

Effect of ER stress inhibitors on the induction of UPR in steatotic and non-steatotic livers in PH + I/R

GRP78. To examine the effect of PH + I/R on the induction of UPR in steatotic and non-steatotic livers, GRP78 levels, a classical ER stress marker, were determined. PH + I/R increased GRP78 mRNA and protein levels in both liver types, whereas GRP78 levels in steatotic livers were lower than in non-steatotic livers ($P < 0.05$) (Figure 5a), indicating

that steatosis is related to ER stress modulation. In the PBA and TUDCA groups, and particularly in the TUDCA group, GRP78 levels were lower in both liver types than in the PH + I/R group. As the induction of GRP78 is indicative of the activation of the UPR, we examined which of the three branches of the UPR (ATF6, IRE and PERK) are activated in both liver types.

The ATF6 pathway. During ER stress, ATF6 is converted from a 90-kDa protein (p90ATF6 α or p90ATF6 β) to a 50-kDa protein (p50ATF6 α or p50ATF6 β).⁶ Thus, we analyzed the effect of PH + I/R on hepatic p50ATF6 α and p50ATF6 β protein expression in steatotic and non-steatotic livers. PH + I/R increased p50ATF6 α and p50ATF6 β levels in both liver types compared with the sham group (Figure 5b). However, the PBA and TUDCA groups of both liver types resulted in p50ATF6 α and p50ATF6 β levels similar to those of the PH + I/R group.

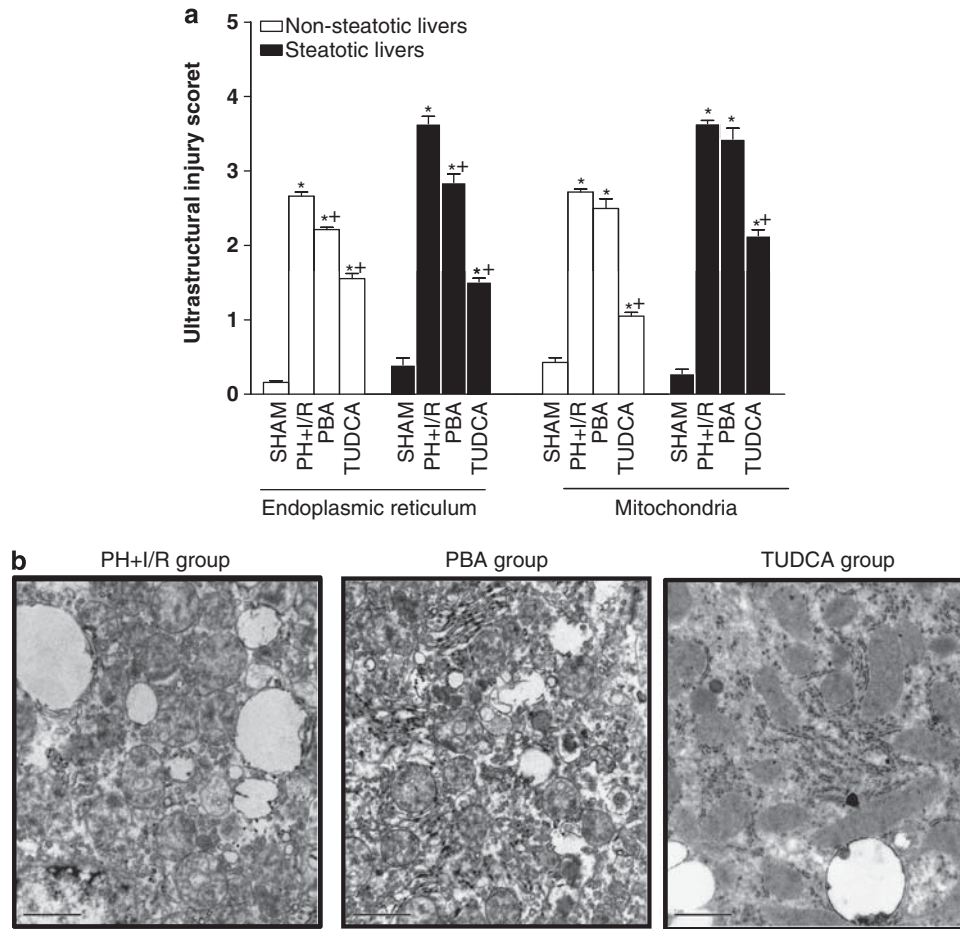


Figure 3 Endoplasmic reticulum and mitochondrial damage. (a) Ultrastructural injury score of the endoplasmic reticulum and mitochondria analyzed in both liver types. * $P < 0.05$ versus SHAM; ** $P < 0.05$ versus PH + I/R (b) Electron microscopic images of steatotic livers. PH + I/R group: evident ultrastructural alterations of RE and the mitochondria. PBA group: minimal structural alterations of RE and mitochondrial damage similar to the PH + I/R group; TUDCA group: no apparent ultrastructural alterations of RE and the mitochondria

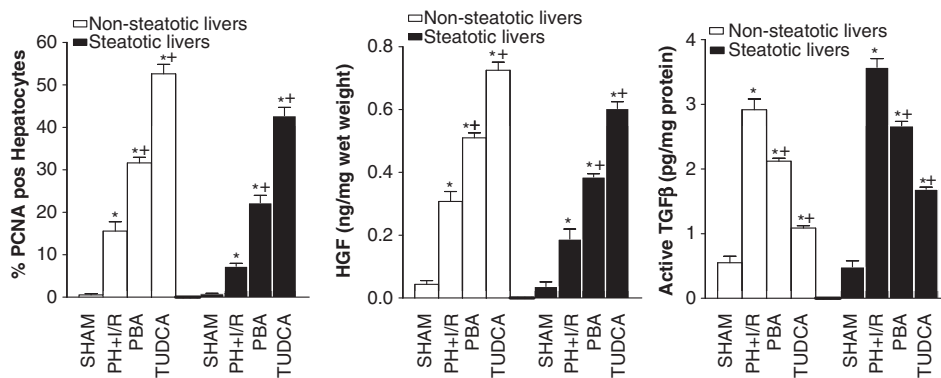


Figure 4 Liver regeneration. The percentage of PCNA-positive hepatocytes was calculated, and HGF and active TGFβ levels were measured in both liver types. * $P < 0.05$ versus sham; ** $P < 0.05$ versus PH + I/R

The IRE1 pathway. IRE1 activation induced by ER stress, was measured by monitoring sXBP-1 and TRAF2. IRE1 cuts the unspliced *XBP1* (*XBP1(U)*) mRNA into spliced *XBP1* (*XBP1(S)*) mRNA, which encodes the transcriptionally active XBP-1(S) protein. Thus, we examined hepatic XBP-1(S) protein expression.³ PH + I/R increased XBP-1(S) protein levels in steatotic and non-steatotic livers when compared

with the sham group (Figure 6a). This confirms that PH + I/R activates the IRE1/XBP-1 pathway in both liver types. XBP-1(S) levels were lower in steatotic livers. In the TUDCA and PBA groups, and particularly in the TUDCA group, reduced protein XBP-1(S) levels were observed in both liver types compared with the PH + I/R group. PH + I/R increased TRAF2 protein levels in both liver types, especially

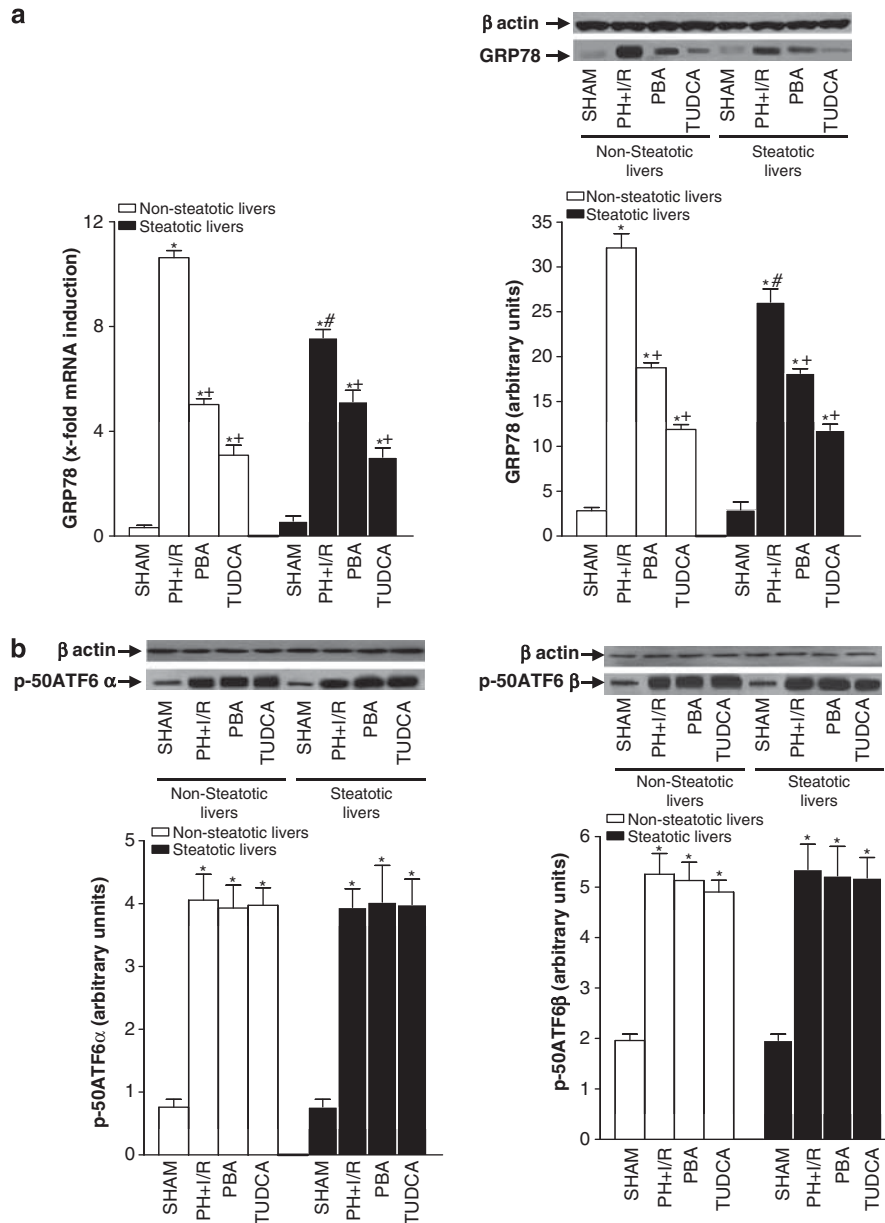


Figure 5 Induction of GRP78 and ATF6 in both liver types. (a) GRP78 mRNA expression and GRP78 protein levels were analyzed in both liver types. For GRP78 mRNA expression in the liver, PCR fluorescent signals for GRP78 were standardized to PCR fluorescent signals obtained from an endogenous reference (β -actin). Comparative and relative quantifications of GRP78 gene products normalized to β -actin and control sham group were calculated by the $2^{-\Delta\Delta CT}$ method. (b) p50ATF6 α and p50ATF6 β protein levels were analyzed in both liver types. For GRP78 and ATF6 protein levels in the liver, representative western blots at the top and densitometric analysis at the bottom. * $P < 0.05$ versus sham; + $P < 0.05$ versus PH + I/R. # $P < 0.05$ versus PH + I/R non-steatotic group. GRP78 mRNA and protein levels are significantly lower in the untreated steatotic livers group than in the untreated non-steatotic liver group (# $P < 0.05$)

in non-steatotic livers (Figure 6a). In the TUDCA and PBA groups, and particularly in the TUDCA group, reduced TRAF2 levels were observed in both liver types when compared with the PH + I/R group. TRAF2 activates JNK, caspase 12 and p38.^{3,5} Similar to the results of TRAF2 (Figure 6a), PH + I/R increased phosphorylated JNK (pJNK) protein expression in both liver types, with lower pJNK levels in steatotic livers (Figure 6b). In the TUDCA and PBA groups, and particularly in the TUDCA group, reduced pJNK levels were observed in both liver types compared with the PH + I/R group. The results of caspase 12 (Figure 2b) followed a

similar pattern to that described for TRAF2. However, this was not the case of phosphorylated p38 (pP38). Thus, in the TUDCA and PBA groups, and particularly in the TUDCA group, pP38 levels increased in both livers when compared with the PH + I/R group (Figure 6b). Total JNK and p38 levels were similar in all groups.

The PERK pathway. PERK phosphorylates eIF2 α in response to ER stress. The phosphorylation of eIF2 α induces a translation of ATF4 mRNA into the corresponding protein, which in turn induces different genes,

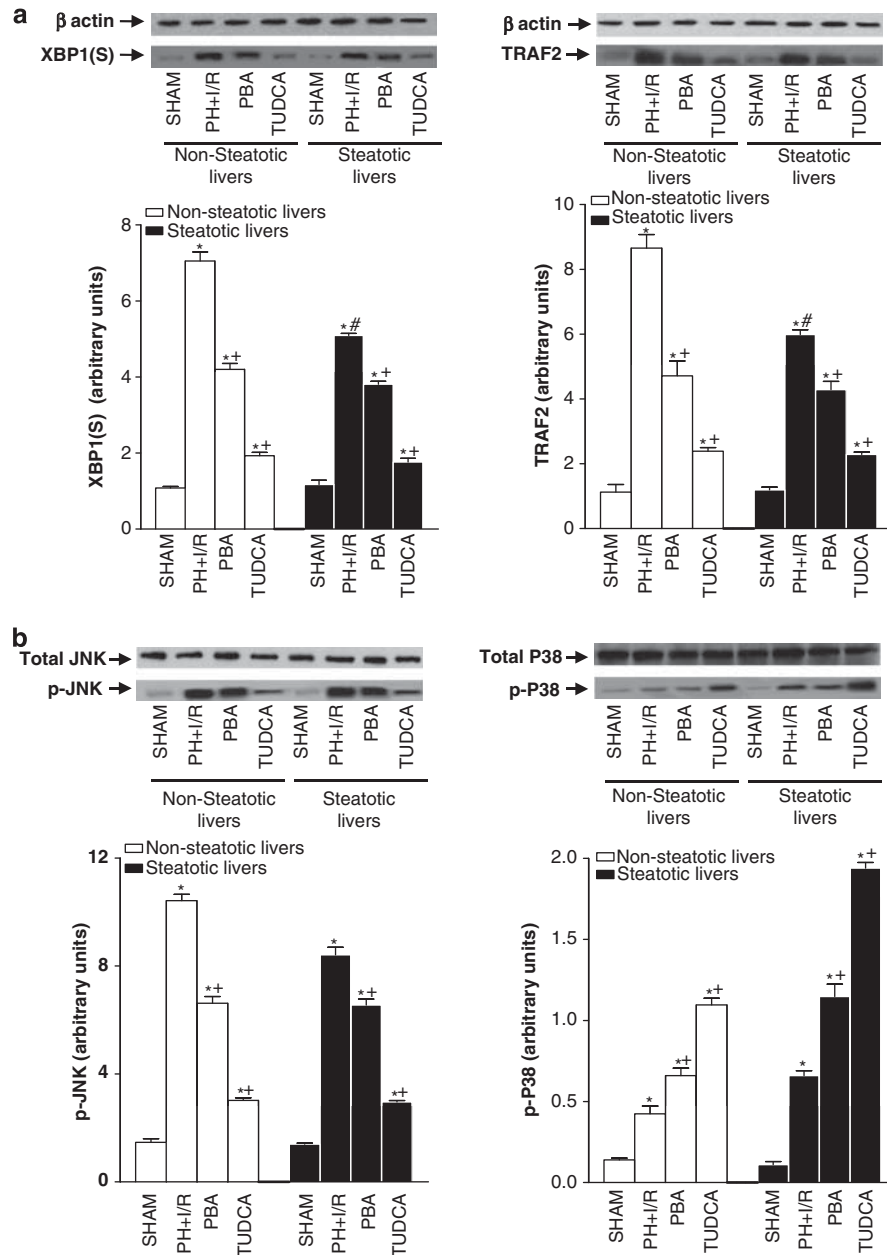


Figure 6 IRE1 pathway. Protein levels of (a) XBP-1 and TRAF2 and (b) pJNK and pP38 were analyzed in both liver types. Representative western blots at the top and densitometric analysis at the bottom. * $P < 0.05$ versus sham; + $P < 0.05$ versus PH + I/R; # $P < 0.05$ versus PH + I/R non-steatotic group. Protein levels of XBP-1 and TRAF2 are significantly lower in the untreated steatotic livers group than in the untreated non-steatotic liver group (# $P < 0.05$)

including CHOP.^{3,6} Thus, we assessed hepatic expression levels of phospho-PERK (pPERK), phospho-eIF2 α (peIF2 α), ATF4 and CHOP by western blotting. PH + I/R induced PERK and eIF2 α phosphorylation (Figure 7a), ATF4 accumulation and increased CHOP protein levels (Figure 7b) in both liver types compared with the sham group. Indeed, PERK and eIF2 α phosphorylation and ATF4 and CHOP protein expression levels in the untreated group were lower in steatotic livers than in non-steatotic livers ($P < 0.05$), confirming the impact of steatosis on ER stress. In the TUDCA and PBA groups, and particularly in the TUDCA group, reduced PERK activation

and downregulation of the levels of peIF2 α , ATF4 and CHOP were observed in both liver types when compared with the PH + I/R group.

ER stress and mitochondrial damage. Considering the potential relationship between GSK3 β and mitochondrial VDAC, we evaluated whether changes in GSK3 β activity induced by ER stress could be associated with changes in mitochondrial VDAC activity. It is well known that ER stress activates GSK3 β (reduces phosphorylated GSK3 β (pGSK3 β)) and activates VDAC (increases phosphorylated VDAC (pVDAC)).^{10–12,15} Our results indicated that PH + I/R

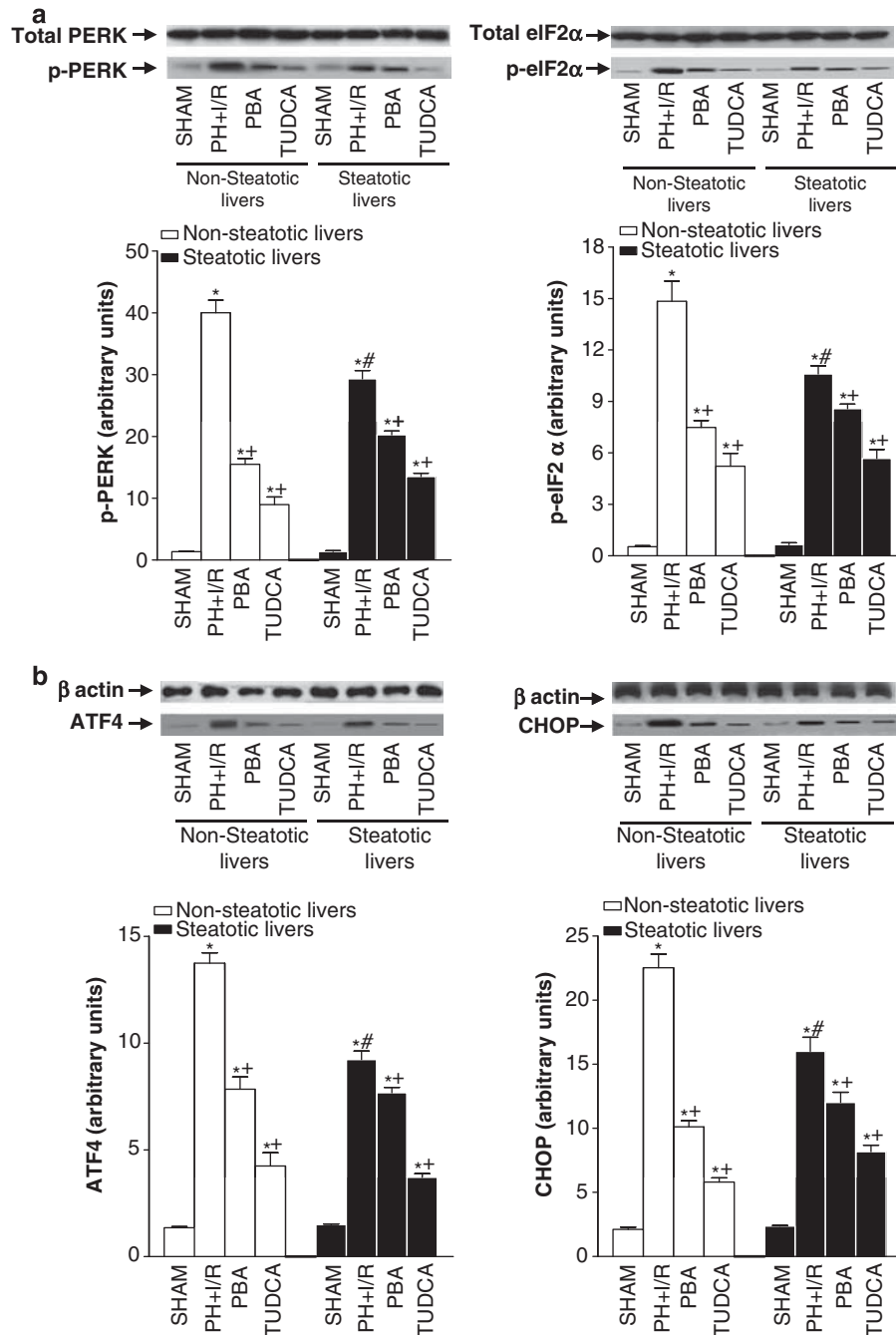


Figure 7 PERK pathway. Protein levels of (a) pPERK and p-eIF2 α and (b) ATF4 and CHOP in both liver types. Representative western blots at the top and densitometric analysis at the bottom. * $P < 0.05$ versus sham; ⁺ $P < 0.05$ versus PH + I/R; [#] $P < 0.05$ versus PH + I/R non-steatotic group protein levels of PERK, eIF2 α , ATF4 and CHOP are significantly lower in the untreated steatotic livers group than in the untreated non-steatotic liver group ([#] $P < 0.05$)

induced activation of GSK3 β and VDAC in both liver types when compared with the sham group. This was evidenced by the reduced pGSK3 β levels and the increased pVDAC levels observed in both liver types of the PH + I/R group compared with the sham group (Figure 8a). In the PBA group, pGSK3 β and pVDAC protein expression levels were similar to those of the PH + I/R group. However, TUDCA reduced the activation of GSK3 β and VDAC in both liver types when compared with the PH + I/R group. This was evidenced by the increased

pGSK3 β levels and the reduced pVDAC levels observed in both liver types of the TUDCA group compared with the PH + I/R group. In addition, these results of GSK3 β and VDAC were in accordance with the results for cytochrome *c* and caspase 9 (Figure 2b), as only in the TUDCA group but not in the PBA group, a reduction in cytochrome *c* and caspase 9 was observed in both liver types when compared with the PH + I/R group. Considering that ERK is a potential inhibitor of GSK3 β ^{10–12,15} and that in our hands, TUDCA

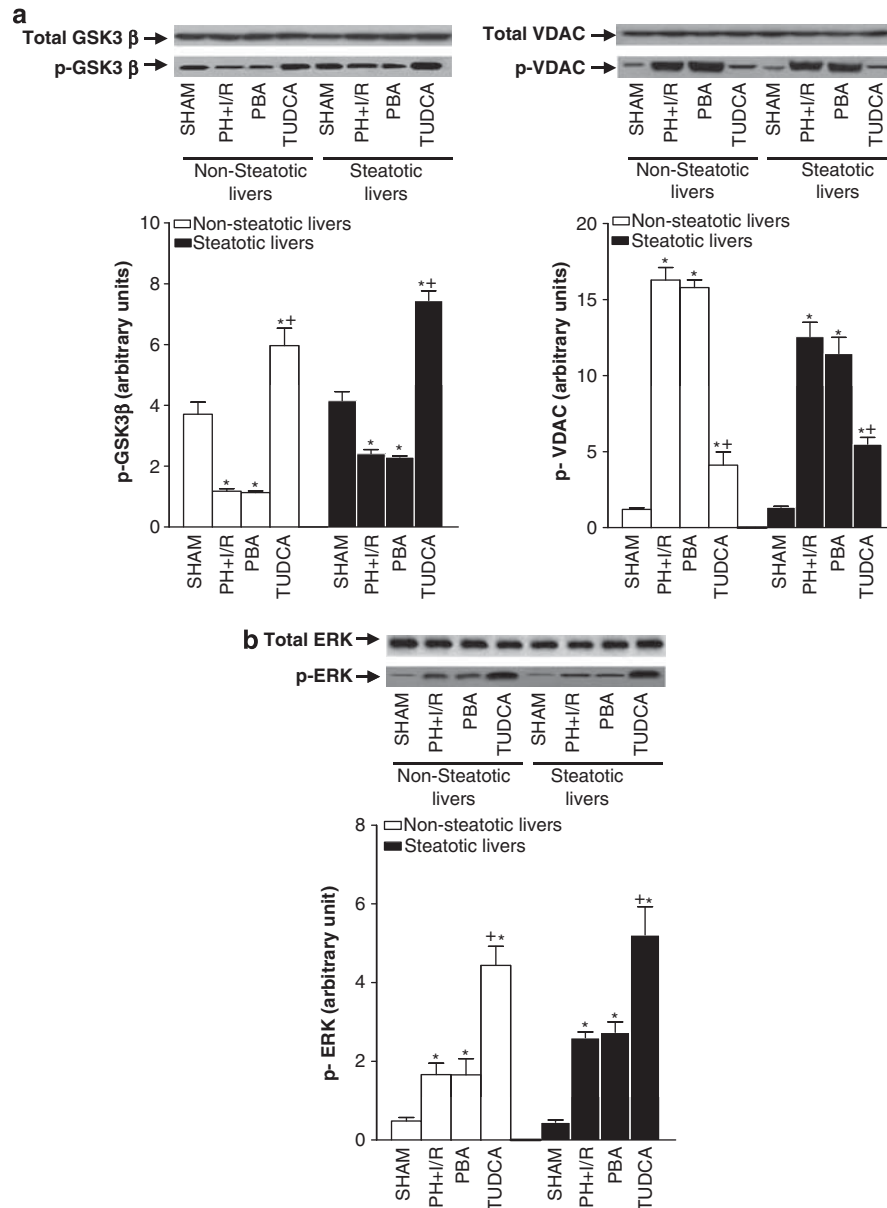


Figure 8 Endoplasmic reticulum stress and mitochondrial damage. Protein levels of (a) pGSK3 β and pVDAC and (b) pERK in both liver types. Representative western blots at the top and densitometric analysis at the bottom. * $P < 0.05$ versus sham; $^+P < 0.05$ versus PH + I/R

reduced GSK3 β activity in both liver types, we also evaluated whether the expression of ERK is upregulated by TUDCA. In the TUDCA group, increased pERK protein expression levels were observed in both liver types when compared with the PH + I/R group (Figure 8b). Total GSK3 β , VDAC and ERK levels were similar in all groups (Figure 8).

Discussion

The data of this study show the activation of all three branches of UPR: PERK, ATF6, IRE1 and their downstream targets, as well as the presence of ER stress, evidenced by the induction of CHOP, GRP78 upregulation and caspase 12 activation in steatotic and non-steatotic livers under conditions of PH + I/R.

Our study shows that steatotic livers differed from non-steatotic livers in their response to UPR and ER stress. Steatotic livers showed a reduced ability to respond to ER stress as the activation of two UPR arms, IRE1 and PERK, was weaker in the presence of steatosis. This could explain why steatotic livers required lower dose of PBA and TUDCA than did non-steatotic livers.

Previous studies in experimental models of hepatic I/R without hepatectomy have indicated that apoptosis and necrosis were the predominant forms of cell death in lean and fat rats, respectively.^{23,26} Different hypotheses, including decreased ATP production and dysfunction of regulators of apoptosis, such as Bcl-2, Bcl-xL and Bax have been proposed to explain the failure of apoptosis in steatotic livers.^{23,26}

The results of this study may throw some light on this question. Reduced proapoptotic factors related to ER stress such as caspase 12, CHOP and JNK^{3,5,6} were observed in steatotic livers under conditions of PH + I/R compared with non-steatotic livers. In addition, reduced caspase 9 activation was observed in the presence of steatosis compared with non-steatotic ones. This may be related to the reduced activation of the two UPR arms, IRE1 and PERK, which are responsible for caspase 9 and 12 activation, JNK activation and CHOP induction.

PBA and TUDCA have been approved for clinical use as protective agents in various diseases, including urea cycle disorders, cholestatic liver diseases and cirrhosis.^{27,28} Considering our results, targeting the ER with PBA or TUDCA may provide a novel therapeutic approach in clinical conditions in which I/R injury and liver regeneration occur, including hepatic resection under I/R and liver transplantation from reduced size liver graft. Blocking only one cell death pathway from the ER may be insufficient to preserve cell survival. PBA and TUDCA reduced activation of the two UPR arms, IRE1 and PERK, in both liver types and this in turn, reduced apoptosis, necrosis and inflammation and improved liver regeneration. PBA and TUDCA reduced the IRE1 pathway and its downstream targets, such as XBP-1(S) and TRAF2. This was associated with reduced caspase 12 and JNK levels. However, the reduction in TRAF2 induced by PBA and TUDCA was associated with increased p38 expression, suggesting that mechanisms other than TRAF2 could be responsible for the effects of these ER stress inhibitors on p38.

The ability of ER stress inhibitors to protect both liver types under conditions of PH + I/R is of clinical interest as numerous strategies that are effective in non-steatotic livers may not be useful in the presence of steatosis.²¹ Considering our results, TUDCA seems to be more effective in reducing hepatic damage and promoting liver regeneration in both liver types than PBA. The reduction in caspase release was more evident in the case of TUDCA. The effect of TUDCA in reducing the inflammatory response such as neutrophil accumulation, oxidative stress and proinflammatory mediators including TNF and IL1 was more evident than in the case of PBA. In addition, the superior ability of TUDCA to enhance p38 expression compared with PBA may explain at least, partially the higher beneficial effects of TUDCA on liver regeneration. The key role of p38 in promoting liver regeneration in both liver types has been previously demonstrated by our group in the same experimental model of PH + I/R.¹ Moreover, only TUDCA protected against mitochondrial damage, which might also contribute to favor liver regeneration. This conclusion is based on numerous reports indicating that the mitochondrial damage induced by I/R is associated with low ATP levels and increased ROS production. It also stems from other studies indicating that ATP is necessary for DNA synthesis and that ROS induce DNA damage and inhibit cell division.^{21,22,29}

ER stress may activate the mitochondrial apoptosis pathway.⁹ ER stress induces activation of GSK3 β , which in turn activates mitochondrial VDAC, and apoptosis and necrosis ensue.^{12,14,15} Considering our results, ER stress activates GSK3 β and mitochondrial VDAC in both liver types under conditions of PH + I/R. This was associated with cytochrome *c*

release from the mitochondria and caspase 9 activation. PBA reduced ER stress in both liver types but did not affect the mitochondrial damage in PH + I/R. However, TUDCA inhibited ER stress in both liver types and this was associated with the inhibition of GSK3 β , VDAC, cytochrome *c* release and caspase 9 activation. Our results indicate that TUDCA increased ERK in both liver types. This could be responsible at least partially for the inhibition of GSK3 β activity. In fact, the inactivation of GSK3 β by ERK has been described in cardiomyocytes.^{12,14,15}

In conclusion, ER stress inhibitors, including PBA and TUDCA could represent effective strategies to reduce hepatic I/R injury and to improve liver regeneration in steatotic and non-steatotic livers under conditions of PH + I/R. The beneficial effects of TUDCA on hepatic I/R injury and liver regeneration were more evident than those observed for PBA.

Materials and Methods

Experimental animals. Homozygous (obese, Ob) and heterozygous (lean, Ln) Zucker rats (Ifa-Credo, L'Arbresle, France), aged 16–18 weeks, were used in the experiments. Ob Zucker rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60–70% steatosis). Ln Zucker rats showed no evidence of steatosis.¹ This study complied with European Union regulations (Directive 86/609 EEC) on animal experiments.

Surgical procedure. A rat model of partial hepatectomy (70%) under 60 min of ischemia was used. After anesthesia with isoflurane and resection of the left hepatic lobe, a microvascular clamp was placed for 60 min across the portal triad supplying the median lobe. Congestion of the bowel was avoided during the clamping period by preserving the portal flow through the right and caudate lobes. At the end of ischemia time, the right lobe and caudate lobes were resected, and reperfusion of the median lobe was achieved by releasing the clamp.¹

Experimental groups. The experimental groups were as follow: (1) sham ($n=20$, 10 Ln and 10 Ob): hepatic hilar vessels were dissected; (2) PH + I/R ($n=20$, 10 Ln and 10 Ob): partial hepatectomy (70%) under 60 min of ischemia;¹ (3) PBA ($n=20$, 10 Ln and 10 Ob): as in group 2, but treated with PBA (200 and 100 mg/kg in Ln and Ob animals, respectively) before surgery; (4) TUDCA ($n=20$, 10 Ln and 10 Ob): as in group 2, but treated with TUDCA (100 and 50 mg/kg in Ln and Ob animals, respectively) before surgery.

Plasma and liver samples corresponding to all experimental groups were collected at 24 h reperfusion. The range of PBA and TUDCA doses used in this study is based on previous studies in non-steatotic livers undergoing I/R without hepatectomy.^{17,30} Preliminary studies from our group used different doses of PBA (50, 100, 200 and 400 mg/kg) and TUDCA (25, 50, 100 and 200 mg/kg) for both liver types and indicated that the doses of PBA and TUDCA chosen for this study were the most effective in protecting both liver types against hepatic injury and increasing liver regeneration.

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) (Applied Biosystems, Foster City, CA, USA). The TaqMan gene expression assay was performed according to the manufacturer's protocol (Applied Biosystems).

Western blotting. The liver tissue was homogenized as described previously.^{31,32} Total liver lysates were then used to quantify GRP78, CHOP, total PERK and pPERK, total and pElF2 α , ATF4, ATF6 α , ATF6 β , sXBP-1, TRAF2, cleaved caspase 3, caspase 12, cytochrome *c*, total and phospho-ERK, total and phospho-p38 MAPK, total and phospho-GSK3 β , total and phospho-JNK, total and phospho-AKT, as well as total and phospho-VDAC by western blot. Cytosolic fractions were used to quantify cleaved caspase 9 and cytochrome *c* by western blot. Proteins were separated by SDS-PAGE and transferred into polyvinylidene fluoride membranes. Membranes were immunoblotted with antibodies directed against GRP78, CHOP, total and pPERK, total and pElF2 α , ATF4, ATF6 α , ATF6 β , sXBP-1, β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TRAF2,

cleaved caspase 3, cleaved 9 and caspase 12, cytochrome *c*, total and phospho-ERK, total and phospho-p38 MAPK, total and phospho-GSK3 β , total and phospho-JNK and total and phospho-AKT (Cell Signalling Technology Inc., Beverly, MA, USA). 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, Hercules, CA, USA). The scanning values for GRP78, CHOP, ATF4, ATF6 α , ATF6 β , sXBP-1, TRAF2, cleaved caspase 3, cleaved caspase 9, caspase 12 and cytochrome *c* were divided by the scanning values for β -actin, and those for phosphorylated PERK, eIF2 α , GSK3 β , VDAC, ERK, JNK and p38 MAPK by the total PERK, eIF2 α , GSK3 β , VDAC, ERK, JNK and p38 MAPK, respectively.

Biochemical determinations. ALT and AST levels, HGF, total and active TGF β , IL1 β , TNF α , MDA, MPO and caspase 3 activity were measured as described elsewhere.^{1,21,22,33}

PCNA labeling index. The proliferation index of proliferating cell nuclear antigen (PCNA)-stained biopsy specimens was determined in 30 high-power fields as reported previously.¹ Data are expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes.

Histology and red-oil staining. To appraise the severity of hepatic injury by optical microscopy, H&E-stained sections were scored 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 hyper eosinophilia and loss of intercellular borders; and grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration.²² Steatosis in liver was evaluated by red-oil staining on frozen specimens, according to standard procedures.

TUNEL assay. DNA fragmentation was determined using a TUNEL assay in deparaffinized liver samples as described elsewhere. TUNEL-positive nuclei were counted.³⁴

Electron microscopy. To appraise the ER and mitochondrial damage by electron microscopy, liver samples were processed as reported previously and stained sections were viewed under an H 600-AB Hitachi electron microscope (Hitachi, Tokyo, Japan).^{35,36} In all, 20 hepatocytes from each specimen were examined. Damage to the mitochondria and ER (rough and smooth) was scored as follows. For the mitochondria: grade 0, normal; grade 1, prominent cristae; grade 2 swelling; grade 3, collection and amorphous material. For ER: grade 0, normal; grade 1, dilatation; grade 2, irregular lamellar organization and vacuolization, grade 3, presence of focal breaks.

Statistics. Data are expressed as means \pm S.E. and were compared statistically by variance analysis, followed by the Student–Newman–Keuls test. $P < 0.05$ was considered significant.

Conflict of interest

The authors declare no conflict of interest.

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1. Ramalho FS, Alfany-Fernandez I, Casillas-Ramirez A, Massip-Salcedo M, Serafin A, Rimola A *et al*. 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**: 130–140.

- Kammoun HL, Chabanon H, Hainault I, Luquet S, Magnan C, Koike T *et al*. GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. *J Clin Invest* 2009; **119**: 1201–1215.
- Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005; **115**: 2656–2664.
- Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO *et al*. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006; **313**: 1137–1140.
- Yoneda T, Imaizumi K, Oono K, Yui D, Gomi F, Katayama T *et al*. Activation of caspase-12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *J Biol Chem* 2001; **276**: 13935–13940.
- Rao RV, Ellerby HM, Bredesen DE. Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ* 2004; **11**: 372–380.
- Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R *et al*. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell* 2000; **6**: 1355–1364.
- Lim JH, Lee HJ, Ho Jung M, Song J. Coupling mitochondrial dysfunction to endoplasmic reticulum stress response: a molecular mechanism leading to hepatic insulin resistance. *Cell Signal* 2009; **21**: 169–177.
- Deniaud A, Sharaf el dein O, Maillier E, Poncet D, Kroemer G, Lemaire C *et al*. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. *Oncogene* 2008; **27**: 285–299.
- Brewster JL, Linseman DA, Bouchard RJ, Loucks FA, Precht TA, Esch EA *et al*. Endoplasmic reticulum stress and trophic factor withdrawal activate distinct signaling cascades that induce glycogen synthase kinase-3 beta and a caspase-9-dependent apoptosis in cerebellar granule neurons. *Mol Cell Neurosci* 2006; **32**: 242–253.
- Takadera T, Fujibayashi M, Kaniyu H, Sakota N, Ohyashiki T. Caspase-dependent apoptosis induced by thapsigargin was prevented by glycogen synthase kinase-3 inhibitors in cultured rat cortical neurons. *Neurochem Res* 2007; **32**: 1336–1342.
- Miura T, Nishihara M, Miki T. Drug development targeting the glycogen synthase kinase-3beta (GSK-3beta)-mediated signal transduction pathway: role of GSK-3beta in myocardial protection against ischemia/reperfusion injury. *J Pharmacol Sci* 2009; **109**: 162–167.
- Feldmann G, Haouzi D, Moreau A, Durand-Schneider AM, Bringuier A, Berson A *et al*. Opening of the mitochondrial permeability transition pore causes matrix expansion and outer membrane rupture in Fas-mediated hepatic apoptosis in mice. *Hepatology* 2000; **31**: 674–683.
- Nishihara M, Miura T, Miki T, Tanno M, Yano T, Naitoh K *et al*. Modulation of the mitochondrial permeability transition pore complex in GSK-3beta-mediated myocardial protection. *J Mol Cell Cardiol* 2007; **43**: 564–570.
- Markou T, Cullingford TE, Giraldo A, Weiss SC, Alsafi A, Fuller SJ *et al*. Glycogen synthase kinases 3alpha and 3beta in cardiac myocytes: regulation and consequences of their inhibition. *Cell Signal* 2008; **20**: 206–218.
- Xie Q, Khaoustov VI, Chung CC, Sohn J, Krishnan B, Lewis DE *et al*. Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. *Hepatology* 2002; **36**: 592–601.
- Vilatoba M, Eckstein C, Bilbao G, Smyth CA, Jenkins S, Thompson JA *et al*. Sodium 4-phenylbutyrate protects against liver ischemia reperfusion injury by inhibition of endoplasmic reticulum-stress mediated apoptosis. *Surgery* 2005; **138**: 342–351.
- Falasca L, Tisone G, Palmieri G, Anselmo A, Di Paolo D, Baiocchi L *et al*. Protective role of tauroursodeoxycholate during harvesting and cold storage of human liver: a pilot study in transplant recipients. *Transplantation* 2001; **71**: 1268–1276.
- Marziani M, Francis H, Benedetti A, Ueno Y, Fava G, Venter J *et al*. Ca²⁺-dependent cytoprotective effects of ursodeoxycholic and tauroursodeoxycholic acid on the biliary epithelium in a rat model of cholestasis and loss of bile ducts. *Am J Pathol* 2006; **168**: 398–409.
- Panella C, Ierardi E, De Marco MF, Barone M, Guglielmi FW, Polimeno L *et al*. Does tauroursodeoxycholic acid (TUDCA) treatment increase hepatocyte proliferation in patients with chronic liver disease? *Ital J Gastroenterol* 1995; **27**: 256–258.
- Massip-Salcedo M, Zaouali MA, Padrisa-Altes S, Casillas-Ramirez A, Rodes J, Rosello-Catafau J *et al*. Activation of peroxisome proliferator-activated receptor-alpha inhibits the injurious effects of adiponectin in rat steatotic liver undergoing ischemia-reperfusion. *Hepatology* 2008; **47**: 461–472.
- Serafin A, Rosello-Catafau J, Prats N, Gelpi E, Rodes J, Peralta C. Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* 2004; **39**: 688–698.
- Selzner M, Rudiger HA, Sindram D, Madden J, Clavien PA. Mechanisms of ischemic injury are different in the steatotic and normal rat liver. *Hepatology* 2000; **32**: 1280–1288.
- Fausto N, Laird AD, Webber EM. Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *FASEB J* 1995; **9**: 1527–1536.
- Bissell DM, Wang SS, Jarnagin WR, Roll FJ. Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation. *J Clin Invest* 1995; **96**: 447–455.
- Selzner N, Selzner M, Jochum W, Clavien PA. Ischemic preconditioning protects the steatotic mouse liver against reperfusion injury: an ATP dependent mechanism. *J Hepatol* 2003; **39**: 55–61.

27. Invernizzi P, Setchell KD, Crosignani A, Battezzati PM, Larghi A, O'Connell NC *et al*. Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. *Hepatology* 1999; **29**: 320–327.
28. Perlmutter DH. Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. *Pediatr Res* 2002; **52**: 832–836.
29. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992; **53**: 957–978.
30. Ishigami F, Naka S, Takeshita K, Kurumi Y, Hanasawa K, Tani T. Bile salt tauroursodeoxycholic acid modulation of Bax translocation to mitochondria protects the liver from warm ischemia-reperfusion injury in the rat. *Transplantation* 2001; **72**: 1803–1807.
31. Ramirez-Ortega M, Zarco G, Maldonado V, Carrillo JF, Ramos P, Ceballos G *et al*. Is digitalis compound-induced cardiotoxicity, mediated through guinea-pig cardiomyocytes apoptosis? *Eur J Pharmacol* 2007; **566**: 34–42.
32. Puri P, Mirshahi F, Cheung O, Natarajan R, Maher JW, Kellum JM *et al*. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology* 2008; **134**: 568–576.
33. Ben Mosbah I, Massip-Salcedo M, Fernandez-Monteiro I, Xaus C, Bartrons R, Boillot O *et al*. Addition of adenosine monophosphate-activated protein kinase activators to University of Wisconsin solution: a way of protecting rat steatotic livers. *Liver Transpl* 2007; **13**: 410–425.
34. Fernandez L, Carrasco-Chaumel E, Serafin A, Xaus C, Grande L, Rimola A *et al*. Is ischemic preconditioning a useful strategy in steatotic liver transplantation? *Am J Transplant* 2004; **4**: 888–899.
35. 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**: 908–914.
36. Peralta C, Bulbena O, Bargallo R, Prats N, Gelpi E, Rosello-Catafau J. Strategies to modulate the deleterious effects of endothelin in hepatic ischemia-reperfusion. *Transplantation* 2000; **70**: 1761–1770.



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