SerpinB3 administration protects liver against ischemia-reperfusion injury

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ABSTRACT

We have investigated the change in SerpinB3 during hepatic ischemia and the potential role of its antiprotease activity in cell protection by the administration of wild-type SerpinB3 (SerpinB3-WT) or active loop-deleted recombinant SerpinB3 protein (SerpinB3-D) in a rat model of ischemia (60 min)/reperfusion (60 min) (I/R). A time-dependent increase of SerpinB3, both at transcription and protein level, was found in ischemic livers after 60, 120 and 180 min. SerpinB3-WT decreased polymorphonuclear cell infiltration and serum enzymes, and increased ATP when compared with I/R group. These events were not obtained using SerpinB3-D. No significant changes in both liver SerpinB3 mRNA and protein were found in all I/R groups considered. The present data show that the administration of SerpinB3-WT reduced the I/R injury and this effect appears to be dependent on its anti-protease activity.

Key words: Ischemia/reperfusion; SerpinB3; inflammation.

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Introduction

Liver ischemia/reperfusion (I/R) injury occurs in liver transplantation, complex liver resection or hemorrhagic shock. The oxygen deprivation followed by reoxygenation are two essential phases occurring in I/R damage. Several cellular and functional changes that take place in the ischemic insult are associated with cellular injury; furthermore, the oxygen restoration results in paradoxical exacerbation of injury.[1] The potential mediators involved in liver I/R injury are numerous and include acute phase reactant cytokines and polymorphonuclear neutrophils (PMN).[2] The modulation of specific survival pathways by appropriate drugs may be useful in reducing liver I/R injury.

It is generally accepted that after acute liver injury, such as ischemia or I/R damage, inflammatory cells, *i.e.*, Kupffer cells (KCs), are activated worsening the initial liver damage. In the early stages of damage, the activation of KCs and overexpression of inflammatory factors are the main causes of liver dysfunction after transplantation.[3] Of note, recent results on activated KCs reported that these cells can be polarized into two subtypes. M1-type KCs play a pro-inflammatory role, and M2-type KCs play an anti-inflammatory role.[4] Interleukin-6 (IL-6) is an acute phase reactant cytokine with pleiotropic biological effects. This cytokine plays a central role in hematopoiesis, host defense, and inflammation and also possesses protective properties in hepatic I/R injury.[2]

SerpinB3 (formerly known as squamous cell carcinoma antigen-1 or SCCA1) is a member of the family of serine-protease inhibitors (Serpin) undetectable in normal hepatocytes and overexpressed in damaged hepatocytes. The protective effect of SerpinB3 is likely due to its ability to prevent oxidative stress-induced apoptotic cell death and it occurs through its direct interaction with the intramitochondrial respiratory complex I, leading to a decreased mitochondrial ROS generation.[5] Another study provided a link between SerpinB3, hypoxia and ROS by showing that hypoxia inducible factor 2α (HIF-2α) acts as a direct transcriptional inducer of the *SerpinB3* gene in an *in vitro* liver model.[6] Moreover, in liver cancer cells, by acting as an autocrine and/or paracrine mediator, SerpinB3 induces also cell proliferation and stemness features, beside apoptosis resistance.[7-9]

The aims of the present study were to investigate the change in SerpinB3 during hepatic ischemia and the potential role of its antiprotease activity in cell protection using an animal model of I/R injury.

Materials and Methods

Animals

Male Wistar rats (Charles-River, Italy) were used in this study. The use and care of animals in this experimental study was approved by the Italian Ministry of Health and by the University Commission for Animal Care: Protocol n. 533/2020 for liver ischemia model, part I; Protocol n. 274/2021 for liver I/R and treatment with two different forms of recombinant SerpinB3, including a wild type form (SerpinB3-WT) and a form lacking its antiprotease activity (SerpinB3-D) obtained from a mutant plasmid (mutant B,[10] kindly provided by Prof. Tim Harrison). The recombinant proteins used in this study were obtained in our laboratory, as previously described.[10]

Ischemia and ischemia-reperfusion (I/R) procedure

The effects of both ischemia and I/R injury were studied using an *in vivo* model of partial normothermic hepatic ischemia. Part I: ischemia to the left and median lobe was induced by clamping with microvascular clips the branch of portal vein and the branch of the proper hepatic artery after the bifurcation to the right lobe, with the abdomen temporary closed with a suture for 60, 120 and 180 min (n= 4 each group). Part II: rats were subjected to partial hepatic ischemia (60 min), as above described, followed by reperfusion period (60 min). SerpinB3-WT or SerpinB3-D isoform, obtained as previously described,[11] were evaluated in I/R damage. In details, 15 min before ischemia, SerpinB3-WT or SerpinB3-D (100 µg/mL) were administered into the portal vein (n=5 each group). Sham animals (n=3 each group in part I; n=5 in part II) were subjected to the same procedure without the clamping of the vessels.

Liver histology

At the end normothermic reperfusion, liver biopsies were rapidly removed, fixed in 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 24 h and processed routinely until they were embedded in Paraplast wax. Sections were cut at 5 µm and stained with Hematoxylin and Eosin (H&E) for histological examination.[12] Histopathological features of hepatic damage were evaluated using a semi-quantitative, histopathology score adapted from the accepted AASLD criteria for steatosis staging (Table 1).[13] For each specimen, five fields were evaluated (10x magnification).

RT-PCR

RNA extraction from livers, cDNA synthesis, quantitative Real-time PCR reactions were performed as previously described using the CFX96 Real-Time instrument (Bio-Rad Laboratories Inc, Hercules, CA, USA). Relative gene expression was normalized to the housekeeping gene and was calculated using the $2^{-\Delta\Delta CT}$ method as previously reported.[14] Primers are reported in Table 2. Samples were run in triplicate and mRNA expression was generated for each sample.

Table 1. Histopathology score of hepatic lesions.

Histological criteria	Severity	Description	Score
Inflammation	None		0
	Moderate	Scattered	1
	Marked	Foci	2
	Severe	Diffuse	3
Necrosis	Absent	0%	0
	Mild	10%	1
	Marked	10-50%	2
	Severe	50%	3

Table 2. Primer sequences.

Gene name	Sequences
SerpinB3	Fw5'- ATGGTCGATGCTTTCAATCC-3' Rv 5'- TGTGGTCCTTGGTGCAGATA-3'
IL-6	Fw5'-AAGCCAGAGTCATTCAGAGCAA-3' Rv 5'-GGTCCTTAGCCACTCCTTCT-3'
TNF-α	Fw 5'-ATGGGCTCCCTCTCATCAGT-3' Rv 5'-GCTTGGTGGTTTGCTACGAC-3'
IL-1β	Fw 5'- AGGCTGACAGACCCCAAAAG-3' Rv 5'- CTCCACGGGCAAGACATAGG-3'
β-Actin	Fw 5'-CACTTTCTACAATGAGCTGCG-3' Rv 5'-CTGGATGGCTACGTACATGG-3'

Serum and liver tissue assays

Hepatic enzymes, alanine transaminase (ALT) and alkaline phosphatase (ALP) were analyzed in serum by the reference laboratory of the Padua Teaching Hospital by hepatic frozen tissues (100 mg). ATP was measured by the luminescence method using the ATPlite luciferin/luciferase kit (Perkin Elmer Inc., Waltham, MA, USA) and expressed as nmol/mg proteins.[15]

Liver was homogenized with suitable lysis buffer as indicated by the collection procedures of the kit supplier, and the concentration of IL-6 (Antibodies-online GmbH, Aachen, Germany) was measured by ELISA kit.

Statistical analysis

Normal data distribution was analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. When data distribution was not normal according to the Kolmogorov-Smirnov test, *t*-test with Welch's correction was used. Spearman rank coefficient (rho) was used in correlation analysis. The value of $p < 0.05$ was considered to indicate statistical significance. Data are reported as the mean value ± standard error of the mean (SEM). Statistical analysis was performed using MedCalc Statistical Software version 18.11.3.

Results

SerpinB3 and inflammation gene profiles during ischemia

A progressive increase of SerpinB3 at transcription and protein level was found in the liver of mice undergoing ischemia after 60, 120 or 180 min, while no changes in SerpinB3 occurred in the corresponding sham groups (Figure 1). The profile of mRNA levels of inflammatory cytokines, including *TNF-α*, *IL-1β*, and *IL-6*, was also analyzed in the corresponding livers. As shown in Figure 1, at variance with the livers of the sham operated rats, the ischemic livers expressed markedly higher mRNA levels of *IL-6*, reaching a peak expression at 120-min ischemia (Figure 1). An earlier increase was observed for the other cytokines, where the difference was detectable at 60 min, lasting up 120 min for *TNF-α*, while for *IL-1β* the raised level was found only at 60 min, decreasing to values lower than those of the sham group at 180 min.

Liver enzymes profile

In ischemic rats, the serum analysis of hepatic enzymes showed a significant increase in ALT at 120 and 180 min, when compared at 60 min (Table 3). In sham-operated rats, no significant changes in ALT was found for all time-points considered.

SerpinB3 is protective against the liver I/R damage

In rats undergoing I/R injury, the histologic characteristics of the livers showed a marked degree of hepatocyte damage, while the livers from sham-operated animals showed a well-preserved hepatic architecture (Figure 2). After liver reperfusion, a marked injury to the parenchyma with sinusoid dilatation and cytoplasmic vacuolation was found as well as wide areas of necrotic cells detached from the parenchyma. Furthermore, an increase in infiltration of polymorphonuclear cells was also detected. These features were not found in the sham-operated group (Figure 2).

In order to evaluate the potential benefit of SerpinB3 in the protection to oxidative damage induced by the I/R process, we have used two different forms of recombinant protein, including a

Table 3. Time-course of serum ALT.

	Sham	Ischemia
60 min	93.0±5.1	89.8±3.7
120 min	100.0±28.4	212.0±66.1*
180 min	128.0±28.2	286.2±84.6*

*p<0.05 *vs* 60-min ischemia.

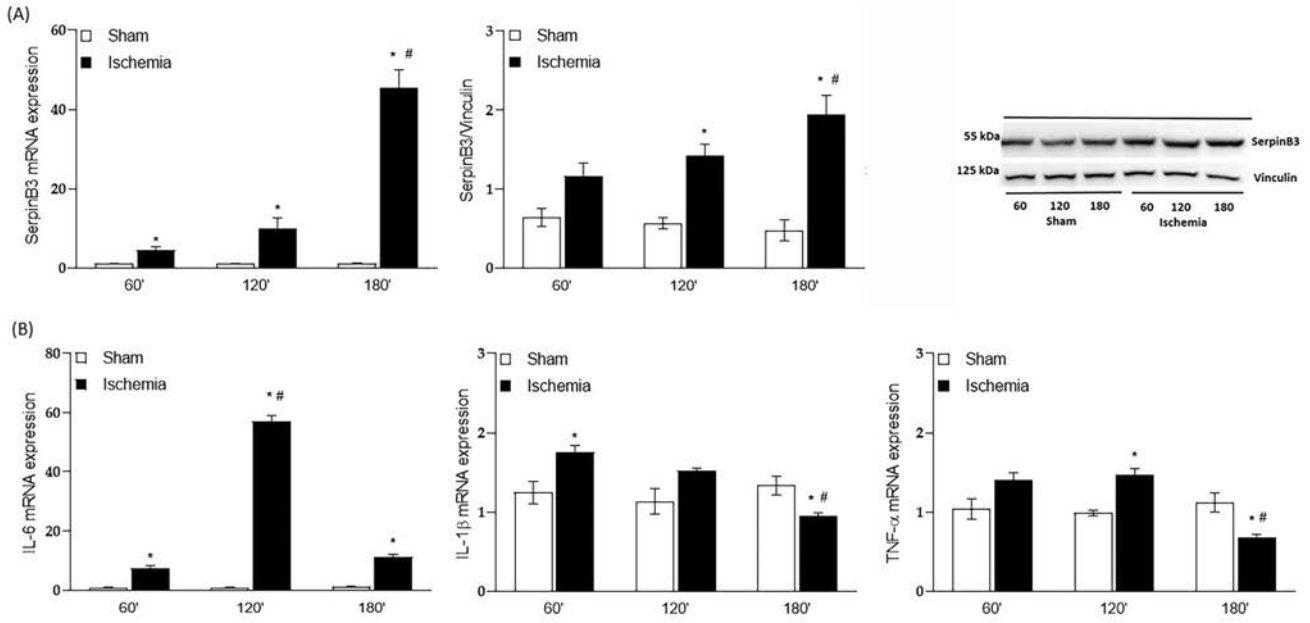

Figure 1. Changes in liver SerpinB3 and inflammatory cytokines after ischemia 60-, 120-, 180-min-ischemia (n=4 in each group) compared to the respective sham groups (n=3 in each group). A) mRNA and protein expression of SerpinB3. B) Gene expression analysis of *IL-6, TNF-α* and *IL-1β* mRNA. The results are reported as the mean ± SEM. *p<0.05 *vs* related Sham; #p<0.05 *vs* 60-min ischemia.

wild type form with a preserved antiprotease activity (SerpinB3-WT) and a mutated form, lacking the antiprotease activity by the deletion of the reactive site loop (SerpinB3-D). We observed that the treatment with SerpinB3-WT was able to decrease inflammation, expressed as polymorphonuclear cell infiltration, and necrosis parameters in the liver, although the latter did not reach statistical significance (Figure 2). These findings were supported by a significant difference in serum transaminases levels comparing I/R SerpinB3-WT *versus* I/R group (Figure 3). On the contrary, SerpinB3-D administration did not protect against I/R damage, as documented by infiltration of polymorphonuclear cells and liver necrosis as well as serum enzymes like those of untreated rats (Figures 2 and 3).

To support the liver histological findings, the quantification of mRNA and protein of IL-6 has been performed. As shown in Figure 4, the administration of SerpinB3-WT determined a significant reduction of this cytokine, as compared with untreated animals, that was unexpectedly similar to that found in animals treated with SerpinB3-D (Figure 4). Considering the liver ability to generate ATP, this energetic compound was synthesized at higher levels in the I/R group treated with SerpinB3-WT compared to the untreated corresponding group, while in rats treated with SerpinB3-D the levels were similar to those of the untreated group (Figure 3).

It is worth to note that the mRNA and protein expression of SerpinB3, evaluated at the end of reperfusion, was unchanged in all considered groups (Figure 4).

Discussion

The present study investigated the change in SerpinB3 and the potential role of its antiprotease activity in liver protection against I/R injury; thus, we compared the effects of two different forms of SerpinB3, including a wild type form SerpinB3-WT and a form lacking its antiprotease activity SerpinB3-D.

The ischemia phase represents the key early source of tissue injury that occurs during I/R injury: when ceasing blood flow, the

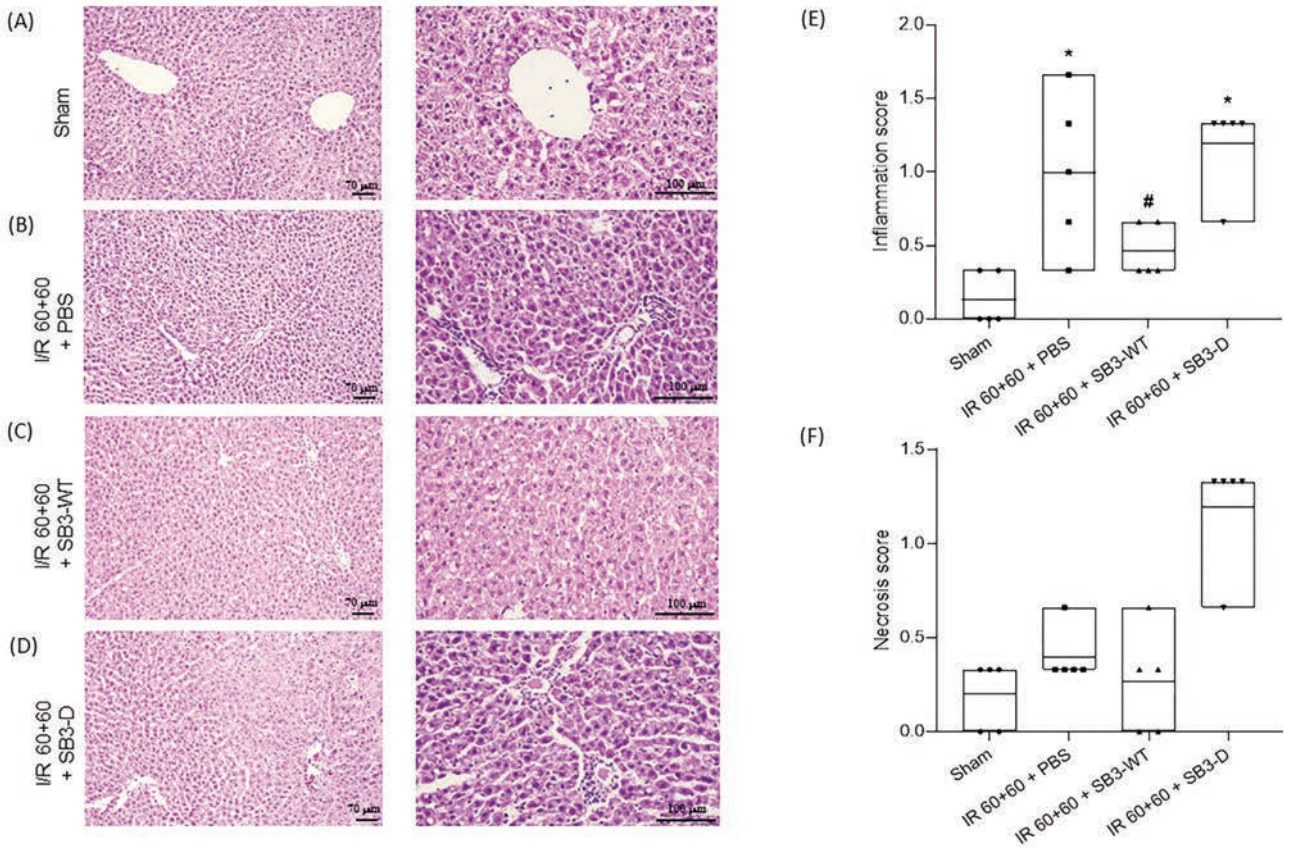

Figure 2. SerpinB3-WT treatment decreases inflammation in livers submitted to I/R injury. Animals were treated with SerpinB3-WT or SerpinB3-D. Sham animals were subjected to the same procedure without the clamping of the vessels. Representative photomicrographs of the morphology (H&E staining) of sham operated and I/R rat livers evaluated at the end of reperfusion. A) Livers from sham operated rats showed a well-preserved parenchymal architecture and good cellular morphology. B) Livers from rats undergoing 60 min ischemia and 60 min warm reperfusion presented pale-stained vacuolated, loss of parenchymal architecture and intercellular border. C) Animals treated with SerpinB3-WT and submitted to I/R differ significantly for inflammation and hepatocyte vacuolation from I/R livers about. D) Animals treated with SerpinB3-D and submitted to I/R livers showed wider areas of necrosis and loss of intercellular borders and parenchymal disarrangement with respect to I/R group. E) Inflammation score. F) Necrosis score. Data are represented as boxplots of n=5 mice for each treatment. The black line indicates the mean and the whiskers show the range from the minimum to maximum value. *p<0.05 *vs* related sham; #p<0.05 *vs* I/R 60+60 +PBS.

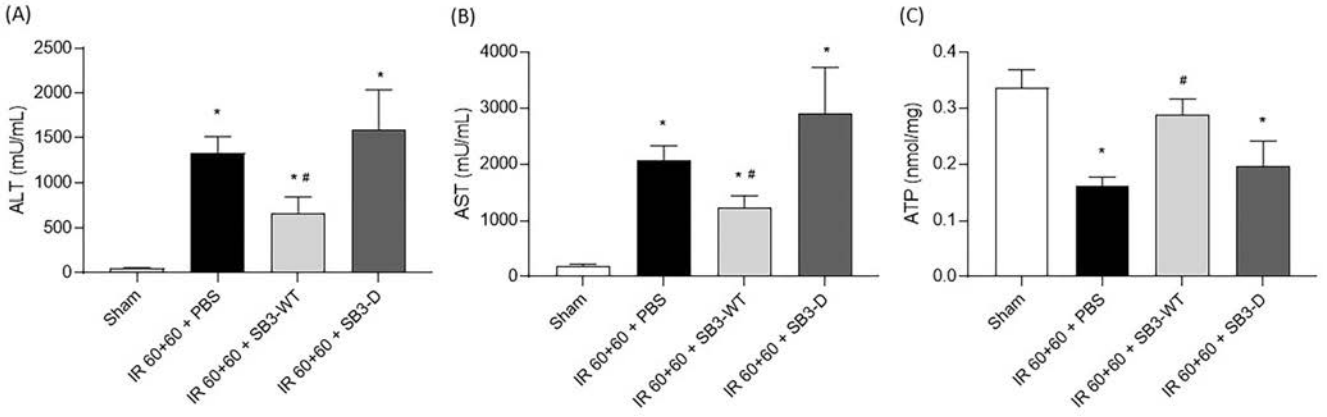

Figure 3. SerpinB3 WT decreases serum ALT (A) and AST (B) and increases liver ATP (C) evaluated in livers from rats undergoing 60 min ischemia and 60 min warm reperfusion. Animals (n=5 in each group) were treated with SerpinB3-WT or SerpinB3-D. Sham animals (n=5) were subjected to the same procedure without the clamping of the vessels. The results are reported as the mean ± SEM *p<0.05 vs related sham; #p<0.05 *vs* I/R 60+60 +PBS.

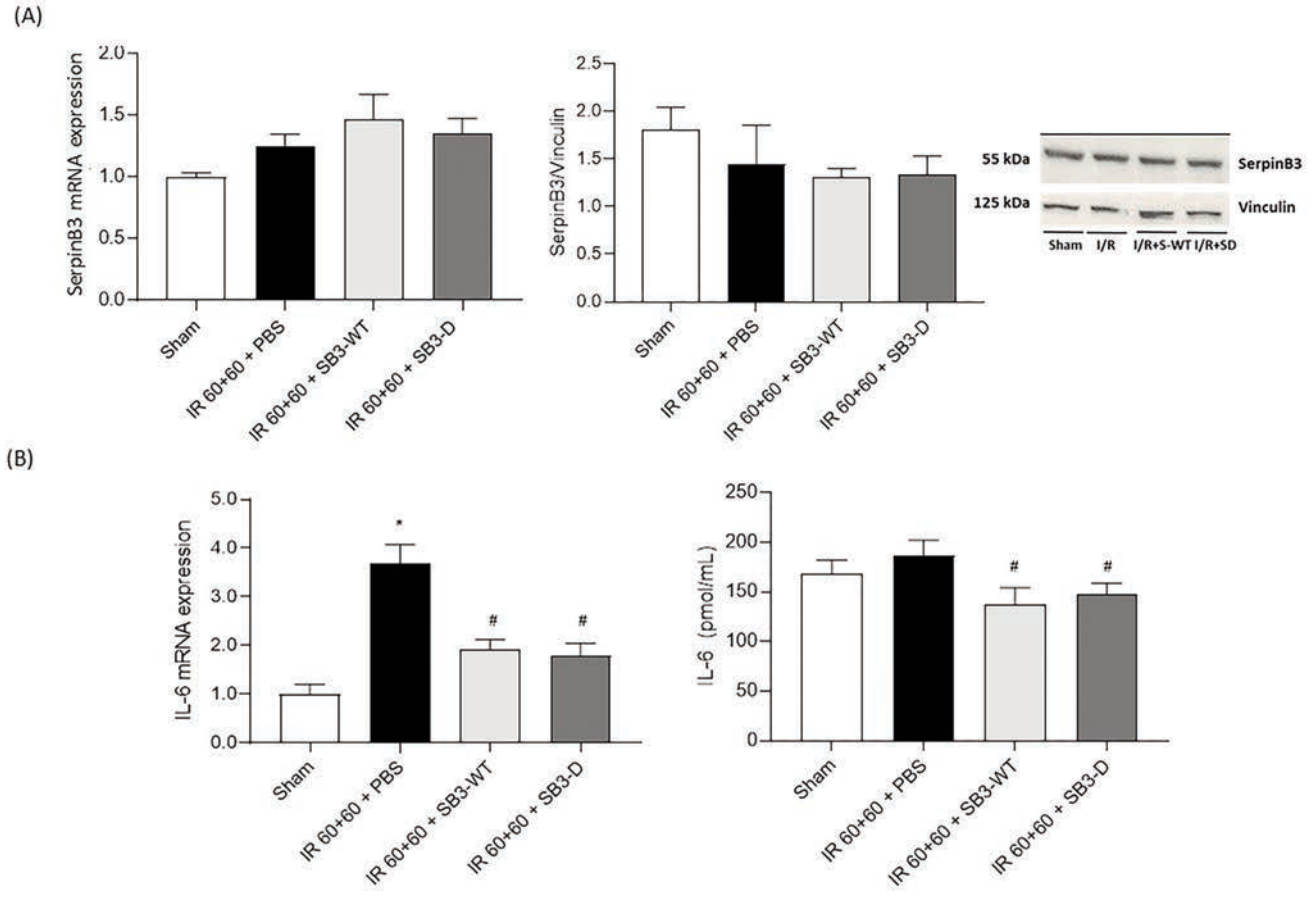

Figure 4. Changes in SerpinB3 (A) and IL-6 (B) mRNA and protein expression evaluated in livers from rats undergoing 60 min ischemia and 60 min warm reperfusion. Animals (n=5 in each group) were treated with SerpinB3-WT or SerpinB3-D. Sham animals (n=5) were subjected to the same procedure without the clamping of the vessels. The results are reported as the mean ± SEM; *p<0.05 *vs* related sham; #p<0.05 *vs* I/R 60+60 +PBS.

lack of oxygen supply triggers cellular glycogen and/or ATP depletion.[16] A novelty of present study has been the identification of profound increase in *SerpinB3* expression that starts at 60 min ischemia and reached 45-fold levels at 180 min ischemia, likely as an attempt of protective response of the damaged liver. In response to ischemic injury, pro-inflammatory cytokines promptly increase in the early development and progression of I/R injury.[17]

Although the results of previous studies showed that inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are increased upon exposure to I/R injury,[18-20] the changes of specific cytokines during only ischemia phase remain to be clarified. In the present study we documented a profound increase in *IL-6* expression during liver ischemia. This result is consistent with those of previous studies: hypoxia but not reoxygenation induces the transcriptional activation of the *IL-6* gene in mouse connective LTK cells. The authors also reported that this event is completely dependent on NF-κB.[21] Furthermore, it was documented that HIF-2α protects against acute liver injury through the production of IL-6.[22] Interestingly, the same factor is also able to induce *SerpinB3* through its direct binding to *SerpinB3* promoter.[6]

In addition to these potential molecular mechanisms, as a response to ischemia, it has been reported that Wnt-β-Catenin and HIF-1 signaling play a relevant protective role during liver ischemia, reducing dramatically the degree of hepatocellular injury.[23] In line with these findings, we have previously found that SerpinB3 is indeed able to upregulate β-catenin.[7,24] In addition, this serpin can operate also as a paracrine mediator able to differentially up-regulate early and transiently HIF-1α, through redox-dependent increased transcription, and in a sustained manner HIF-2α levels, through selective NEDDylation.[25] These data support a crucial role of SerpinB3 in the activation of protective signaling pathways in response to ischemia.

In order to support the preventive use of SerpinB3 for graft preservation in the organ transplant setting, the potential protective effect of SerpinB3-WT or reactive site loop-deleted recombinant SerpinB3-D has been quantified in I/R injury. We observed that SerpinB3-WT administration reduced liver injury as documented in a reduction of polymorphonuclear infiltration and IL-6 levels as well as a decrease in liver enzymes. It's noteworthy that SerpinB3-D administration does not protect against inflammation and enzyme release after I/R damage. The present data suggest that this specific effect is dependent on the SerpinB3 anti-protease activity and this is in keeping with the previously published results [e.g., Unfolded Protein Response (UPR)[8] and TGF-beta modulation [10]].

Instead, it should be noted that in the present study the modulation of IL-6 cytokine occurs equally using both the two SerpinB3 isoform; further investigation is needed to clary this event. In addition, the high levels of tissue IL-6 in sham animals probably are due to the operative trauma of laparotomy as already reported by Ogura *et al*.[26] and Wanner *et al*.[27]

High levels in liver SerpinB3 appear as a typical event of ischemia because at the end of the reperfusion a moderate increase in SerpinB3 was found. Since hypoxia up-regulates SerpinB3 in HepG2 cells through transcriptional inducer HIF-2α, we suppose that the oxygen restoration, that occurs during reperfusion, inactivates HIF-2α by posttranslational hydroxylation of specific amino acid residues within its α-subunits;[28] this is a possible explanation of low levels of SerpinB3 found after I/R injury.

The results presented in this study also indicate that a recovery in tissue ATP occurred only in SerpinB3-WT treated group and this event was not found using SerpinB3-D. Depletion in ATP during ischemia causes loss of transcellular electrolyte gradients, influx of free calcium followed by phospholipase activation and, therefore, it is the main contributor of cell swelling and lysis.[29] In the liver the administration of SerpinB3-WT confers protection as documented by reduced hepatic enzymes possibly through preservation and restoration of tissue ATP contents. Previously data demonstrated a close correlation between hepatic ATP and serum AST levels in I/R injury: ischemic preconditioning resulted in doubling of the ATP levels and significantly lower AST levels after reperfusion.[30] Of note, previous data demonstrated that a fraction of SerpinB3 is located in the inner mitochondrial compartments where it binds respiratory Complex I and down-modulates its activity. Because mitochondrial dysfunction, with decreased activities of the complex enzymes in the respiratory chain has been reported in I/R injury,[31] we suppose that SerpinB3-WT is able to protect hepatic mitochondria during the reperfusion period.

In addition, it has been reported that lysosomal membranes are vulnerable to insults such as ischemia and reperfusion injury and oxidative stress.[32] Moreover, SerpinB3 is a known inhibitor of cathepsin L, a cysteine protease that relocalizes to the cytoplasm after disruption of the lysosomal membrane[33] and recent data demonstrated the role of SerpinB3 in protecting cathepsin L-mediated cell death after lysosomal membrane permeability.[34]

In conclusion, molecular processes occurring during hepatic I/R are diverse, and continuously include new and complex mechanisms. Although other studies are requested, we documented changes in SerpinB3 during ischemia and the positive effects of early administration of SerpinB3-WT that may be considered as a new strategy against hepatic I/R injury thus supporting its preventive use for graft preservation in the organ transplant setting.

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