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Therapeutic benefits of SYK inhibitor administration on binge drinking-induced alcoholic liver injury, steatosis and inflammation in mice

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Abstract

Background—Binge drinking is increasingly recognized as an important cause of liver disease with limited therapeutic options for patients. Binge alcohol use, similar to chronic alcohol consumption, induces numerous deregulated signaling events that drive liver damage, steatosis and inflammation. In this report, we evaluated the role of spleen tyrosine kinase (SYK), which modulates numerous signaling events previously identified linked in the development alcohol-induced liver pathology.

Methods—A three day alcohol binge was administered to C57BL/6 female mice and features of alcoholic liver disease (ALD) were assessed. Some mice were treated daily with intraperitoneal injections of a SYK inhibitor (R406) [5–10mg/kg body weight] or drug vehicle control. Liver and serum samples were collected and were assessed by western blotting, biochemical, ELISA, EMSA, RT-qPCR and histopathological analysis.

Results—We found that binge drinking induced significant SYK activation (SYK^{Y525/526}) with no change in total SYK expression in the liver. Functional inhibition of SYK activation using a potent SYK inhibitor, R406, was associated with a significant decrease in alcohol-induced hepatic inflammation as demonstrated by decreased phospho-NF-κB p65, NF-κB nuclear binding, TNF-α and MCP1 mRNA in the liver. Compared to vehicle controls, SYK inhibitor treatment decreased alcohol binge-induced hepatocyte injury indicated by histology and serum ALT. Strikingly, SYK inhibitor treatment also resulted in a significant reduction in alcohol induced liver steatosis.

Conclusion—Our novel observations demonstrate the role of SYK activation in the pathomechanism of binge drinking induced liver disease highlighting SYK a potential multifaceted therapeutic target.

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Introduction

Binge drinking, characterized by heavy episodes of alcohol consumption driving blood alcohol levels to 0.8g/dl or higher (Courtney and Polich, 2009), is becoming an alarming global health problem. In the United States, it is estimated about 1 in 6 adults binge drinks about four times a month (Centers for Disease and Prevention, 2012). While binge drinking has been mostly linked to young adults between the ages of 18–34, it is now being realized that individuals over the age of 65 also binge drink (Centers for Disease and Prevention, 2012). While chronic alcohol consumption leads to devastating health consequences especially on the liver, reports including ours have shown that binge drinking can be equally damaging and amplify liver damage in chronic alcoholics (Shukla et al., 2013, Bala et al., 2014). Binge drinking, just like chronic alcohol consumption, has been shown to induce molecular pathophysiologic changes in the liver that drive liver inflammation, steatosis and cell death (Szabo, 2015, Bala et al., 2012, Tuma and Casey, 2003). There are currently very limited treatment options for patients affected by binge drinking and chronic alcoholic liver disease. Given the broad spectrum of pathophysiologic changes associated with binge drinking, the identification of potent multifunctional therapeutic agents is highly needed.

In this report, we assessed the therapeutic potential of a spleen tyrosine kinase (SYK) inhibitor to combat binge drinking induced alcoholic liver disease (ALD). Spleen tyrosine kinase is a cellular non-receptor tyrosine kinase, which has been shown by numerous studies to modulate disease conditions characterized significant inflammation, steatosis and cell which are major hall marks of ALD (Mocsai et al., 2010, Dennehy et al., 2008, Lee et al., 2015, Miller et al., 2012, Sanderson et al., 2009, Yi et al., 2014). Functional inhibition of SYK in these conditions has been shown to alleviate inflammation and cell death (Bajpai, 2009, Genovese et al., 2011, Lapchak et al., 2012, Lin et al., 2010, Scott, 2011, Thorarensen and Kaila, 2014). The role of SYK in the pathomechanism of or use of SYK inhibitors in binge drinking induced ALD has not been studied.

Using a well characterized mouse model of binge drinking (Brandon-Warner et al., 2012, Bala et al., 2012), we found a significant increase of hepatic activated SYK but no change in total SYK expression. Using a potent SYK inhibitor we found that SYK inhibitor treatment protected mice from binge alcohol induced liver disease. These observations suggest a role of SYK in the pathomechanism of ALD highlighting SYK inhibitors as a potential treatment in alleviating liver disease due to binge drinking.

Methods

Animals

Acute/binge ethanol experiments made use of 10–12 week old C57BL/6 female mice purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed at UMASS Medical School (Worcester USA) animal facility. Mice were maintained on unrestricted chow diet and received oral gavage of alcohol at 5g/kg body weight (20% vol/vol in sterile phosphate buffer saline) as previously described (Bala et al., 2012). Animals in the control experimental groups received oral gavage of sucrose calorie matched for the corresponding binge alcohol group. Two hours prior to alcohol or sucrose gavage mice received i.p.

injections of the SYK inhibitor (5mg/kg body weight in 200 μ L volume) (Selleckchem cat # S2194) or i.p. injections vehicle (200 μ L volume) over a 3 day period. Following three days of ethanol gavage with and without SYK inhibitor treatments, blood samples were obtained from mice by cheek bleed into serum collecting tubes (BD Biosciences, San Jose, CA USA). Serum samples were separated from blood within the hour from cheek bleeds. Following blood collections, mice were euthanized and liver samples harvested and stored appropriately for further analysis.

Kinetic Assays

ALT analysis was performed on mouse serum samples using a kinetic assay as previously described (Petrasek et al., 2012).

Total liver triglyceride levels were determined using the L-Type Triglyceride H kit (Wako Chemicals USA Inc., VA) (Petrasek et al., 2012).

Blood alcohol concentration

The amount of alcohol in the serum samples was measured by an Analox Alcohol Analyzer as previously described (Bala et al., 2012).

Liver Histopathology

Formalin fixed liver specimens from mice were stained with Hematoxylin/eosin (H&E) using the UMASS Medical School DERC histology core facility.

ELISA of cell culture supernatants

Serum tumor necrosis factor alpha (TNF- α), MCP1, IL-1 β concentrations were measured using a sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, 100 μ L of 1/10 diluted serum samples were added into flat-bottom 96-well micro titer plates pre-coated overnight with a capture antibody against TNF- α , followed by incubation for 2 h at room temperature. Plates were washed three times, followed by the addition of 100 μ L/well antibody against TNF- α conjugated with horseradish peroxidase. After 1 hour of incubation, plates were washed three times with wash buffer. Then 100 μ L substrate solution was added, and the plates were incubated for 30 min at room temperature in the dark. The reaction was terminated with acid stop solution (50 μ L). Optical densities of plates were read at 450 nm in a micro plate reader. ERK1/2 (pT202/Y204) ELISA was measured from liver protein lysates using the Abcam ERK1/2 (pT202/204) SimpleStep ELISA kit (cat # ab17+6640) according to the manufacturers guide.

Electrophoretic mobility shift assays (EMSA)

Nuclear protein extracts were obtained from mice liver samples using methods described recently. 4 μ g of total liver nuclear protein with concentrations determined using the BioRad protein assay. NF- κ B nuclear binding assay was performed as described previously (Ganz et al., 2015).

Real time quantitative mRNA expression analysis

Total liver RNA was extracted from liver samples using the Qiagen RNeasy kit according to the manufacturer's specification. 1µg of total RNA extracted from liver tissue was transformed to cDNA using iScript cDNA Synthesis Kit (BIO-RAD) according to the manufactures specifications. Real time qPCR was performed using Sybr-Green reagent (BIO-RAD) and CFX96 Touch Real-Time PCR detection system (Bio-Rad). Relative comparison of target gene expressions made use of the comparative Ct method with no treatment control serving as baseline reference for comparisons. The 18s RNA was used gene normalisation control similar to our previous reports (Ganz et al., 2015, Bukong et al., 2016). The following primer pairs were used.

Mouse Primers	Forward sequence 5' to 3'	Reverse sequence 5' to 3'
TNF α	CACCAC CATCAA GGACTC AA	AGGCAACCTGACCAC TCTCC
MCP1	CAGGTCCCT GTCATGCTTCT	CAGGTCCCTGTC ATGCTTCT
IL-1 β	CTTTGAAGTTGACGGACCC	TGAGTGATACTGCCTGCCTG
18s	GTA ACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
PRDM16	CCCCACATTCCGCTGTGAT	CTCGCAATCCTTGCACTCA
FASN	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
UCP1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
ADRP	CTGTCTACCAAGCTCTGCTC	CGATGCTTCTCTTCCACTCC

Western Blot analysis and antibodies

Total liver protein was extracted from mice liver using RIPA buffer (Boston Bio-products cat # BP-115) supplemented with protease inhibitor (Roche Cat. # 11836153001) and phosphor-Stop (Roche cat # 04906837001). 50ug of total protein was used for western blot analysis and resolved proteins transferred unto nitrocellulose membranes and visualized by chemiluminescent methods using the Fujifilm LAS-4000 luminescent image analyzer. The following antibodies were used: SYK (cell Signaling cat. # 2712), phospho-SYK^{Y525/526} (Cell signaling cat. # 2710), phospho-SYK^{Y525/526} (Abcam Cat. # ab58575), Phospho-NF- κ B p65 (Ser536) (Cell signaling Cat. # 3031 and β -Actin (Abcam cat. # ab6276).

Statistical Methods used

GraphPad prism was used for graphical presentations and statistical analysis was done using the student t test and analysis of variance with p<0.05% considered to be statistically significant. Graphical data are expressed as Mean +SEM.

Results

Binge alcohol induced liver damage is associated with significant hepatic SYK activation

Binge drinking has been shown to induce a wide spectrum of pathophysiologic changes similar to chronic alcohol consumption (Shukla et al., 2013, Bala et al., 2014, Bala et al.,

2012). In both conditions there are limited treatment options for patients thereby necessitating an urgent need for novel therapeutic discovery studies. We found that binge alcohol consumption in mice resulted in a significant hepatic injury as determined by H&E histology (Fig. 1A) and significant increase of serum ALT (Fig. 1A). Further, binge alcohol use in mice was associated with significant hepatic steatosis as determined by Oil-red-O staining (Fig. 1B) and increased liver triglyceride levels (Fig. 1B). We found that binge alcohol-induced liver injury associated with an increase in liver TNF- α (Fig. 1C), MCP1 (Fig. 1C) and IL-1 β (Fig. 1C). While there was no change in total SYK (Fig. 1D), strikingly a significant increase of activated SYK (phospho-SYK^{Y525/526}) expression was found in the liver after binge drinking (Fig. 1E). These observations suggested a potential role of SYK activation in binge alcohol-induced liver disease thereby necessitating further investigations.

Functional inhibition of SYK significantly reduces binge alcohol-induced activated SYK and phospho-ERK1/2 in the liver

Binge alcohol-induced liver disease is associated with deregulated signaling events that drive downstream signaling processing including ERK1/2 activation leading to significant hepatic inflammation (Aroor et al., 2011). To test the therapeutic potential of SYK inhibitor treatment in binge alcohol-induced liver disease, mice were injected intra-peritoneally with the SYK inhibitor, R406 (5mg/kg) or not as detailed in the experimental schematic (Sup. Fig. 1A). We found that SYK inhibitor treatment did not have any effects on blood alcohol levels (Sup. Fig. 1B) or on total hepatic SYK expression (Fig. 2A), but significantly reduced binge alcohol-induced SYK activation (phospho-SYK^{Y525/526}) (Fig. 2B). Further, SYK inhibitor treatment was associated with a significant reduction in binge alcohol-induced liver phospho-ERK1/2 as determined by ELISA method (Fig. 2C).

Therapeutic administration of a SYK inhibitor significantly reduces pro-inflammatory cytokine induction and liver injury induced by binge alcohol drinking in mice

A major hallmark of binge alcohol induced liver disease is the induction of hepatic injury caused by the direct effect of alcohol, alcohol metabolites, gut derived endotoxins and increased pro-inflammatory cytokines (Shukla et al., 2013, Szabo, 2015, Szabo and Bala, 2010, Szabo and Mandrekar, 2010). To identify potential mechanisms of how SYK inhibitor treatment ameliorated binge-alcohol induced liver disease, we assessed the effect of in-vivo treatment with R406 on phospho-NF κ B p65, NF κ B nuclear binding, which has been shown previously to be significantly increased during binge alcohol drinking in mice (Bala et al., 2012). We found that SYK inhibitor treatment significantly reduced binge alcohol induced liver phospho- NF- κ B p65 (Fig. 3A) and NF- κ B DNA binding (Fig. 3B). Further we found that SYK inhibitor treatment significantly reduced the NF κ B targets, TNF- α (Fig. 3C) and MCP1 (Fig. 3D) mRNA and protein liver expression of these cytokines that was significantly induced following binge alcohol drinking in mice. SYK inhibitor treatment led to a significant reduction in liver injury as demonstrated by H&E histology (Fig. 3E) and alteration of serum ALT (Fig. 3E) compared to binge alcohol vehicle treated group.

Inhibition of SYK activation in-vivo suppresses binge alcohol-induced hepatic steatosis by suppressing lipid biogenesis and enhancing fatty acid oxidation

Binge alcohol, similar to chronic alcohol, is characterized by significant hepatic steatosis (Petrasek et al., 2013). To further assess the therapeutic effects SYK inhibitor treatment in binge alcohol induced liver disease, we assessed hepatic steatosis in mice following binge alcohol drinking with and without SYK inhibitor or vehicle treatment as indicated. We found that SYK inhibitor treatment significantly reduced the alcohol-induced hepatic steatosis as demonstrated by liver Oil-Red O staining (Fig. 4A). Further, SYK inhibitor treatment was also associated with a significant reduction of binge alcohol induced liver triglyceride accumulation (Fig. 4B). Given that hepatic steatosis involves increased lipid biogenesis and storage, we next assessed the effects of SYK inhibitor treatment on these components. We found that SYK inhibitor treatment reduced liver Adipose differentiation-related protein (ADRP) (Fig. 4C) and fatty acid Synthase (FASN) (Fig. 4D) mRNA expression that was induced by binge alcohol drinking in mice. Strikingly, SYK inhibitor treatment was also associated with a significant increase in genes that enhance lipid energy dissipation (Seale et al., 2007, Seale et al., 2011, Baluom et al., 2013) as demonstrated by increased PR Domain Containing 16 (PRDM16) (Fig. 4E) and Uncoupling Protein 1 (UCP1) (Fig. 4F) mRNA expression.

Discussion

Excessive alcohol use is a major cause of many social and health problems including liver disease. While long term alcohol use was previously thought to be a prerequisite for alcoholic liver disease (ALD) (Hatton et al., 2009), different drinking patterns are present in alcoholics and the significance of different alcohol consumption patterns on liver disease is yet to be understood. Some patterns are characterized by heavy drinking episodes (binge drinking) that are increasingly recognized to be just as detrimental to the liver (Shukla et al., 2013, Bala et al., 2014). Similar to chronic alcoholic liver disease, the spectrum of liver pathology due to binge drinking is characterized by metabolic changes that cause steatosis, hepatic inflammation and cell death (Szabo, 2015, Szabo and Bala, 2010, Louvet and Mathurin, 2015, Tuma and Casey, 2003). Given the broad spectrum of deregulated events that characterize binge drinking induced ALD, the identification of a multi-targeted therapeutic option is a major imperative. Here we assessed the role of spleen tyrosine kinase (SYK) as a potential multifunction and therapeutic target for binge drinking induced ALD.

Binge alcohol drinking in humans and mouse models is characterized by increased serum endotoxins and pro-inflammatory cytokines including TNF- α , IL-6 and MCP-1 (Bala et al., 2014, Szabo, 2015, Szabo and Bala, 2010). Serum endotoxin, lipopolysaccharide (LPS), is a potent trigger of hepatic inflammation by activation of TLR4 expressing cells in the liver (Szabo, 2015, Szabo and Bala, 2010). TLR4 has been shown to bind constitutively to spleen tyrosine kinase and induces pro-inflammatory cytokine production including TNF- α and MCP-1 (Miller et al., 2012) when activated. SYK is a non-receptor tyrosine kinase which is expressed on almost all cell types in the body with diverse regulatory functions (Mocsai et al., 2010). SYK plays an important modulatory role in immune recognition receptors triggering multiple downstream signaling pathways modulating both the innate and adaptive

immune responses (Mocsai et al., 2010, Liao et al., 2013). SYK has been implicated in the pathogenesis of numerous diseases characterized by inflammation, steatosis and cell death which are classical features of ALD (Andre et al., 2011, Frommhold et al., 2007, Iwata et al., 2012, McAdoo et al., 2015, Puissant et al., 2014, Hong et al., 2014) (Beier and McClain, 2010). Further, SYK inhibitor treatments using rationale design of inhibitors targeting SYK phosphorylation provided significant therapeutic benefits in various disease models. Further, several SYK inhibitors are currently under clinical trial in patients with rheumatoid arthritis, autoimmune thrombocytopenic purpura, B-cell lymphomas, allergic rhinitis and asthma with varying success (Liao et al., 2013, Moretto et al., 2012). However, the role of SYK or SYK inhibitors in binge drinking ALD has not been studied.

Here we show for the first time that SYK plays a modulatory role in binge drinking induced liver pathology. Using a mouse model for binge drinking characterized by hepatic injury, steatosis and inflammation, we found that liver phosphorylated SYK^{Y525/526} was significantly increased without any change in total SYK expression. Phosphorylated SYK^{Y525/526} expression has been shown to be an indication of SYK activation and a pre-requisite for its downstream modulatory function. Using a potent SYK inhibitor, R406 (Brasemann et al., 2006), we found that SYK inhibitor treatment significantly reduced SYK^{Y525/526} expression that was induced by binge drinking in the liver. Binge drinking induced serum LPS binds TLR4 on liver cells which in turn induces SYK activation of its downstream target including ERK1/2 (Miller et al., 2012, Chaudhary et al., 2007, Lin et al., 2010). We found that functional inhibition of SYK activation that was induced by binge drinking was associated by a significant decrease of phospho-ERK1/2. Further, SYK inhibitor treatment resulted in decreased in NF- κ B nuclear binding, and decreased hepatic inflammatory cytokine TNF- α and MCP-1 production. In addition to hepatic inflammation, binge drinking also causes significant hepatic cell death and steatosis (Petrasek et al., 2013). We found that SYK inhibitor treatment significantly reduced alcohol induced hepatocyte death and steatosis as demonstrated by H&E histology, serum ALT and oil red O histology. Strikingly we found that SYK inhibitor treatment reduced steatosis by decreasing lipid biogenesis as demonstrated by liver ADRP and triglyceride expression while at the same time enhancing lipid energy dissipation (Seale et al., 2007, Seale et al., 2011) as demonstrated by increased PRDM16 and UCP1 expression.

These observations suggest a diverse role of SYK in the pathomechanism of liver inflammation after binge drinking. Our novel findings identified SYK as a central signaling molecule in binge drinking-induced liver damage. Future studies with SYK inhibitors may aid therapeutic discoveries for binge drinking induced ALD. Given that there is almost complete resolution of binge drinking induced liver damage and steatosis by SYK inhibitor treatment, it would be important to further assess the therapeutic potential of SYK inhibitor treatment in clinical studies. Our findings additionally underscore the need to assess the role of SYK inhibitor treatment in chronic ALD which currently has no sufficient treatments for patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Acute/Binge alcohol induced liver injury in mice induces SYK activation

Female C57BL/6 mice received sugar or ethanol gavage daily for three days. Liver samples were stained by H&E (A) or Oil-red-O (B), and liver injury and steatosis was quantified by measuring serum ALT (A) and liver triglycerides (B), respectively. Total liver expression of TNF- α , MCP-1 and pro-IL-1 β in the liver was analyzed by qPCR (C). Total liver protein was analysed for total SYK (D) and activated SYK^{Y525/526} (E) expression by western blotting using β -actin as loading control. * $p < 0.05$ compared to baseline sugar control was considered statistically significant by student t-test for 7–10 mice per experimental group.

Figure 2. SYK inhibitor treatment decreased acute/binge alcohol induced activated SYK and ERK1/2 expression in the liver

C57BL6 mice received alcohol or isocaloric sugar gavage daily for three days with and without SYK inhibitor or vehicle treatment (5mg/kg) as indicated. Total liver protein was analysed for total SYK (A), activated SYK^{Y525/526} (B) by western blotting using β -Actin as normalization control, phospho ERK1/2 [pThr202/Tyr204] by ELISA (C). */#p<0.05 compared to baseline sugar gavage control or alcohol gavage test group was considered statistically significant by ANOVA for 7–10 mice per experimental group.

Figure 3. SYK inhibitor treatment reduces acute/binge alcohol induced phospho-NF-κB p65, NF-κB nuclear binding, hepatic inflammatory cytokine production and hepatocyte injury

Female C7BL/6 mice received daily alcohol or isocaloric sugar gavage over three days with or without SYK inhibitor treatment (5mg/kg) as indicated. Total liver protein was analysed for phospho- NF-κB p65 by western blotting using β-Actin as protein loading control (A). Nuclear liver extracts were analysed for NF-κB promoter binding by EMSA (B). Total liver RNA or protein was analysed for TNF-α (C) and MCP-1 (D) by quantitative RT-PCR using 18s RNA as normalization control and ELISA. Hepatocyte injury was analysed by H&E histology and serum ALT (E). A representative slide of 6–8 mice per experimental group is presented. */#p<0.05 compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 6–8 mice per experimental group.

Figure 4. Acute/binge alcohol induced hepatic steatosis is ameliorated with SYK inhibitor treatment

C57BL/6 mice received daily alcohol or isocaloric sugar gavage for three days with or without SYK inhibitor treatment (5mg/kg) as indicated. Liver steatosis was evaluated by Oil-red-O staining (A) and liver triglyceride assay (B). Liver RNA was analysed for ADRP (C), FASN (D), PRDM16 (E) and UCP1 (F). */#p < 0.05 compared to baseline or EtOH vehicle group was considered statistically significant by ANOVA for 6–8 mice per experimental group.