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ISCHEMIA-REPERFUSION INJURY IN RAT STEATOTIC LIVER IS DEPENDENT ON NFκB P65 ACTIVATION

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Abstract

Background—Steatotic liver grafts tolerate ischemia-reperfusion (I/R) injury poorly, contributing to increased primary graft nonfunction following transplantation. Activation of nuclear factor kappa-B (NFκB) following I/R injury plays a crucial role in activation of pro-inflammatory responses leading to injury.

Methods—We evaluated the role of NFκB in steatotic liver injury by using an orthotopic liver transplant (OLT) model in Zucker rats (lean to lean or obese to lean) to define the mechanisms of steatotic liver injury. Obese donors were treated with bortezomib to assess the role of NF-κB in steatotic liver I/R injury. Hepatic levels of NF-κB and pro-inflammatory cytokines were analyzed by ELISA. Serum transaminase levels and histopathological analysis were performed to assess associated graft injury.

Results—I/R injury in steatotic liver results in significant increases in activation of NF-κB (40%, $p < 0.003$), specifically the p65 subunit following transplantation. Steatotic donor pretreatment with proteasome inhibitor bortezomib (0.1 mg/kg) resulted in significant reduction in levels of activated NF-κB (0.58 ± 0.18 vs. 1.37 ± 0.06 O.D./min/10μg protein, $p < 0.003$). Bortezomib treatment also reduced expression of pro-inflammatory cytokines MIP-2 compared with control treated steatotic and lean liver transplants respectively (106 ± 17.5 vs. 443.3 ± 49.9 vs. 176 ± 10.6 pg/mL, $p = 0.02$), TNF-α (223.8 ± 29.9 vs. 518.5 ± 66.5 vs. 264.5 ± 30.1 pg/2μg protein, $p = 0.003$) and IL-1β (6.0 ± 0.91 vs. 19.8 ± 5.2 vs. 5 ± 1.7 pg/10μg protein, $p = 0.02$) along with a significant reduction in ALT levels (715 ± 71 vs. 3712.5 ± 437.5 vs. 606 ± 286 U/L, $p = 0.01$).

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Conclusion—These results suggest that I/R injury in steatotic liver transplantation are associated with exaggerated activation of NF- κ B subunit p65, leading to an inflammatory mechanism of reperfusion injury and necrosis. Proteasome inhibition in steatotic liver donor reduces NF- κ B p65 activation and inflammatory I/R injury, improving transplant outcomes of steatotic grafts in a rat model.

Keywords

hepatic steatosis; I/R injury; liver transplantation; NF- κ B; PS-341; bortezomib; obese; marginal graft

1. INTRODUCTION

Liver transplantation is an effective treatment option for a variety of end stage liver diseases. Though the number of patients on waiting lists for liver transplantation has increased the number of organs available for transplantation has remained stagnant. Hepatic steatosis remains one of the major reasons accounting for liver organ discards.

Hepatic steatosis, especially macrosteatosis, is associated with increased rates of primary graft non-function and operative mortality post-transplantation[1–4]. Transplant centers routinely discard 25% of the grafts offered that exhibit greater than 30% macrosteatosis, and nearly all decline grafts with >50% steatosis[5]. It has been hypothesized that steatotic grafts are particularly vulnerable to I/R injury. However the mechanisms that contributes to increased susceptibility of steatotic grafts to I/R injury remains poorly defined. In this study we transplanted livers from lean and obese Zucker rats following two hours of cold storage into lean recipients and examined at 2 and 24 hours post transplantation to define the underlying mechanisms that contribute to the enhanced I/R injury. We utilized a short cold ischemia time to define the signaling events since steatotic livers exhibit severe I/R injury and high mortality.

Transcription factor NF- κ B is central in mediating hepatic I/R injury through increased expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6 and IL-8), cell adhesion molecules (ICAM-1 and VCAM-1) and acute phase proteins[6, 7]. Increased levels of TNF- α and IL-1 β in circulation induces necrosis in several *in vitro* and *in vivo* models. Fatty livers have also been shown to develop massive necrosis following I/R insults [8], whereas lean livers display apoptosis. In the current study we used the Leptin deficient Zucker rat model which exhibits relatively uniform steatosis to test our hypothesis. Macrovesicular steatosis is the major reason for discarding potential livers for transplantation. Previous studies have shown that animals in the Zucker model develop macrovesicular steatosis that closely resembles the clinical setting and was used for this study. Moreover, the mechanisms that mediate I/R injury are similar in the Zucker rat and the dietary induced model. Based on its key role in mediating I/R injury, we postulated that activation of the NF- κ B inflammatory pathway might be a major mechanism responsible for the altered pathophysiology observed in the steatotic liver. We further hypothesized that inhibition of NF- κ B activation might improve results in steatotic liver grafts by decreasing acute inflammation and attenuating I/R injury. In this study we demonstrate that transplantation of steatotic liver results in increased activation of NF- κ B resulting in a significant increase in levels of pro-inflammatory cytokines TNF- α , IL-1 β and MIP-2 that are associated with necrosis of transplanted steatotic liver and increased serum transaminases. Bortezomib (PS-341), a selective inhibitor of 26s proteasome is an FDA approved drug for NF- κ B inhibition, has been successfully employed to treat multiple myeloma and mantle cell lymphoma. PS-341 mediated blockade of NF- κ B activation has been shown to induce immunomodulatory changes that inhibit the functions of CD4 T cells, dendritic cells, and

plasma cells through the modulation of cytokine secretion (IFN- γ , TNF- α , IL-6, IL-5, SDF-1 etc). We had selected PS-341 to use in this study because of its significant effect of apoptosis and immune functions, the two major contributors of I/R injury. Using the proteasome inhibitor, bortezomib we further demonstrate that donor pretreatment results in significant reduction in levels of activated NF- κ B, leading to decreased pro-inflammatory cytokines. We also demonstrate markedly improved histology in transplanted steatotic grafts, without evidence of necrosis, and improvement in serum transaminase levels.

2. OBJECTIVE

The overall objective of the study is to define the early molecular events that contribute to the exacerbated I/R injury in the steatotic liver following transplantation and to determine the ability of NF- κ B inhibition to ameliorate I/R injury in the steatotic liver transplantation model.

3. MATERIALS AND METHODS

3.1 Animal model

Zucker rats represent a well-characterized model of leptin receptor deficiency induced obesity [9–12] and were used in this study. Animals were housed in pathogen-free conditions with 12-hour diurnal light cycle and access to standard rodent chow and water ad libitum. Surgical procedures were performed under aseptic conditions approved by the Washington University Animals Studies Committee and in accordance with the National Institutes of Health guidelines in “The Guide for the Care and Use of Laboratory Animals.”

3.2 NF κ B inhibition using bortezomib

Homozygous (Obese, Ob) Zucker rats aged 9–11 weeks and weighing 250–350g were chosen as donors with weight-matched heterozygous (Lean, Ln) rats used as recipients. Lean donors to lean recipients were matched for sex and weight as controls. Intravenous bortezomib (0.1mg/kg) (Millenium Pharmaceuticals, Boston, MA) was given to obese donors immediately prior to operation, while control animals received intravenous saline.

3.3 Surgical Techniques

Orthotopic liver transplantation was performed under isoflurane (Baxter, IL) inhalational anesthesia using Kamada's modified cuff technique[13]. Prior to organ procurement, 1 mL of saline containing 200 units of heparin was given intravenously, and the donor liver perfused via portal vein with 10mL of cold physiological saline, followed by 10mL of cold University of Wisconsin(UW) solution. Following cuff preparation, the graft was stored in UW solution at 4°C for 2 hours. Following storage, the graft was slowly flushed with 20mL of cold saline prior to transplantation.

3.4 Histological grading

Following sacrifice, paraffin embedded liver tissue was cut into 5–7 μ m sections, stained with hematoxylin and eosin and examined by two independent, blinded investigators using semi-quantitative point counting system[14]. Briefly, 100 adjacent fields were scored per slide at 200 $\times$ magnification to determine the percentage of necrosis using an ordinal scale, with grade 0 equaling no evidence of injury, grade 1 indicating mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis, grade 2 indicating moderate to severe injury with extensive nuclear pyknosis, grade 3 indicating severe necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration.

3.5 Serum transaminases & cytokine measurement

Liver injury was quantified by serum alanine aminotransaminase (ALT) using serum analyzer (Biotron, CA). Serum MIP-2, TNF- α and IL-1 β levels were measured by ELISA (Biosource, CA).

3.6 Quantification of NF κ B activation by EMSA and ELISA

Hepatic NF κ B levels was determined using electrophoretic mobility shift assay (Promega, WI) using DNA binding probe (5'-AGTTGAGGGGACTTCCCCAGGC-3'). Trans-Am NF κ B Family (p65, p50, c-Rel, p52, RelB) and p65 ELISA kits (Active Motif, Carlsbad, CA) were used to quantify hepatic NF κ B activation. Briefly, 10 μ g of whole protein liver extract was incubated in duplicate in 96-well plates coated with immobilized oligonucleotide(5'-GGGACTTTCC-3') binding site for NF κ B p65 subunit, developed using anti-IgG horseradish peroxidase, developed and absorbance measured at 450nm.

3.7 Statistical Analysis

Data were analyzed using Graphpad prism 4 software, statistical difference between groups were analyzed by ANOVA, when significant differences were observed Bonferroni's multiple comparison was performed. Results are presented as mean $\pm$ standard error, and considered significant when $p < 0.05$.

4. RESULTS

4.1 Activated NF κ B p65 subunit is significantly increased in steatotic grafts

Analysis of the steatotic and lean liver grafts demonstrated a significant increase in NF- κ B activation at both 2 and 24 hours post-reperfusion in steatotic grafts (Fig. 1A lanes 1 and 4) compared to lean. Activation was markedly decreased by donor treatment with bortezomib (Fig. 1A, lanes 2 and 5), resulting in levels comparable to lean grafts. Analysis of individual members of NF- κ B family using a NF- κ B profile ELISA revealed that NF κ B activation in steatotic grafts was due to the p65 subunit (Fig. 1B) whereas no significant differences were observed in the p50, c-Rel, p52 and Rel-B subunits, with a 40% increased activation in steatotic versus lean grafts at early reperfusion ($p < 0.003$). By 24 hours post-reperfusion, activated NF κ B remained numerically but not statistically elevated in steatotic grafts ($p = \text{NS}$).

4.2 Proteasome inhibition by bortezomib is specific for NF- κ B p65

Analysis of hepatotoxicity by AST and ALT in lean and obese rats (n=5 each) treated with 0.5, 0.25, 0.1 mg/kg body weight of bortezomib at 24 hrs demonstrated that treatment with 0.5 and 0.25 mg/kg resulted in a 2 fold increase in the levels of AST and ALT ($p < 0.05$) in the obese animals when compared to untreated controls and lean animals. However treatment with 0.1 mg/kg did not result in any significant increase in the AST and ALT levels compared to basal level and this dose was used for further studies. Proteasome inhibition with bortezomib in steatotic livers specifically inhibited p65 without affecting p50, c-Rel, p52 and Rel-B (Fig. 1B). Bortezomib treatment resulted in effective inhibition of p65 in steatotic versus lean livers at both early and late reperfusion (2 hours: $p < 0.003$, 24 hours: $p < 0.05$), similar to levels expressed by lean grafts $p = \text{NS}$ (Fig. 1C).

4.3 Transplant recipients of steatotic liver grafts demonstrate a significant increase in pro-inflammatory cytokines and chemokines and is reduced by steatotic donor treatment with bortezomib

MIP-2 mediates hepatic I/R injury through active neutrophil recruitment and activation, including induction of respiratory burst and CD11b up-regulation (8,17,31). We found that

MIP-2 levels in liver protein homogenates were increased 2.5-fold in steatotic versus lean grafts ($p<0.001$) 2 hours post-reperfusion (Fig. 2A). Proteasome inhibition in steatotic donors reduced hepatic MIP-2 more than 4-fold at 2 hours reperfusion in bortezomib treated Ob to Ln groups compared to saline treated Ob to Ln pairs ($p=0.02$) as well as lean transplant pairs ($p<0.001$) (Fig. 2A). Hepatic MIP-2 levels were also reduced at 24 hours reperfusion, but did not reach significance.

TNF- α is well known to play a critical role in hepatic I/R injury (40,49), by inducing apoptotic cell death and contributing to a pro-inflammatory response via activation of JNK and NF κ B pathways. Hepatic TNF- α levels showed a 2-fold increase measured in steatotic versus lean grafts ($p=0.003$) at early reperfusion and 1.3-fold increase at late reperfusion ($p=ns$). Proteasome inhibition with bortezomib resulted in a 2-fold TNF- α reduction in steatotic versus lean grafts at early reperfusion ($p=0.003$), such that bortezomib-treated obese rats showed local TNF- α levels similar to Ln to Ln controls ($p=NS$). At 24 hours post-reperfusion, bortezomib treatment of obese donors resulted in a 30% reduction in hepatic TNF- α ($p=0.03$), again similar to lean transplant pairs ($p=NS$) (Fig. 2B).

IL-1 β , another important mediator of hepatic and systemic I/R injury (7,21,28), was elevated 3-fold in recipients of steatotic versus lean liver grafts at early reperfusion ($p<0.05$). Differential elevation in fatty grafts approached 4-fold by 24 hours post-reperfusion ($p<0.005$, Fig. 2C). Analysis of the steatotic livers transplanted following treatment with bortezomib resulted in a significant reduction in the hepatic IL-1 β by at least 3-fold at both 2 and 24 hours post-reperfusion in bortezomib treated Ob to Ln transplant pairs (2h, $p=0.02$; 24h, $p=0.001$) when compared to untreated controls. Measurement of local IL-1 β levels in bortezomib-treated steatotic grafts were not different than lean grafts at both early and late reperfusion, $p=NS$ (Fig. 2C).

4.4 Proteasome inhibition abrogates ALT increases observed in recipients of steatotic grafts

ALT release is an established marker of hepatocellular damage. There were no differences in serum ALT following early reperfusion, but greater than 6-fold elevation was observed in animals that received steatotic grafts when compared to animals receiving lean grafts at 24 hours post-reperfusion ($p<0.01$, Fig. 3). This marked release of transaminases correlated with massive necrosis observed histologically (Fig. 4A). ALT elevation was abrogated by bortezomib treatment in obese to lean transplant pairs at 24 hours post-reperfusion ($p=0.01$). ALT levels in bortezomib-treated Ob to Ln transplant pairs were indistinguishable from lean transplant pairs ($p=NS$).

In a separate set of experiments to assess mortality, we performed $n=6$ pairs of liver transplants per group and followed animals to 24 hours post-transplant. Ln to Ln pairs showed the best graft function, followed by bortezomib treated pairs. Not surprisingly, untreated Ob to Ln transplant pairs fared the worst, with a graft function rate of 33%. Although statistical significance was not achieved, possibly due to small sample sizes or other factors such as the high degree of macrosteatosis observed in this model, these initial results suggest a trend towards better graft function when donors of steatotic livers are treated by bortezomib and needs to be further tested in animals with varying degrees of fibrosis including dietary models.

4.5 Severe necrosis is the predominant form of hepatocellular death in steatotic grafts

Apoptosis results in controlled cell death with efficient clearance of the dead cells whereas necrosis results in release of the cellular contents that leads to activation of potent pro-inflammatory signals. Histopathological analysis of the livers demonstrated severe necrosis

and architectural obliteration predominating in grafts from obese donors to lean recipients (Fig. 4A). Nuclei were distorted, with indistinct cellular borders and a markedly hypereosinophilic tissue. Injury grading (10) revealed nearly 70% Grade 3 necrosis in steatotic versus lean grafts at 24 hours reperfusion (65.6 ± 5.5 vs. $13.3 \pm 3.5\%$ Grade 3 necrosis/100hpf $p < 0.001$) (Fig. 4D), indicating irreversible hepatocellular death. There were no appreciable histological differences among groups at 2 hours post-reperfusion (data not shown).

On the other hand, bortezomib treated steatotic grafts appeared viable, with well-preserved nuclear and cellular morphology (Fig. 4B), appearing similar to lean liver grafts (Fig. 4C). The histological injury observed in saline-treated Ob to Ln grafts was abrogated by bortezomib treatment ($17 \pm 3\%$ vs. $65.6 \pm 5.5\%$, $p = 0.0007$), comparable to the scoring of Ln to Ln controls ($13.3 \pm 3.5\%$, $p = \text{NS}$).

5. DISCUSSION AND REFERENCES

Hepatic steatosis remains one of the major impediments in the under-utilization of marginal livers for transplantation. Hepatic steatosis and its associated higher rates of primary graft non-function, morbidity and mortality are increasingly complicating the current practice of liver surgery and transplantation [2, 3, 15–17]. The lack of understanding of the mechanisms of injury in the steatotic liver results in the inability to initiate specific therapeutic targeting of the steatotic liver to improve its function and has remained a difficult challenge. In the present study we demonstrate that activation of the NF- κ B signaling cascade induces an inflammatory cascade as a major mechanism in the pathophysiology of steatotic liver response to I/R injury. We also demonstrate that NF κ B inhibition through a proteasome inhibitor, Bortezomib, in the steatotic donor results in a significant reduction of acute inflammatory injury. Our findings demonstrate that p65 is the predominant NF κ B subunit expressed by the steatotic graft in response to I/R injury (Fig. 1B and 1C, $p < 0.003$). Pharmacological blockade of NF κ B by proteasome inhibitor bortezomib appears to be specific for p65, resulting in attenuation of the inflammatory cascade, and improved structure and function of the steatotic liver following transplantation.

Strategies known to effectively attenuate I/R injury in lean liver have thus far been ineffective or inconclusive in steatotic livers [18, 19]. In evaluating a pan-caspase inhibitor, Selzner et al. described the failure of activation of apoptotic cascade in steatotic rat liver leading to the prominent role of necrosis, whereas in lean hepatocytes I/R injury results in activation of apoptosis [8, 20]. Histopathological analysis of transplanted steatotic liver demonstrated significant increases in the amount of necrosis when compared to lean livers (Fig. 4A, D) and support a prominent role for necrosis [8] in the mechanism of cell death (Fig. 4D) [1, 4, 8, 15]. It is unclear whether necrosis occurs by default following failure to activate apoptosis [8, 21], suggesting the possibility that NF- κ B activation may be a common upstream event to the necroapoptotic signaling pathway [22–24].

Analysis of the steatotic livers immediately post transplantation by EMSA demonstrates that there is a significant increase in the activation of NF- κ B in the steatotic liver when compared to lean controls (Fig. 1). Analysis of the individual subunits of the NF- κ B family further demonstrated that p65 was the predominant subunit activated in the steatotic liver graft. Several previous studies using LPS have demonstrated that activation of p65 results in the increased production of pro-inflammatory cytokines. Based on our data (Fig. 2A–C), another possibility is that NF κ B activated transcription in steatotic animals is primarily inflammatory, and the necrotic injury observed (Fig. 4A and 4D) is due to an overwhelming inflammatory response rather than the alternative outcome of failed apoptosis. Histopathology assessment using standard morphology did not show any significant changes

in the liver architecture in rats treated with PS-341 in our studies. In addition, we believe that NF- κ B dosing immediately prior to donor liver recovery would be unlikely to allow for changes in macrosteatosis sufficient to induce a significant change in microcirculation. On this basis, we believe the improvements seen with PS-341 in the setting of severe macrosteatosis are more likely resulting from proteasome inhibition of inflammatory cytokine upregulation.

Our results also demonstrate that p65 is the predominant NF κ B subunit expressed in steatotic graft in response to I/R injury (Fig. 1B and 1C, $p < 0.003$). Also known as RelA, p65 is a critical subunit in activation of the canonical NF κ B pathway[22–26]. NF κ B is known to be a crucial regulator of the inflammatory response, triggering production of pro-inflammatory cytokines (IL-1, IL-6, TNF- α), chemokines (IL-8, MIP-1 α , MCP-1, RANTES), and adhesion molecules (ICAM, VCAM, E-selectin) with activation of Kupffer cells and recruitment and sequestration of activated neutrophils to the liver[7, 24, 27–30]. Subsequent release of reactive oxidants and proteases results in parenchymal injury and organ necrosis[30], complemented by microcirculatory disturbances and Kupffer cell induced oxidant stress[29]. Our results (Fig. 2–3, 4A and D) favor these observations, pointing to exaggerated NF κ B activation and inflammatory response in the steatotic graft. NF κ B activation in steatotic grafts following I/R injury is 2-fold greater than in lean counterparts (Fig. 1A and 1C, $p = 0.001$), resulting in 2, 3 and 4 fold higher expression of hepatic pro-inflammatory cytokines IL-1 β , TNF- α and MIP-2 ($p < 0.005$, < 0.01 , < 0.001 , respectively).

Aside from its role as the strongest transcriptional activator of NF κ B(41), recent studies have shown that posttranslational modifications of p65 are critical in regulating NF κ B transcriptional activity, including TNF- α and IL-1 induced phosphorylation, and TNF- α induced acetylation[22, 24, 31]. Our data strongly suggest that p65 may represent an important link between hepatic steatosis and its poor tolerance to ischemic stress. While many methods of NF κ B modulation have been described, [32–35], it has been argued that the control of I κ B degradation is the most important point of regulation[36]. Accordingly, we found that donor treatment with bortezomib, a 26S selective proteasome inhibitor, reduced p65 activation at an early (2hours, Fig. 1C, $p < 0.003$) time point following reperfusion in steatotic graft, such that there was no difference in expression when compared to lean grafts or at late time points (Fig. 1C, $p = \text{NS}$). These observations are similar to those observed in other models where there is a significant increase in the NF- κ B immediately following injury but returns to normal levels after 4 hours. These observations demonstrate that blocking of NF- κ B activation in steatotic livers results in a significant reduction in the activation of pro-inflammatory cascade. NF- κ B has been shown to play a central role in both the inflammatory injury phase and the regeneration phase following liver I/R injury[37]. NF- κ B has been shown to play a pivotal role in the increased production of both pro-inflammatory cytokines (TNF- α , IL-1 β , etc) that damages the cells as well as anti-inflammatory cytokines (IL-10, TGF- β) that are cytoprotective. Though in the current study we found significant decreases in the pro-inflammatory cytokine levels (TNF- α , IL-1 β and KC1, Fig. 2) we did not find any significant differences in the anti-inflammatory cytokines (IL-10, TGF- β , data not shown) following NF- κ B inhibition by bortezomib. These observations suggest that there could be other potential mechanisms such as increased number of regulatory T cells, decreased dendritic cell activation, decreased TLR activation as well as decreased CD4 T cell functions could contribute to the protective effects of PS-341 used in this study.

In summary, our data indicate a key role for NF- κ B subunit p65 in pathogenesis of I/R injury in steatotic livers. Defining the mechanisms by which p65 mediates NF- κ B activation leading to activation of inflammation may provide clues to the mechanism of I/R injury in

hepatic steatosis, and a precise target for reduction of I/R injury. The current study demonstrates that one such strategy is pharmacological inhibition of NF- κ B with proteasome inhibitor bortezomib, resulting in decreased activation of p65 NF- κ B subunit and decreased pro-inflammatory cytokine secretion. Reduction in levels of TNF- α , IL-1 β and MIP-2 was associated with a significant reduction in necrosis of steatotic livers and significant reductions in AST and ALT levels, similar to those observed in transplanted lean livers. Analysis of benefits with bortezomib treatment in this model demonstrated that there was a reduction in I/R associated graft injury in Bortezomib treated fatty livers when compared to untreated controls (Fig. 3). Further experiments using bortezomib and other proteasome inhibitors are warranted to explore potential strategies in human liver transplantation with steatotic liver grafts.

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List of Abbreviations

IL-1β	interleukin-1 beta
I/R	ischemia-reperfusion
Ln	lean
MIP-2	macrophage inflammatory protein-2
NFκB	nuclear factor kappa-B
PS-341	bortezomib
Ob	obese
OLT	orthotopic liver transplant
TNF-α	tumor necrosis factor alpha

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Highlights

Steatotic livers exhibit exacerbated I/R injury

I/R injury in steatotic liver results in increased activation of NF- κ B p65 subunit

Proteasome inhibition in steatotic liver reduces NF κ B p65 activation and I/R injury

Bortezomib reduces expression of inflammatory cytokines in steatotic and lean liver

Figure 1A

Figure 1B

Figure 1C

Figure 1. NFκB activation in steatotic livers is mediated by p65 subunit and treatment with bortezomib (PS-341) results in a 2-fold reduction in activation

A. Electrophoretic mobility shift assay for NFκB showed significant activation at both 2 and 24 hour reperfusion time points in liver extracts from obese (Ob) donors of steatotic grafts. Activation was visibly decreased by donor treatment with bortezomib, appearing comparable to lean (Ln) controls. **B.** ELISA for NFκB family members showed that observed NFκB activation is due almost exclusively to the p65 subunit. Treatment with bortezomib (PS-341) results in a 2-fold reduction in activation, $p < 0.05$. **C.** ELISA for NFκB p65 subunit showed greater than 2-fold reduction in activation in bortezomib (PS-341) treated obese (Ob) to lean (Ln) transplant pairs when compared to saline treated pairs at the 2 hours post-reperfusion, ($p = 0.001$). At 24 hours post-reperfusion, bortezomib treated Ob to Ln pairs again showed significant reduction in NFκB activation ($p < 0.05$), demonstrating effective inhibition of NFκB activation at both early and late reperfusion in our model.

Figure 2A

Figure 2B

Figure 2C

Figure 2. Increased levels of MIP-2, TNF- α , and IL-1 β observed in steatotic livers following I/R injury are significantly reduced by donor treatment with bortezomib

A. ELISA for hepatic MIP-2 levels showed a 4-fold reduction at 2 hours post-reperfusion in bortezomib treated obese (Ob) donor to lean (Ln) recipients as compared to saline treated Ob to Ln rats ($p=0.0003$). Hepatic levels of MIP-2 were lower in bortezomib treated Ob to Ln rats when compared to Ln to Ln transplants ($176 \pm 10.6 \text{ pg}/2 \mu\text{g protein}$, $p=0.0009$). **B.** ELISA for hepatic TNF- α levels showed a 2-fold reduction in bortezomib (PS-341) treated obese (Ob) to lean (Ln) pairs as compared to saline treated pairs at early (2hr) reperfusion, ($p=0.003$), such that bortezomib treated rats showed local TNF- α levels similar to lean control pairs ($p=\text{NS}$). At 24 hours post-reperfusion, bortezomib treatment again resulted in a 30% reduction in hepatic TNF- α ($p=0.03$), similar to that of Ln to Ln controls. **C.** ELISA for hepatic IL-1 β was reduced 3-fold in at both 2 and 24 hour reperfusion time points in bortezomib treated obese (Ob) to lean (Ln) transplant pairs as compared to saline treated Ob to Ln pairs. ($p=0.02$; $p=0.001$). Bortezomib treated pairs showed local IL-1 β levels comparable to Ln controls at both 2 and 24 hours post-reperfusion.

Figure 3.

Serum ALT measurement showed an elevation at early reperfusion (2 hours) in obese (Ob) to lean (Ln) transplant pairs compared to Ln to Ln controls, but bortezomib treatment did not affect ALT. At 24 hours post-reperfusion, there was a marked ALT elevation in saline treated Ob to Ln transplant pairs, correlating with massive necrosis seen on histology. This elevation was abrogated by bortezomib treatment ($p=0.01$), reducing ALT by greater than 5-fold and returning ALT to the level of Ln to Ln controls. There was no difference in ALT between bortezomib treated Ob to Ln animals compared to Ln controls.

Figure 4.

H&E photos at 40X. **A.** Hepatic graft appearance after 2 hours of ischemia and 24 hours of reperfusion from obese donors showed significant areas of necrosis and architectural obliteration, as indicated by the arrow. Nuclei are distorted, no cellular borders can be discerned, and the tissue is markedly hypereosinophilic. **B.** Bortezomib (PS-341) treated steatotic grafts appear viable at 24 hours, with well-preserved nuclear and cellular morphology, appearing grossly similar to controls from lean donors as pictured in **C.** There were no appreciable histological differences between groups at 2 hours post-reperfusion (not shown). **D.** Histological scoring. Saline treated obese (Ob) to lean (Ln) grafts at 24 hours reperfusion showed nearly 70% Grade 3 necrosis: disintegration of hepatic cords, severe hemorrhage, marked neutrophil infiltration. This histological injury was completely abrogated by bortezomib treatment, $p=0.0007$, and similar to Ln to Ln controls ($p=NS$).