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BASIC STUDIES

Endoplasmic Reticulum stress induces hepatic stellate cell apoptosis and contributes to fibrosis resolution

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Abstract

Background: Survival of hepatic stellate cells (HSCs) is a hallmark of liver fibrosis, while the induction of HSC apoptosis may induce recovery. Activated HSC are resistant to many pro-apoptotic stimuli. To this issue, the role of Endoplasmic Reticulum (ER) stress in promoting apoptosis of HSCs and consequently fibrosis resolution is still debated. *Aim:* To evaluate the potential ER stress-mediated apoptosis of HSCs and fibrosis resolution *Methods:* HSCs were incubated with the ER stress agonists, tunicamycin or thapsigargin. *In vivo*, HSC were isolated from normal, bile duct-ligated (BDL) and bile duct-diverted (BDD) rats. *Results:* In activated HSC, the specific inhibitor of ER stress-induced apoptosis, calpastatin, is significantly increased vs. quiescent HSCs. Calpain is conversely reduced in activated HSCs. This pattern of protein expression provides HSCs resistance to the ER stress signals of apoptosis (apoptosis-resistant phenotype). However, both tunicamycin and thapsigargin are able to induce apoptosis in HSCs *in vitro*, completely reversing the calpain/calpastatin pattern expression. Furthermore, *in vivo*, the fibrosis resolution observed in rat livers subjected to bile duct ligation (BDL) and subsequent bile duct diversion (BDD), leads to fibrosis resolution through a mechanism of HSCs apoptosis, potentially associated with ER stress: in fact, BDD rat liver shows an increased number of apoptotic HSCs associated with reduced calapstatin and increased calpain protein expression, leading to an apoptosis-sensible phenotype. *Conclusions:* ER stress sensitizes HSC to apoptosis both *in vitro* and *in vivo*. Thus, ER stress represents a key target to trigger cell death in activated HSC and promotes fibrosis resolution.

Liver fibrosis represents the pathological response of the liver to all forms of chronic injuries and is associated with significant morbidity and mortality worldwide (1,2). The excessive accumulation of extracellular matrix in fibrotic liver diseases is a dynamic process mainly regulated by hepatic stellate cells (HSCs), which activate in response to chronic liver injury, developing a myofibroblast-like phenotype associated with increased proliferation and collagen synthesis (1,3–5).

Survival of HSC is a hallmark of liver fibrosis, while the induction of HSC apoptosis has been shown to induce recovery from fibrosis (6–8). Apoptosis can occur either through the caspase/mitochondria system or through the death-receptor pathways (9–11). Additionally, apoptosis may occur through the cellular response of the Endoplasmic Reticulum (ER) stress (12–15). Activated human HSC are resistant to many pro-apoptotic stimuli, such as serum deprivation, Fas-ligand (FasL) and toxic levels of bile acids (5,16,17).Thus, understanding the mechanisms by which HSCs became vulnerable to apoptosis signal would be very important.

The endoplasmic reticulum (ER) is an important organelle required for cell survival and normal cellular function. In the ER, nascent proteins are folded with the assistance of ER chaperones (i.e. molecular chaperones and folding enzymes). Subsequently, on the one hand, folded proteins are transported to the Golgi apparatus; on the other hand, unfolded and misfolded proteins are retained in the ER, retro-translocated to the cytoplasm by the machinery of ER-associated degradation (ERAD), and degraded by the proteasome. (18,19) Accumulation of unfolded and/or misfolded proteins in the ER lumen, creating the condition of ER stress, results in the activation of the unfolded protein response (UPR). (20) The UPR is a complex and coordinated adaptive signalling mechanism aimed at re-establishing homoeostasis of ER functions. However, under conditions of severe and prolonged ER stress, the UPR is unable to restore

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normal cellular function. (21) Subsequently, cell death, usually occurring by apoptosis, is triggered.

Signalling through the ER stress sensors can trigger pro-apoptotic signals during prolonged ER stress. However, the ER stress sensors do not directly cause cell death, but rather initiate the activation of downstream molecules such as CHOP or JNK, which further push the cell down the path of death. (22) This signal results in caspase activation, the execution phase of ER stress-induced apoptosis, and finally in the ordered and sequential dismantling of the cell. (23) Caspases are cysteine proteases that exist within the cell as inactive proforms or zymogens and are cleaved to form active enzymes following the induction of apoptosis. ER stress activates both intrinsic and extrinsic apoptotic pathways (24–26). Currently, three main pathways of ER stress-induced apoptosis are identified: (i) the pro-apoptotic pathway of CHOP/GADD153 transcription factor, which is mainly induced via PERK/eIF2, (ii) IRE1-mediated activation of apoptosis signal-regulating kinase 1 (ASK1)/c-Jun NH2-terminal kinase (JNK) and (iii) activation of the ER-localized cysteine protease, caspase-12 (21,27,28).

Several molecules are involved in the regulation of apoptotic process in cells: among these, calpain/calpastatin levels could play an interesting role. Calpains are Ca2-dependent cysteine proteases that are ubiquitously expressed in cells. Calpains have been characterized as pivotal mediators of both necrotic and apoptotic cell death following acute hypoxia, traumatic brain injury, chronic degeneration, or excitotoxicity. In addition, calpastatin represents the specific endogenous inhibitor of calpains and is provided with four inhibitory domains (CAST 1, 2, 3 and 4) simultaneously acting in calpains inhibition (29).

Two important inducers of ER stress-mediated apoptotic signals are tunicamycin and thapsigargin. Tunicamycin blocks the synthesis of all N-linked glycoproteins (N-glycans) and causes cell cycle arrest in G1 phase, while thapsigargin raises cytosolic calcium concentration by blocking the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticulum, which causes these stores to become depleted. The role of these molecules in inducing HSCs apoptosis, their correlation with the calpain/calpastatin expression and the subsequent contribution of this process to hepatic fibrogenesis remain still unclear.

Although liver fibrogenesis and its reversibility are currently debated issues, the potential mechanisms involved in this process and leading to a reversal of fibrosis are still limited. Specifically, the role of ER stress in promoting apoptosis of HSCs and consequently promoting the degradation and reduction in cellular matrix deposition is still debated (30,31).

The apoptotic activity of HSC, counterbalanced by the hepatocytes survival, could represent a consistent setting for fibrosis reversibility. The aim of the current study was therefore to evaluate the potential apoptotic process of HSCs associated with ER stress mechanisms and the resolution of fibrosis in '*in vitro*' and '*in vivo*' models.

Materials and methods

In vitro study

Reagents

Tunicamycin and thapsigargin were purchased from Sigma Chemical Co. (Saint Louis, MO). Recombinant rat TNF-α was obtained from PeproTech EC (London, UK). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): calpain 1 and calpastatin (1:200), phospho-JNK (Thr-185 and Tyr-185, dil. 1:500), phospho-ERK1/2 and phospho-AKT (1:1000), phospho-p53, Bax and caspase-12 (1:200), CHOP (1:500). All antibodies of Santa Cruz were diluted in 5% non-fat dry milk TBS-Tween solution and incubated over night. The cleaved caspase-3 was obtained from Cell Signaling (Milano, Italy) (1:1000 in 5% BSA TBS-Tween). The acetoxymethyl ester of bis-(2-amino-5-phenoxy) ethane-N,N,N',N'-tetraacetic acid (MAPTA-AM) was purchased from Calbiochem (La Jolla, CA).

Hepatic stellate cells isolation and culture, and ER stress induction

HSCs were isolated from male Wistar rats (200–250 g), using the pronase–collagenase perfusion technique as previously described (32). HSCs were activated by culture on plastic dishes for 7–14 days with medium supplemented with 10% fetal bovine serum (FBS). Experiments were performed both on quiescent HSCs (freshly isolated, 24 h plastic culture) and on activated HSCs (between the first and third serial passages). ER stress was induced in activated HSCs by a 24-h treatment with two different drugs: tunicamycin (2 μg/ml), an inhibitor of glycosylation, and thapsigargin (2 μg/ml), a tight-binding inhibitor of intracellular Ca^{2+} pumps. Activated HSCs were previously serum-starved at 0% FBS Iscove's medium for 12 h and subsequently exposed to tunicamycin or thapsigargin in 10% FBS Iscove's medium at the above-mentioned concentrations.

Protein extracts and cell fractionation

Freshly isolated and cultured HSCs were lysated in ice-cold RIPA buffer: 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, containing the protease inhibitors PMSF (1 mM), aprotinin (5 μg/ml) and sodium orthovanadate (1 mM). Cell lysate was centrifuged at 10 000 *g* for 10 min and the supernatant was removed and stored at −20°C.

For detection of Bax translocation from cytosol to mitochondria, cells were disrupted with a homogenizer

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in 10 mM Hepes/KOH (pH 7.6), 10 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol and protease inhibitors. After homogenization, sucrose (250 mM) was added to the lysate and then centrifuged at 3000 *g* for 3 min for removing nuclei and unbroken cells. The mitochondria-rich fraction was obtained by centrifugation of the supernatant at 9000 *g* for 20 min. (33).

Western blotting analysis

Proteins were measured by the Bradford method (Bio-Rad Laboratories, S.r.l. Milano). The samples, containing equivalent amounts of proteins, were boiled for 5 min in Laemmli buffer and analysed by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). After electrophoresis, the proteins were transferred into nitrocellulose membranes (Schleicher and Schuell BioScience, Keene, NH). The separated proteins were detected by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by developing the blot with Luminol reagent (Santa Cruz Biotechnology) according to the manufacturer's recommended protocol. After detection of protein expression, the blots were re-probed with a mouse anti-β-actin monoclonal antibody (dilution 1:5000, Sigma Chemical Co, Saint Louis MO), used as a sample loading control. Densitometric evaluation of the blots was performed by computerized imaging analysis method.

Apoptotic cell death analysis

Exposure of phosphatidylserine upon the outer leaflet of the membrane, the early event of a programmed cell death, was quantified by surface annexin-V/biotin staining (Roche, Mannheim, Germany) as previously described (34). Briefly, HSCs were plated on microscopy slide flasks and then incubated with thapsigargin or tunicamycin [2 μM] in 10% FBS-supplemented medium for 24 h. Annexin V-positive cells counting was performed by using a computerized image analysis system, connected to a Olympus microscope (Olympus Vanox AHBT3, Japan). Data were expressed as percentage of annexin V-positive cells.

Determination of caspase-8 activity

Pro-apoptotic stimuli induced by tunicamycin or thapsigargin were evaluated in activated HSCs by using the Caspase-Glo 8 Assay (Promega Italia S.r.l., Milano) for measuring the caspase-8 activity. Briefly, HSCs were plated in white-walled multiwell plates and incubated in presence or in absence of 2 μg/ml of tunicamycin or thapsigargin or 20 ng/ml of TNFα, for 4 h in 10% serum-supplemented medium. The luminescence signal was measured by using a multiwell plate reader (Perkin-Elmer Multilabel Counter Victor^2, Boston, MA).

Transient transfection with calpastatin siRNA

The short-interfering RNAs (siRNAs) were purchased from Dharmacon (Epsom, UK). The four sense sequences specifically targeted the Calpastatin mRNA; negative control was performed using a non-targeting pool non-coding for human or murine gene sequences. Activated HSCs were transfected at 30–40% of confluence by using the INTERFERin reagent (PolyPlus-transfection, Illkirch, France) according to the manufacturer's recommendation. 10 nmol/L of siRNA was transfected in 10% FBS-enriched medium, which after 12 h was replaced by fresh medium. After 48 h, cells were incubated with tunicamycin for additional 24 h and processed for analysis(35).

In vivo study

Bile duct ligation (BDL) and diversion (BDD)

To induce HSCs activation and apoptosis, rats were subjected to BDL for 2 weeks (n of rats = 20). In parallel experiments, a group of rats (n = 10) underwent also a bile duct diversion (BDD) procedure, to reconstitute the biliary tree through an anastomotic connection between the small bowel and the main bile duct, as previously described (36). HSCs from BDD rats were isolated after 1, 2 and 7 days from the BDD procedure, while immunohistochemistry staining was performed in rat liver specimens after 7 days from the BDD procedure.

Histology of liver samples

At the time of sacrifice, the liver was excised and then small pieces (0,5 cm) were fixed in buffered formalin for 24 h and embedded in paraffin. Four micrometre sections were mounted on glass slides coated on with 0.1% poly-L-lysine and a double immunostaining for αSMA/TUNEL was performed to evaluate apoptosis of activated HSCs.

Apoptotic cells were identified using In Sito Cell Death Detection Kit, POD, supplied by Roche, a kit for immunohistochemical detection and quantification of apoptosis (programmed cell death) at single cell level, based on labelling of DNA strand breaks (TUNEL technology). Positive αSMA cells were identified through Monoclonal Mouse Anti-Human Smooth Muscle Actin M0851 supplied by Dako. The secondary antibody was provided by Santa Cruz, goat anti-mouse HRP conjugated. Analysis was performed by light microscopy. *In situ* detection of apoptosis on single cells was performed counting positive cells to Annexin V on the liver section.

Moreover, Sirius red staining for collagen deposition, as previously described, was performed in total liver samples of rats(36).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was conducted using the

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analysis of variance (ANOVA) followed by the Student–Newman–Keuls test if the former was significant. A P value <0.05 was considered statistically significant.

Results

Calpain and calpastatin expression is modulated by HSCs activation

Calpains are calcium-activated proteases that trigger ER stress-induced apoptosis, while calpastatin is their endogenous inhibitor. Protein expression of calpain (Fig. 1a) and calpastatin (Fig. 1b) is detected both in quiescent and activated HSCs. The levels of each protein differ between the two phenotypes: in freshly isolated HSCs (0 days), characterized by quiescent phenotype, the calpain is more expressed than calpastatin, while in activated HSCs (14 days of primary culture and passage-1), calpain protein expression is significantly reduced in comparison with the specific endogenous inhibitor, calpastatin. Trans-differentiation of HSCs into a myofibroblastic phenotype is confirmed by western blot for the αSMA (α-smooth muscle actin) (Fig. 1c). Thus, in the course of HSCs activation, the specific inhibitor of ER stress-induced apoptosis, calpastatin, is significantly increased in comparison with quiescent status.

Tunicamycin and thapsigargin-induced CHOP, Caspase-12 expression and JNK activation

We analyzed the expression of CHOP (also known as GADD153). This molecule is actively involved in the pathway of ER stress-induced apoptosis, and while being normally undetectable in proliferating cells, it becomes highly synthesized in cells exposed to conditions that perturb the homeostasis of ER and is linked to the development of apoptosis (37). Immunoblots of CHOP were obtained from lysates of untreated HSCs and tunicamycin-treated HSCs (tunicamycin concentration 2 μg/ml). At different time points, the western blot showed that the protein expression started increasing at 3 h of treatment until 24 h, if treated with the above-mentioned ER stress agonists (Fig. 2a). Similar results were observed testing Caspase-12 expression by western blot analysis in Ac-HSCs incubated with tunicamycin (Fig. 2b) or thapsigargin (data not shown). In the same experiment, we observed that tunicamycin induced an early activation of JNK (Fig. 2c), a kinase that is responsive to stress stimuli, while no modifications were produced in AKT and ERK1/2 activity (Fig. 2d). Similar results were obtained also when HSCs were incubated with thapsigargin (2 μg/ml) (data not shown).

Tunicamycin- and thapsigargin-induced ER stress leads to HSCs apoptosis

Activated HSCs are resistant to apoptosis, but whether the ER stress agonists, such as tunicamycin and thapsigargin, are able to interfere with HSC survival is not known yet. Thus, we tested whether ER stress may induce apoptosis in activated HSCs. After 24-h incubation of activated or quiescent HSCs with either tunicamycin or thapsigargin (2 μg/ml), we performed

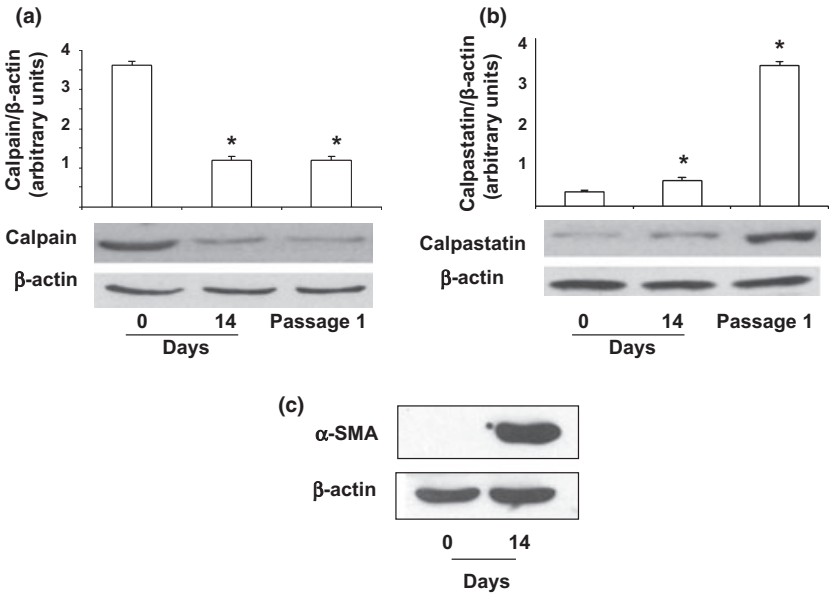

Fig. 1. *In vitro* western blot analysis of quiescent and activated hepatic stellate cells (HSCs) (a) Calpain, (b) calpastatin and (c) αSMA (alpha smooth muscle actin) protein expression in freshly isolated HSCs (0 days) and in activated HSCs (14 days and passage 1). Means ± SD of three independent experiments (P* <0,05 vs. 0 days).

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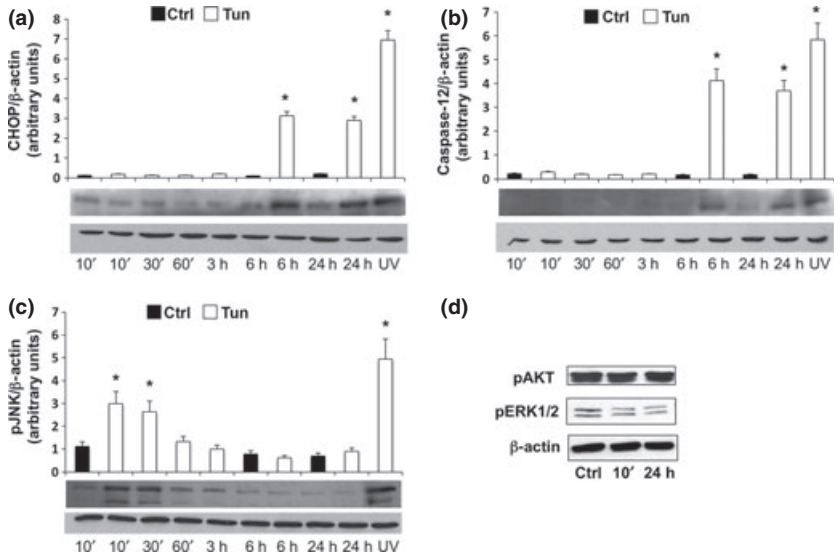

Fig. 2. *In vitro* induction of Endoplasmic Reticulum (ER) stress. Hepatic stellate cells (HSCs) were incubated at different time points in the presence or absence of tunicamycin (2 μg/ml) in 10% serum-supplemented medium. (a) CHOP expression induction, (b) JNK phosphorylation, (c) Caspase-12 and (d) survival pathway activation (AKT and ERK1/2) were detected by western blot analysis. Data are expressed as fold-increase over the control value (untreated cells). Means ± SD of three independent experiments (*P** <0,05 vs. ctrl, controls).

apoptosis analysis. Compared with control cells, in both tunicamycin- and thapsigargin-incubated HSCs, we observed an increased number of apoptotic cells as shown by counting the annexin V-positive elements (Fig. 3a). Notably quiescent HSCs were more sensitive to apoptotic stimuli, in comparison with activated HSCs. Moreover, the programmed cell death was associated with the activation of p53, an important mediator of cell death (38–40) (Fig. 3b), with the migration of pro-apoptotic Bax to mitochondria (41, 42) (Fig. 3c), and the activation of caspase-3, the downstream effector of caspases (43) (Fig. 3d), as demonstrated by western blot analysis. Similar result in p53, Bax and caspase-3 were obtained in quiescent HSCs (data not shown).

Apoptosis induced by tunicamycin and thapsigargin is not death receptor-dependent

To verify if the induction of apoptosis of HSCs occurs only through the ER stress, we measured the caspase-8 activity. In fact, caspase-8 is activated in the extrinsic pathway that involves receptor engagement or cross-linking of the tumour necrosis factor (TNF) superfamily of death receptors. It is known that TNFα may exert an anti-apoptotic effect on activated HSCs (44).

The absence of variation in caspase-8 activity in activated HSCs, incubated for 4 h with placebo (control) or with the ER stress inducers (thapsigargin or tunicamycin both at the concentration of 2 μg/ml) or TNFα (at the concentration of 20 ng/ml), used as negative control, indicates that the death receptor pathway of apoptosis is not involved (Fig. 4a).

ER stress modulates calpastatin and calpain expression in activated HSC

To examine whether ER stress-induced apoptosis may have an effect on calpain and calpstatin expression in activated HSC, immunoblots were performed with lysates from tunicamycin- or thapsigargin-treated HSCs. Specifically we treated, serum-starved HSCs, with tunicamycin or thapsigargin and incubated for 24 h. Subsequently, we performed protein extraction. We observed that the protein levels of calpastatin were significantly reduced in ER-stressed cells (Fig. 4b), while an increase of calpain occurred (Fig. 4c). Thus, ER stress was able to reverse the increased calpastatin/calpain ratio previously observed during the process of HSCs activation.

Calpastatin downregulation sensitizes HSCs to apoptosis

Activated HSCs showed an increased level of calpastatin and a lower level of calpain in comparison with quiescent HSCs. Furthermore, the Calpastatin/Calpain ratio was notably modified by the incubation of HSCs with tunicamycin or thapsigargin. To evaluate whether ER stress-induced apoptosis is influenced by calpatatin/calpain ratio, we downregulated the expression of calpastatin, by specific siRNA, and tested the apoptotic potential. As shown in Fig. 5a, transfection of Ac-HSCs with calpastatin siRNA was able to significantly reduce the protein expression of calpastatin in comparison with cells transfected with non-targeting pool. Furthermore, siRNA-transfected cells showed increased sensitivity to apoptotic signals mediated by ER stress. Notably,

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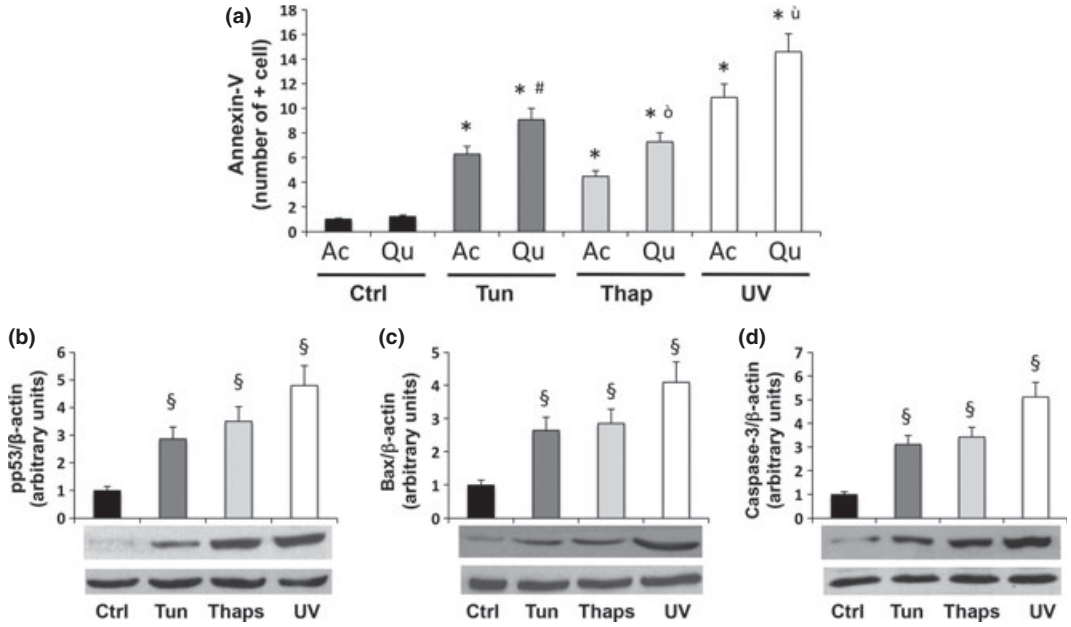

Fig. 3. Tunicamycin and thapsigargin induce apoptosis through Endoplasmic Reticulum (ER) stress. Activated- (Ac) and Quiescent-(Qu) hepatic stellate cells (HSCs) were exposed to a 24-h treatment with tunicamycin or thapsigargin (2 μg/ml) in 10% serum-supplemented medium, positive control cells subjected to UV exposure (40J/m^2). (a) Counting of annexin-V-positive cells; (b) p53, (c) mitochondrial Bax and (d) cleaved caspase-3 analysis by western blot. Data are expressed as fold-increase over the control value (untreated cells). Means ± SD of three independent experiments (P* <0,05 vs. ctrl Ac HSCs; $P^{\#}$ <0,05 vs. Ac HSCs+tun; $P^{ò}$ <0,05 vs. Ac HSCs+tha; $P^{ù}$ <0,05 vs. Ac HSCs+UV; $P^{§}$ <0,05 vs. ctrl).

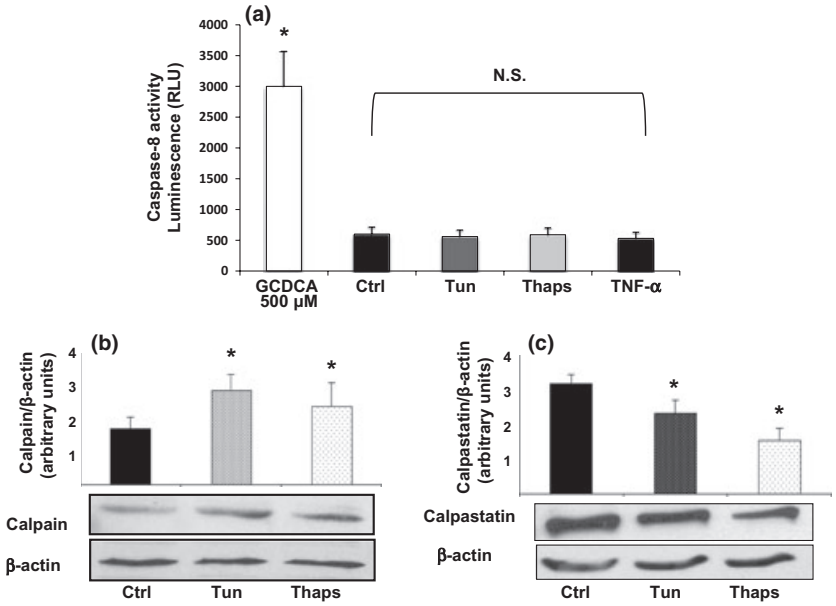

Fig. 4. Different regulations of calpain and calpastatin expression in Endoplasmic Reticulum (ER)-stressed hepatic stellate cells (HSCs). (a) HSCs were incubated for 4 h in the presence of 2 μg/ml of tunicamycin or thapsigargin, or 20 ng/ml of TNFαor 500 μM of GCDCA (glycochenodeoxycholic acid), in 10% serum-supplemented medium, and then processed caspase-8 activity measurement. The luminescence signal generated is proportional to the amount of caspase-8 activity. Means ± SD of three independent experiments (N.S., no significant difference vs. ctrl). Western blot analysis shows the effect of ER stress on calpastatin (b) and calpain (c) protein expression. Data are expressed as fold-increase over the control value (untreated cells). Means ± SD of three independent experiments. Data are expressed as fold-increase over the control value (untreated cells). Means ± SD of three independent experiments (P*<0,05 vs. ctrl, controls).

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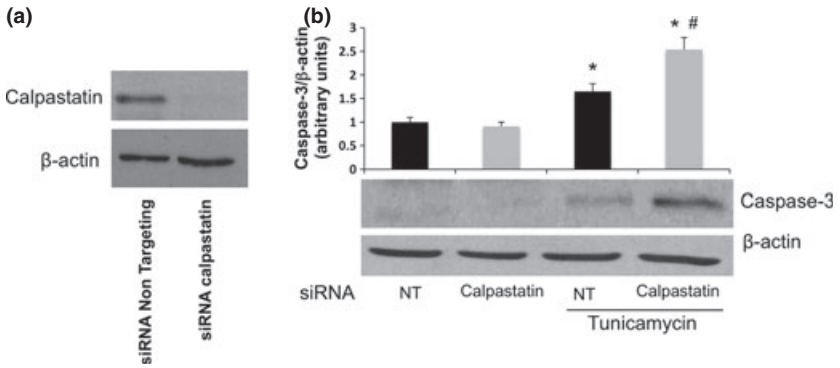

Fig. 5. Calpastatin downregulation sensitizes activated HSCs to apoptosis. Ac-HSCs were transfected overnight (12h) with non-targeting (NT) or Calpastatin siRNA at the concentration of 10 nM, kept in culture for 48 h and subsequently incubated with 2 μg/ml tunicamycin for 24 h. (a) Calpastatin protein expression in NT or Calpastatin siRNA transfected HSCs; (b) cleaved caspase-3 protein expression by western blot analysis in the presence or absence of tunicamycin. Means ± SD of three independent experiments (P^* <0,05 vs. NT, $P^{\#}$ <0,05 vs. NT+tunicamycin).

downregulation of calpastatin expression was able to increase protein expression of cleaved caspase-3, when cells were treated with tunicamycin (Fig. 5b). Similar results were obtained using thapsigargin (data not shown).

In vivo reduction in calpastatin in BDD-isolated HSCs is associated with fibrosis resolution

To study the potential role of ER stress in inducing HSCs apoptosis *in vivo*, we used the model of fibrosis reversion that occurs after biliodigestive anastomosis following bile duct ligation as previously described (36).

HSCs activation was induced in rats by bile duct ligation (BDL) model for 2 weeks. As recovery from fibrosis is associated with a reduction in the number of activated HSCs and with HSCs apoptosis, groups of BDL rats were subjected to biliary digestive diversion (BDD) and then sacrificed at 1, 3 and 7 days. HSCs were isolated from normal, BDL- and BDD-rats, and then lysated for western blot analysis. Immunoblots for calpastatin (Fig. 6a) and calpain (Fig. 6b) showed a decreased calpain/calpastatin ratio in activated HSCs from BDL compared with quiescent HSCs from normal rats. As expected, the BDD liver of rat subjected to 2 weeks BDL and 1 week BDD showed a significant reduction in collagen deposition in comparison with BDL rat not subjected to BDD, as demonstrated by the Sirius red staining (Fig. 6c). Furthermore, after BDD, the resolution of fibrosis and the observed increase in calpain/calpastatin ratio were strictly associated with HSCs apoptosis, as shown by double immunostaining for αSMA/TUNEL, indicating that after BDD, some HSCs (positive for αSMA) were also positive for TUNEL and therefore subjected to the apoptosis process (Fig. 6d).

Discussion

Saturated fatty acids promote Endoplasmic Reticulum Stress and Liver Injury in rats with hepatic steatosis (45). Furthermore, recent studies have implicated the ER in obesity, insulin resistance and diabetes (46–50). While the physiological function of the ER is mainly represented by the synthesis and processing of secretory and membrane protein (51), several pathological stresses (e.g. calcium homoeostasis, protein glycosylation and oxidative and reductive stress) disrupt ER homoeostasis and lead to the accumulation of unfolded proteins and protein aggregates in the ER lumen, which can be detrimental to cell survival (52–54). Disruption of ER homoeostasis, collectively termed 'ER stress', activates the UPR, a signalling pathway that links the ER lumen with the cytoplasm and nucleus (55–58). If the UPR is not sufficient to mitigate the imposed stress, caspase-dependent and -independent programmed cell death ensues (59).

The key experiment of the entire study is highly connected to a fundamental observation on the mechanisms that drive apoptosis: calpain protein expression decreases, while calpastatin expression increases in activated HSCs, in comparison with the same cells in the quiescent status (Fig. 1).

As extensively demonstrated in literature, it is well known that resistance of HSCs to apoptosis represents one of the mechanisms involved in fibrosis promotion.

In fact, the possibility to specifically induce apoptosis in HSCs has been hardly proposed as a promising antifibrotic therapy. Calpastatin is highly expressed in activated HSCs and is, by definition, the main inhibitor of ER stress-induced apoptosis in cells. These considerations provide solid basis to suppose a certain activity in the mechanisms that mediates apoptosis and specifically, in the processes that protect HSCs from death.

In activated HSCs, the decreased protein levels of calpain are counterbalanced by an increase in calpastatin expression. The increase in calpastatin expression is even higher in cells subjected to one passage in culture, as expected by the acquisition of a myofibroblast phenotype.

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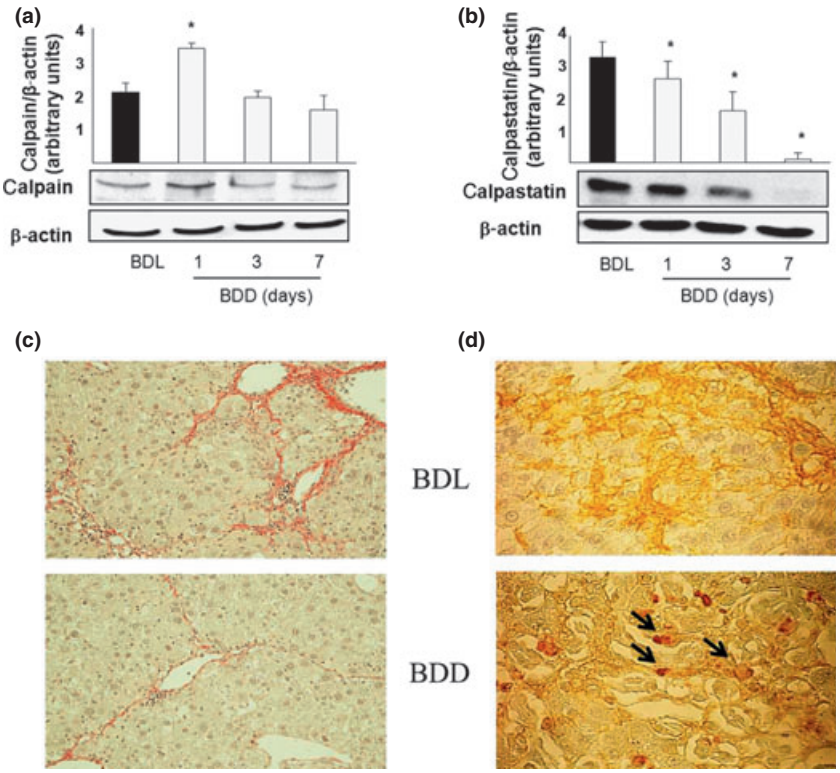

Fig. 6. *In vivo* detection of calpastatin and calpain expression and analysis of liver fibrosis. Activation of hepatic stellate cells (HSCs) was induced in rats by the bile duct-ligation (BDL) for 2 weeks, while the apoptosis mechanisms was induced by the bile duct-diversion (BDD): animal were sacrificed after 1, 3 and 7 days from surgery. HSCs were isolated from normal, BDL and BDD rat livers. Calpastatin (a) and calpain (b) proteins expression by western blot in total rat liver samples. Means ± SD of three independent experiments (*P**<0,05 vs. BDL). Sirius red staining (c) and Double immunostaining for αSMA/TUNEL (d) on liver sections respectively 20x and 40x magnification from BDL and BDD rats. (black arrows indicate TUNEL-positive HSCs).

The experiments performed in '*in vitro*' cultured HSCs, after a 24 h of serum starvation, showed that both tunicamycin and thapsigargin incubations determine a phosphorylation of JNK and an increased expression of CHOP and Caspase-12, as main indices in the process of cell apoptosis.

The issue that ER stress induces apoptosis in hepatocytes of damaged liver is well known. In fact, previous studies demonstrated the presence of ER stress during fibrosis, the consequently expected apoptosis of hepatocytes and the existence of a certain level of HSC resistance to apoptosis *in vivo* (16). Our study demonstrates that activated HSCs have a certain level of resistance to apoptosis signal due to the increased expression of calpastatin. However, *in vitro*, under exposure of molecules able to induce ER stress, and in the absence of the micro-environment that normally surround cells *in vivo* providing the stimuli for active fibrogenesis, HSCs are responsive to ER stress-induced apoptosis. Conversely, *in vivo*, during fibrogenesis, HSCs are continuously exposed to chemokines and cytokines, and also to a number of additional fibrogenic factors that are able to guarantee a resistance of the cells to apoptotic signals. Specifically, the increased level of calpastatin observed in our study seems to explain the resistance to apoptosis and the ability of these cells to even proliferate, while hepatocytes undergo apoptosis.

A better explanation needs to be developed to specifically characterize the calpastatin expression and the mechanisms underlying the resistance to apoptosis. However, what is easily demonstrable is that HSCs, *in vitro*, in the absence of fibrogenic stimuli and exposed to ER stress inducers, undergo apoptosis.

In addition to this finding, it is interesting to evaluate whether the apoptosis induction observed in HSCs incubated with thapsigargin or tunicamycin is completely dependent on ER stress induction, without any involvement of death receptor activity. To demonstrate this issue, we performed an experiment evaluating one of the upstream pathways involved in the process of receptor-mediated cell death. Caspase-8 activity was completely not detectable in HSCs, demonstrating that thapsigargin and tunicamycin only act through the ER stress induction in a death receptor-independent manner.

Furthermore, our experiments demonstrated that ER stress, induced by tunicamycin or thapsigargin, is able to promote HSCs apoptosis, consequently leading to experimental fibrosis resolution and reversibility.

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Fibrosis resolution is one of the major issues in the field of liver diseases. A number of studies try to explain the pathological condition that, in the course of liver fibrosis, leads to hepatocytes apoptosis and conversely to HSCs proliferation and activation.

Certainly, the HSC resistance to apoptosis is mainly related to their activation process and changes in phenotype. Although the condition characterizing fibrosis is well known, the mechanisms driving the resistance of HSCs to this phenomenon are still unknown.

Our experiments show that HSCs may undergo apoptosis if exposed to ER stress inducers: tunicamycin exposure of HSCs, in a condition of serum deprivation, is therefore able to determine cells apoptosis.

To translate this hypothesis *in vivo*, we performed an experiment with rats subjected to bile duct ligation for 2 weeks and subsequently to bile duct diversion, resulting in the blockage of fibrotic stimulus. This surgical technique allows to recreate, *in vivo*, what was observed *in vitro* in serum-starved HSCs. In fact, while during bile duct ligation condition, HSCs cells are continuously exposed to cytokines and chemokines that drive the activation process, in the course of bile duct diversion, HSCs are not exposed anymore to fibrogenic activity. This experiment allowed to demonstrate, also '*in vivo*', that HSCs exposed to ER stress in a condition of non-active fibrotic damage are able to undergo fibrosis reversibility, through an ER stress-mediated apoptotic pathway. In summary, HSCs are constitutively resistant to death receptor-mediated apoptosis, but ER stress sensitizes HSC to apoptosis both *in vitro* and *in vivo*. The standard pattern of calpain/calpastatin protein expression that results in significant increase in calpastatin expression in activated HSCs (apoptosis-resistant phenotype) is completely reversed both *in vitro*, following ER stress agonists such as tunicamycin and thapsigargin, and *in vivo*, following the BDD procedure after the induction of fibrosis (BDL) (apoptosis-sensible phenotype). HSCs from BDD rats acquire the same pattern of calpain/
calpastatin protein expression as observed *in vitro* with tunicamycin or thapsigargin-stimulated HSCs. Thus, either tunicamycin *in vitro* or BDD *in vivo* is able to significantly decrease the protein level of calpastatin, resulting in a general sensitization of cells to apoptotic signals. These data suggest that ER stress-mediated apoptosis, occurring both *in vitro* and *in vivo*, may represent a key target to trigger cell death in activated HSC and promote fibrosis resolution.

Author contributions

Dr De Minicis was the main coordinator for the study concept and design; he collected the results and performed analysis and interpretation of data. He also wrote the manuscript.

Dr Rychlicki, Dr Agostinelli and Dr Candelaresi were involved in the technical support, processing samples for molecular biology and related experiments; they were also involved in the critical revision of the manuscript.

Dr Trozzi and Dr Saccomanno were involved in the technical and material support, mainly characterized by immunohistochemistry staining and statistical analysis.

Dr Taffetani contributed to the collection and processing of the human samples and also provided important intellectual observation for the study.

Dr Marzioni, Prof Benedetti and Dr Svegliati-Baroni (corresponding author) provided important intellectual observation, participated in the development of the study and contributed to the manuscript drafting. They also contributed to the study supervision.

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