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Integrin inhibitor cycloRGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice.

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**INTEGRIN INHIBITOR CYCLOGDRIV BLUNTS ENHANCED LPS-INDUCED LIVER INJURY
CAUSED BY ETHANOL IN MICE**

By

Veronica L. Massey
B.S., Xavier University, 2010

A Thesis
Submitted to the Faculty of the
School of Medicine of the University of Louisville
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for the Degree of

Master of Science

Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

December 2012

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DEDICATION

This thesis is dedicated to my parents

Dr. Evan Massey

and

Dr. Janet Lynch

for their constant encouragement and support

in all my endeavors.

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I would like to thank my mentor, Dr. Gavin Arteel, first for his guidance and support in my thesis research and also for his confidence in my abilities as a scientific researcher. His encouragement during my first scientific investigations as an undergraduate researcher and throughout my graduate career has been instrumental in the success of my research. Many thanks to my committee, Dr. Jesse Roman, Dr. Chris States, Dr. Igor Lukashevich, and Dr. Aruni Bhatnagar, for their comments and assistance.

ABSTRACT

INTEGRIN INHIBITOR CYCLORGDFV BLUNTS ENHANCED LPS-INDUCED LIVER INJURY CAUSED BY ETHANOL IN MICE

Veronica L. Massey

11/15/2012

Background. Progression of alcoholic liver disease (ALD) is associated with an increase in fibrin extracellular matrix (ECM) and inflammation. Previous studies have shown that this accumulation of fibrin in ALD is mediated by impaired fibrinolysis. Additionally, it is known that fibrin(ogen) interacts with the $\alpha\beta3$ integrin of endothelial cells. The purpose of this study was to test the hypothesis that hepatic inflammation caused by alcohol is mediated, at least in part, by activation of integrin $\alpha\beta3$ by fibrin(ogen). To test this hypothesis, a study was designed to determine the effect of cycloRGDFV, a peptide inhibitor of integrin $\alpha\beta3$, in a mouse model of lipopolysaccharide (LPS)-induced inflammation enhanced by ethanol pre-exposure. Methods. Accordingly, male C57BL/6J mice were exposed to ethanol (6 g/kg i.g.) or isocaloric/isovolumetric maltose dextrin solution for 3 days and injected with lipopolysaccharide (LPS; 10 mg/kg i.p.) 24 hours after the last dose of ethanol. Some animals received the $\alpha\beta3$ integrin inhibitor (cycloRGDFV; 3 mg/kg i.p.) 1 hour prior to LPS administration. Animals were sacrificed 8 or 24 hours after LPS administration. Liver damage was assessed by plasma (ALT, AST) and histological indices of liver damage (hematoxylin and eosin; H&E) and inflammation (chloroacetate esterase; CAE). Results. Ethanol pre-exposure enhanced liver injury due to LPS, as indicated by a significant increase in plasma ALT levels. cycloRGDFV significantly attenuated the increase in plasma ALT caused by ethanol pre-exposure. These results were supported by histological changes. Animals that received ethanol and LPS had both larger and more numerous necroinflammatory foci compared to animals that received LPS alone. The number

and severity of necroinflammatory foci was reduced by cycloRGDFV compared to LPS + ethanol. Ethanol pre-exposure enhanced the increase in neutrophil migration caused by LPS, as determined by CAE staining. Animals administered cycloRGDFV had fewer infiltrating neutrophils compared to those that received a combination of ethanol and LPS. Conclusions. These data suggest that cycloRGDFV administration protects against liver injury caused by a combination of alcohol and LPS exposure. CycloRGDFV also blunts ethanol-enhanced neutrophil infiltration caused by LPS in this model. Therefore, integrin $\alpha\beta3$ may play an important role in enhanced hepatic inflammation and injury due to LPS after ethanol pre-exposure.

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INTRODUCTION

Alcoholic liver disease (ALD) affects more than 10 million Americans a year at a cost of more than \$166 billion annually to treat the medical consequences of alcohol abuse(1). ALD is a spectrum of disease states that includes steatosis, steatohepatitis, and fibrosis and cirrhosis. Steatosis is the least severe stage of disease, characterized by fat accumulation in the liver. In some cases, continued alcohol consumption results in development of steatohepatitis, a disease state distinguished by chronic inflammation and the persistence of accumulated fat. The most severe stages of alcoholic liver disease include fibrosis and cirrhosis, which are characterized by collagen matrices and regenerative nodules, respectively. Despite the vast amount of money spent on treating alcoholic liver disease, therapeutic options are limited to palliative care, as no Food and Drug Administration (FDA)-approved therapy currently exists to halt or reverse disease progression. If a rational targeted therapy is to be developed, currently accepted mechanisms behind disease progression must be better understood and novel targets identified.

The mechanisms involved in the various stages of alcohol-induced liver injury have been extensively investigated using animal models that recapitulate the specific stages of the disease (i.e., steatosis, inflammation and fibrosis). However, the mechanisms behind progression of ALD from earlier stages (i.e., steatosis) to later stages (i.e., steatohepatitis), remain unclear although critical to our understanding of how to blunt progression. 2-hit models of alcohol-induced liver injury can be used to study inflammatory injury due to alcohol. In such models, alcohol serves as a 'first hit' that sensitizes the liver to a 'second hit' such as lipopolysaccharide (LPS) resulting in organ injury. For example, the acute ethanol 2-hit model of alcohol-induced liver injury and inflammation is characterized by enhanced lipopolysaccharide (LPS)-induced liver injury and inflammation caused by alcohol pre-exposure (2). This acute alcohol exposure model has been used to show that fibrin deposition strongly correlates to enhanced LPS-induced liver injury and inflammation in mice. Furthermore, blocking fibrin accumulation protected against enhanced liver

injury and inflammation caused by alcohol in this model. Other studies have shown that fibrin accumulation is an early change during chronic alcohol exposure (Arteel et al, unpublished data). These data implicate fibrin as an important player in enhanced inflammation and injury caused by acute alcohol exposure. Fibrin(ogen) is a structural component of the extracellular matrix (ECM) regulated by the coagulation cascade. Fibrin's role in inflammation has been highlighted by its ability to exert a myriad of inflammatory effects. For example, Qi and colleagues showed that fibrin induces IL-8 expression by vascular endothelial cells (3). Fibrin matrices also enhance migration of inflammatory cells in vitro, including IL-8 stimulated neutrophils (4). Whereas fibrin's pro-inflammatory effects are well documented, the mechanism by which this extracellular matrix component mediates these effects remains unclear.

Fibrin matrices could mediate inflammatory effects either via their structural role as a component of the ECM or by acting as signaling molecules. Fibrin serves as a ligand of the integrin family of cell surface adhesion receptors, particularly the integrin $\alpha\beta3$ (5). Integrins are heterodimeric receptors expressed on many cell types, including endothelial cells, inflammatory cells including macrophages and neutrophils, and platelets (6). Integrins are a diverse superfamily with at least 24 different heterodimers that are important in a variety of cell processes including proliferation (7), angiogenesis (8, 9), and inflammation (10). Upon interaction with extracellular components, such as fibrin, the β subunit of an integrin can undergo a rapid conformational change. This change in integrin structure allows for transfer of information across the plasma membrane by activation of intracellular signaling pathways. Adaptor proteins, such as integrin linked kinase (ILK), paxillin and focal adhesion kinase (FAK), are important mediators in signal transduction downstream of integrin activation and play a role in specifying which signaling cascades are activated within the cell (11-15). Owing to their myriad of effects on growth, angiogenesis and inflammation, integrin inhibitors have been investigated for therapeutic use. For example, small peptide inhibitors of integrins, including integrins $\alpha4\beta1$, $\alpha4\beta7$, and $\alpha\beta3$ are currently in clinical trial for treatment of asthma, chronic obstructive pulmonary disease (16), and certain types of cancer (17).

The role of integrin $\alpha\beta3$ in tumor angiogenesis and metastasis is well documented and stimulation of integrin $\alpha\beta3$ also has been shown to contribute to inflammation (18). Integrin $\alpha\beta3$ is strongly expressed by endothelial cells (19) including those that line the hepatic sinusoids where fibrin accumulates after alcohol exposure. Moreover, integrin $\alpha\beta3$ expression has been correlated temporally to fibrin deposition (20). Integrin $\alpha\beta3$ binds fibrin(ogen) via an arginine-glycine-aspartic acid (RGD) sequence on the fibrin molecule (5), allowing the interaction between fibrin and this integrin to be studied using small, commercially available peptide antagonists (21).

The experiments described here were performed to test the hypothesis that hepatic inflammation caused by alcohol is mediated, at least in part, by activation of integrin $\alpha\beta3$ by fibrin(ogen). The goal of this study was to determine the effect of a peptide inhibitor of integrin $\alpha\beta3$, cycloRGDfV, in an acute ethanol 2-hit mouse model of hepatic inflammatory injury.

MATERIALS AND METHODS

Animals and Treatments

Eight week old male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville's Institutional Animal Care and Use Committee. Food and tap water were provided ad libitum. Mice received ethanol (6 g/kg i.g.) or isocaloric/isovolumetric maltose-dextrin solution for 3 days and were injected with LPS (*E. coli*, serotype 055:B5; Sigma, St. Louis, MO; 10 mg/kg i.p.) 24 hours after the last dose of ethanol (2) (Figure 1). The $\alpha\beta3$ integrin inhibitor cycloRGDFV (Enzo Life Sciences, Plymouth Meeting, PA; 3 mg/kg i.p.) or vehicle (PBS) was injected 1 hour prior to LPS administration. Animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.m.) 8 or 24 hours after LPS administration. Blood was collected from the vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were snap-frozen in liquid nitrogen, frozen-fixed in OCT-Compound (Sakura Finetek, Torrance, CA), or were fixed in 10% neutral buffered formalin for subsequent sectioning and mounting on microscope slides.

Clinical Chemistry and Pathologic Evaluation

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (ThermoTrace, Melbourne, Australia). Plasma hyaluronic acid (HA) was quantitated using an ELISA kit (Corgenix, Broomfield, CO). Formalin fixed, paraffin embedded sections were cut at 5 μm and mounted on glass slides. Sections were deparaffinized and stained with hematoxylin and eosin (H&E). Chloroacetate esterase (CAE) staining for neutrophils was performed using the naphthol AS-D chloroacetate

Figure 1

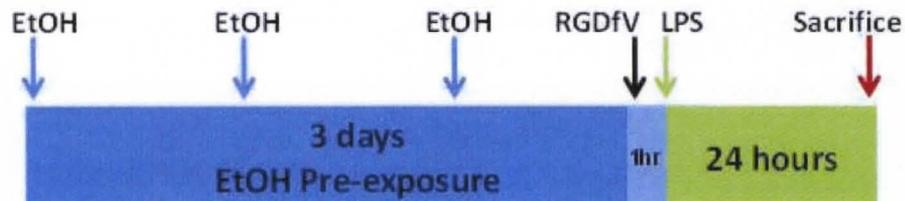


Figure 1: Acute ethanol 2-hit model of liver injury.

Mice received a dose of ethanol (6 g/kg i.g.) or isocaloric/isovolumetric maltose-dextrin solution for 3 d and were injected with LPS (10 mg/kg i.p.) or vehicle (saline) 24 hours after the last dose of ethanol or control diet. The $\alpha\beta3$ integrin inhibitor cycloRGDfV (3 mg/kg i.p.) or vehicle (PBS) was injected 1 hour prior to LPS administration.

esterase kit (Sigma, St. Louis, MO) on paraffin embedded samples. The extent of CAE staining was quantified by blinded counting of CAE-positive neutrophils per 1000 hepatocytes (22). CAE-positive cells counted during quantification were characterized by light pink (i.e. CAE-positive) staining and bi-lobed nuclear morphology.

RNA Isolation and Quantitative Reverse-Transcriptase Polymerase Chain Reaction

The hepatic mRNA expression of select genes was detected by quantitative reverse-transcriptase polymerase chain reaction (rt-PCR), which is routine for this group (2, 23-25). PCR primers and probes for TNF α , PAI-1, IL-6, IL-10 and β -actin were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers and probes for CD31, integrin α v, integrin β 3, C-fos, C-myc, and SOCS3 were bought from Applied Biosystems as kits (Foster City, CA). All primers were designed to cross introns to ensure that only cDNA and not genomic DNA was amplified. Total RNA was extracted from liver tissue by a guanidinium thiocyanate-based method (RNA STAT 60 Tel-Test, Ambion, Austin, TX). RNA concentrations were determined spectrophotometrically and 1 μ g of total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg, MD). PerfeCta qPCR Fast Mix (Quanta Biosciences, Gaithersburg, MD) was used to prepare the PCR reaction mixture. This 2X mixture is optimized for TaqMan reactions and contains MgCl₂, dNTPs, and AccuFast Taq DNA Polymerase. Amplification reactions were carried out using the ABI StepOne Plus machine and software (Quanta Biosciences, Gaithersburg, MD) with initial holding stage (95°C for 30 seconds) and 50 cycles of a 2-step PCR (95°C for 30 seconds, 60°C for 20 seconds). Fluorescent intensity of each sample was measured at each cycle to monitor amplification of the target gene. The comparative CT method was used to determine fold changes in mRNA expression compared to an endogenous reference gene (β -actin). This method determines the amount of target gene, normalized to an endogenous reference and relative to a calibrator ($2^{-\Delta\Delta C_t}$).

Immunostaining

For immunofluorescent detection of the platelet endothelial cell adhesion molecule (CD31), formalin-fixed, paraffin-embedded sections (5 μm) were deparaffinized and then rehydrated with graded ethanol solutions. Slides were incubated in primary antibody for CD31 (Abcam, Cambridge, MA). A secondary antibody, goat anti-rat Alexa 488-IgG was used (Invitrogen, Carlsbad, CA). Samples were mounted and counterstained with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescent detection of fibrin deposition was performed as described in (22). Briefly, frozen liver sections (6 μm thick) were fixed in 10% buffered formalin containing 2% acetic acid to solubilize all but cross-linked fibrin. Sections were blocked with PBS containing 10% horse serum before an overnight incubation at 4°C with affinity purified rabbit anti-human fibrinogen IgG (Dako North America, Inc. Carpinteria, CA) diluted in blocking solution. Donkey anti-rabbit antibody conjugated to Alexa 488 (Invitrogen, Carlsbad, CA) was used as secondary antibody. Tissue was counterstained with Hoechst. Fluorescence for Alexa 488 and Hoechst was detected at excitation and emission wavelengths of 490 nm and 520 nm, and 350 nm and 460 nm, respectively. Immunofluorescence was visualized using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) and Metamorph software.

Immunoblots

All buffers used for protein extraction contained protease, tyrosine phosphatase, and serine-threonine phosphate inhibitor cocktails (Sigma, St. Louis, MO). Liver samples were homogenized in CHAPs buffer (50 mM Tris/Cl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1% (w/v) PMSF). Homogenized samples were loaded onto SDS-polyacrylamide gels containing 10% (w/v) acrylamide, followed by electrophoresis and Western blotting onto nitrocellulose membranes (Hybond P, GE Healthcare, Piscataway, NJ). Primary antibodies against phosphorylated and total extracellular-signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases 1 and 2 (JNK1/2), signal transducer and activator of transcription 3 (STAT3), focal adhesion kinase (FAK), and paxillin (Cell Signaling Technology, Beverly, MA) were used. Bands were visualized using an ECL kit (Pierce, Rockford, IL) and Hyperfilm (GE Healthcare, Piscataway, NJ). Densitometric analysis was performed using UN-SCAN-IT Gel (Silk Scientific, Orem, UT) software.

Statistical Analyses

Results are reported as means \pm standard error mean (SEM; n = 4-7). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for nonparametric data) was used for the determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance.

RESULTS

Hepatic integrin expression and fibrin deposition.

The purpose of these studies was to investigate the role of integrins in mediating the pro-inflammatory effects of fibrin in the pre-fibrotic phase of alcoholic liver disease (ALD). Therefore, it was necessary to verify that the effects of cycloRGDfV administration were not due to changes in hepatic integrin expression or fibrin accumulation in the liver. To determine effects on integrin expression, quantitative rt-PCR was performed for the αv and $\beta 3$ integrin subunits. LPS alone did not significantly affect hepatic mRNA levels of either the αv or $\beta 3$ integrin subunits (Figure 2). The combination of ethanol and LPS significantly increased levels of integrin αv and $\beta 3$ integrin subunit mRNA expression (by ~2-fold and ~1.4 fold, respectively) compared to LPS alone and naïve controls, respectively. CycloRGDfV did not have a significant effect on the mRNA expression of either integrin subunit.

The effect of LPS and ethanol on hepatic accumulation of fibrin was determined by immunofluorescence. LPS robustly increased hepatic fibrin deposition 8 hours after LPS administration (Figure 3). Fibrin deposition was primarily located in the hepatic sinusoidal space. As has been observed previously (2), ethanol pre-exposure enhanced the accumulation of fibrin caused by LPS. CycloRGDfV administration did not alter the effect of LPS or ethanol on fibrin accumulation in mouse liver.

CycloRGDfV protects against enhanced liver injury and neutrophil infiltration.

LPS administration resulted in inflammatory foci in mouse liver compared to animals (Figure 4). As has been observed previously, ethanol significantly increased the detrimental effect of LPS as evidenced by necroinflammatory foci in the hepatic tissue of animals that received the combination of ethanol and LPS. Administration of cycloRGDfV blunted the effect of

Figure 2

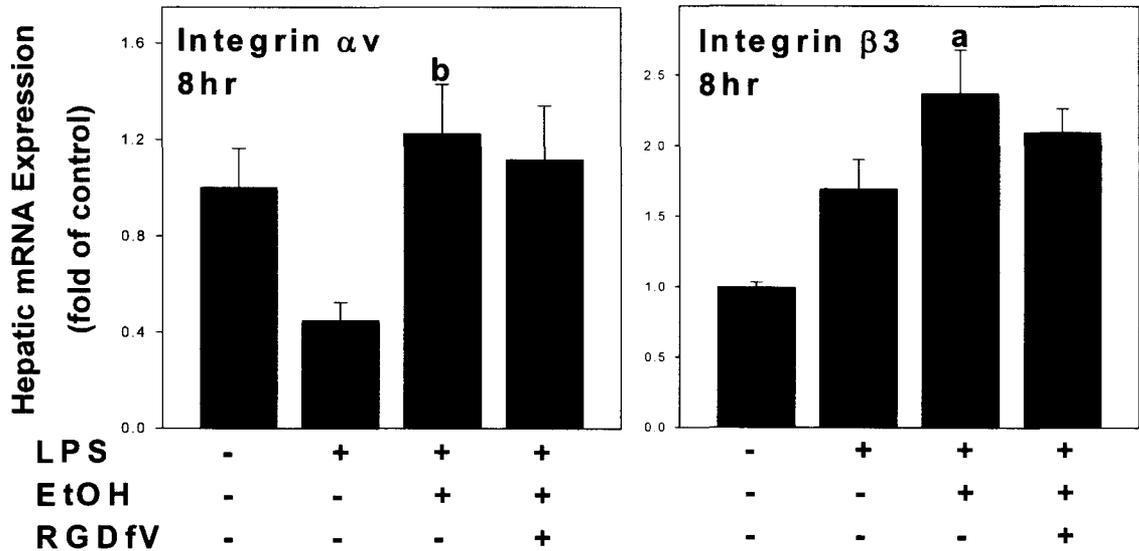


Figure 2: Effect of cycloRGDFV on integrin mRNA expression.

Male C57BL/6J mice were given ethanol or isocaloric, isovolumetric control diet 3 days and injected with LPS or saline vehicle either 8 or 24 h after the last dose of ethanol, with some animals receiving cycloRGDFV 1 h prior to LPS as described in Methods. Hepatic mRNA expression of the αv and $\beta 3$ integrin subunits were determined by real time RT-PCR. Data are means \pm SEM (N=4-6). ^a $p < 0.05$ compared to maltose dextrin controls; ^b $p < 0.05$ compared to LPS alone.

Figure 3

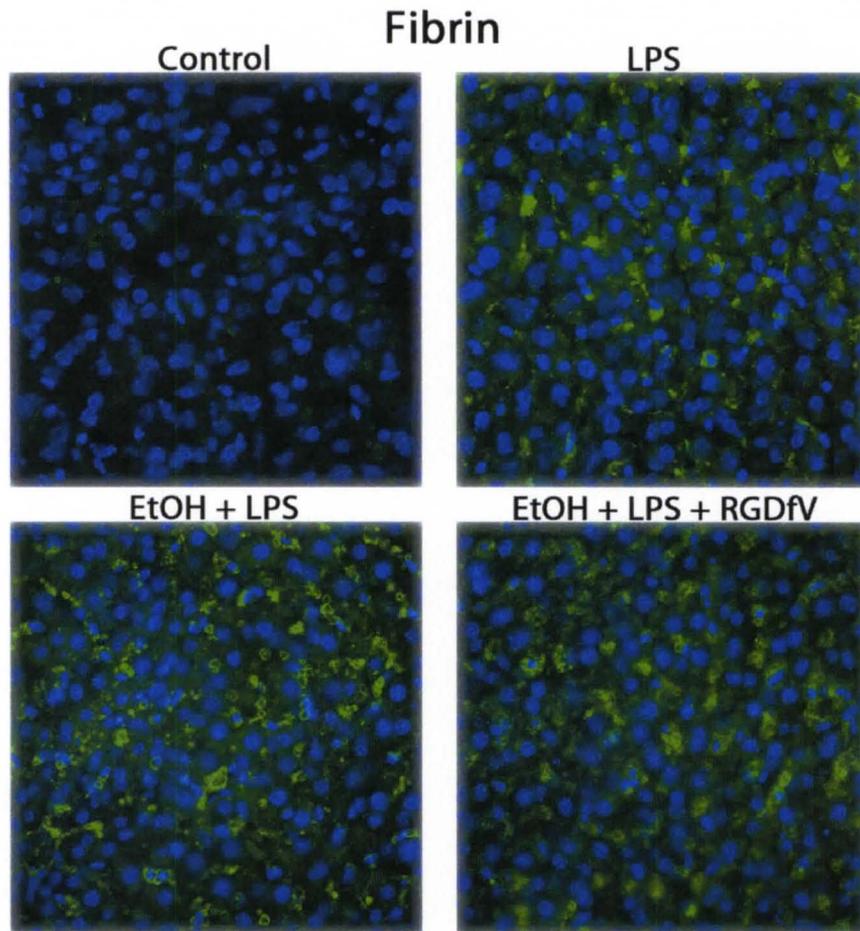


Figure 3: Effect of cycloRGDFV on hepatic fibrin deposition.

Animals were treated as described in Methods. Representative photomicrographs (400x) of immunofluorescent detection of fibrin deposition at the 8 hour time point are shown.

Figure 4

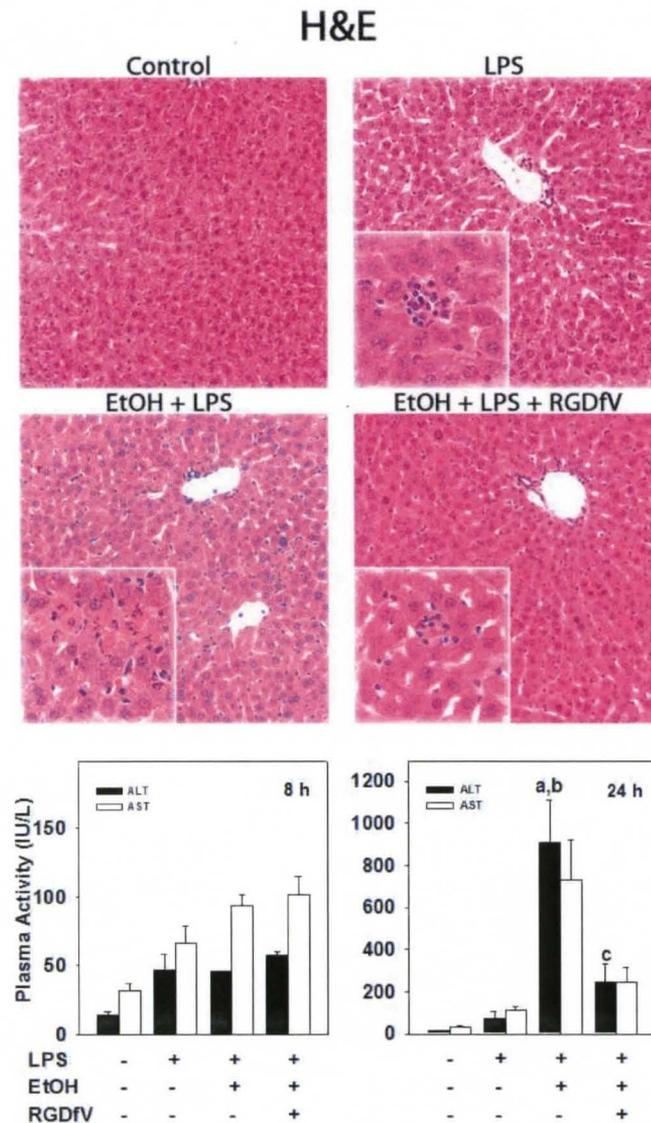


Figure 4: Effect of cycloRGDFV on liver injury.

Representative photomicrographs (200x; 400x insets) of hematoxylin & eosin staining are shown (top panel). Inflammatory foci were seen in the liver of animals that received LPS and a combination of ethanol, LPS, and cycloRGDFV. Necroinflammatory foci were observed in animals that received ethanol and LPS without the integrin inhibitor. The plasma ALT and AST levels were analyzed by spectrophotometric analysis (lower panels). Data are means \pm SEM (n = 4-6). ^a $p < 0.05$ compared to MD controls; ^b $p < 0.05$ compared to LPS alone; ^c $p < 0.05$ compared to LPS + ethanol.

ethanol under these conditions and livers resembled those of animals exposed to LPS alone. Administration of LPS alone significantly increased the number of infiltrating neutrophils 24 hours after LPS administration (Figure 5). Ethanol pre-exposure enhanced the increase in neutrophil migration caused by LPS by ~2 fold. CycloRGDFV blunted this effect of ethanol pre-exposure, with the average number of infiltrating neutrophils in this group similar to those treated with LPS alone. Ethanol pre-exposure enhanced liver injury due to LPS 24 hours after LPS administration, as indicated by a significant increase in plasma ALT levels (Figure 4, bottom left). CycloRGDFV protected against enhanced levels of circulating transaminases, significantly blunting serum levels of ALT and AST. There were no significant differences in serum levels of ALT or AST or gross liver morphology due to alcohol pre-exposure or cycloRGDFV 8 hours after LPS (Figure 4, bottom right) (2).

CycloRGDFV did not affect mRNA expression of cell adhesion and inflammatory genes.

In order to further evaluate the effect of cycloRGDFV on inflammation in this model, hepatic mRNA expression of a panel of inflammatory cytokines and cell adhesion proteins was determined (Table 1). The pro-inflammatory cytokines TNF α and IL-6 as well as the anti-inflammatory cytokine IL-10 were significantly increased in all groups compared to maltose-dextrin fed controls by ~50-, 150-, and 25-fold, respectively; cycloRGDFV did not affect the expression of TNF α , IL-6, or IL-10 compared to animals that received LPS or a combination of LPS and ethanol. PAI-1 expression was significantly increased in all groups compared to controls. Expression of intracellular adhesion molecule-1 (ICAM-1) was significantly increased in all groups compared to controls. The hepatic mRNA expression of the cell surface receptor CD31 was significantly decreased by LPS administration compared to saline administration. Ethanol preexposure and cycloRGDFV did not significantly affect mRNA expression of CD31 compared to LPS alone. Integrins affect intracellular signaling via interaction with adaptor proteins and kinases, including the integrin-linked kinase (ILK). Hepatic mRNA expression of the integrin linked kinase (ILK) was significantly decreased in the groups that received LPS alone or the combination of ethanol and LPS whereas animals receiving cycloRGDFV in combination with

Figure 5

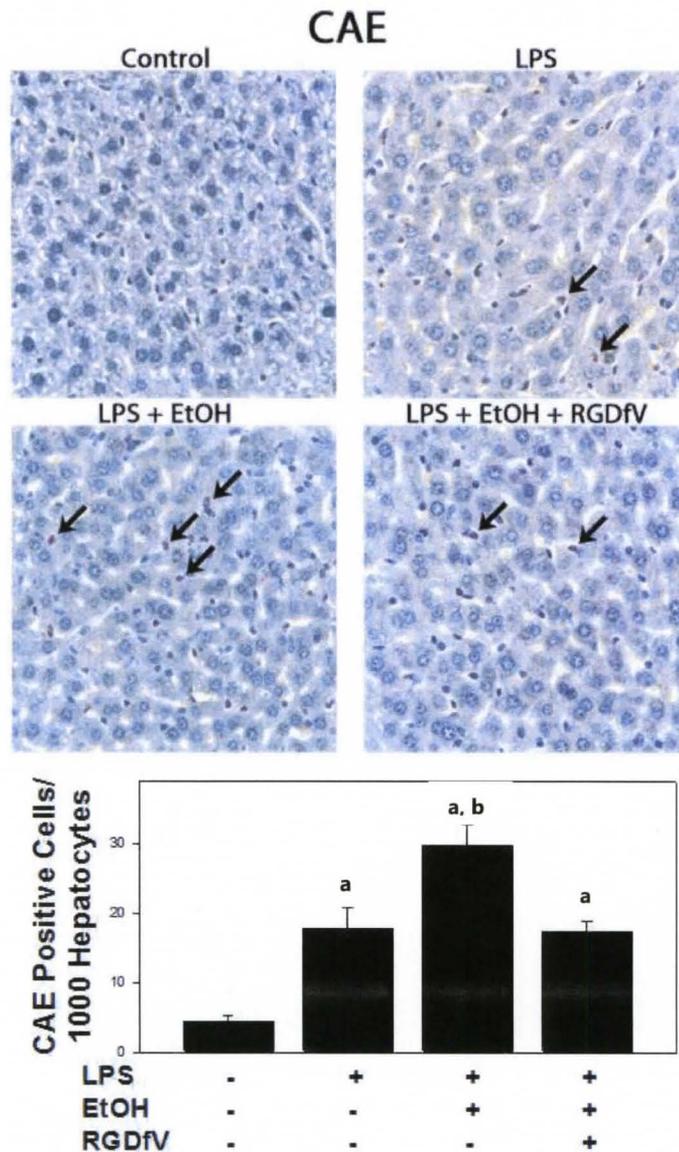


Figure 5: Effect of cycloRGDfV on neutrophil infiltration.

Representative photomicrographs (400x) of chloroacetate-esterase staining are shown (top panel). Number of neutrophils were quantitated as described in Methods (bottom panel). Data are means \pm SEM (n = 4-6). ^a $p < 0.05$ compared to MD controls; ^b $p < 0.05$ compared to LPS alone; ^c $p < 0.05$ compared to LPS + ethanol.

Table 1: Hepatic mRNA Expression. ^a indicates p>0.5 compared to control; ^b indicates p>0.5 compared to LPS

	MD Control	MD + LPS	EtOH + LPS	EtOH + LPS + RGDfV
8 hours				
TNFalpha	1.0±0.37	42.9±8.5 ^a	45.1±5.3 ^a	76.9±21.9 ^a
IL-6	1.0±0.08	131±15.1 ^a	138±23.2 ^a	177±38.7 ^a
IL-10	1.0±0.32	23.8±2.1 ^a	26.37±3.8 ^a	26.7±5.5 ^a
PAI-1	1.0±0.43	1443±278 ^a	1835±210 ^a	1327±296 ^a
ICAM	1.0±0.24	10.6±1.8 ^a	11.3±1.2 ^a	9.5±3.5 ^a
CD31	1.0±0.32	0.26±0.04 ^a	0.40±0.06 ^a	0.40±0.07 ^a
ILK	1.0±0.06	0.35±0.04 ^a	0.56±0.08 ^a	0.69±0.15
c-Fos	1.0±0.15	1.2±0.16	1.11±0.17	2.2±1.02
c-Myc	1.0±0.19	2.3±0.35	1.9±0.25	1.7±0.24
SOCS3	1.0±0.23	4.0±0.58	8.4±1.4 ^{a,b}	5.4±0.94 ^{a,c}
24 hours				
c-Fos	1.0±0.03	0.75±0.14	1.6±0.14 ^{a,b}	1.1±0.17 ^c
c-Myc	1.0±0.21	0.97±.21	0.89±.097	1.3±0.14
SOCS3	1.0±0.23	1.71±0.60	2.6±0.80	1.2±0.15

ethanol and LPS were protected from this decrease in ILK expression.

Endothelial cell dysfunction

Platelet endothelial cell adhesion molecule (CD31) is a cell surface receptor expressed on endothelial cells which may be important in neutrophil migration as well as integrin activation (26). The expression of CD31 was decreased by more than half in all groups compared to controls (Table1). However, immunofluorescent staining for CD31 was increased by LPS, and this increase was blunted by ethanol pre-exposure (Figure 6). CD31 expression in animals administered cycloRGDFV was greater than that in animals that received a combination of ethanol and LPS without the drug. LPS exposure significantly elevated levels of circulating hyaluronic acid (HA) (Figure 7). Neither ethanol preexposure nor cycloRGDFV administration significantly affected levels of HA compared to animals that received LPS alone.

Phosphorylation of signaling molecules downstream of integrin activation

Activation of integrins by extracellular ligands causes a rapid conformational change in the cytosolic domain of the integrin's beta subunit allowing association of adaptor proteins and activation of subsequent signaling cascades, including ERK and JNK (27). The phosphorylation status of ERK1/2 and JNK1/2 were determined by Western blot (Figure 8). Phosphorylation of ERK1/2 was significantly decreased in all groups compared to controls. Levels of phosphorylated JNK1/2 were not significantly affected by any treatment. To explore further the effect of blocking integrin signaling, the phosphorylation status of the adaptor proteins focal adhesion kinase (FAK) and paxillin was also determined. Phosphorylation of FAK was significantly decreased in all experimental groups compared to controls (Figure 8). However, the phosphorylation status of paxillin was not significantly affected in any of the treatment groups.

Effect of cycloRGDFV on STAT3 phosphorylation and STAT3 regulated genes

Despite no significant effect of ethanol pre-exposure or cycloRGDFV on ligands of

Figure 6

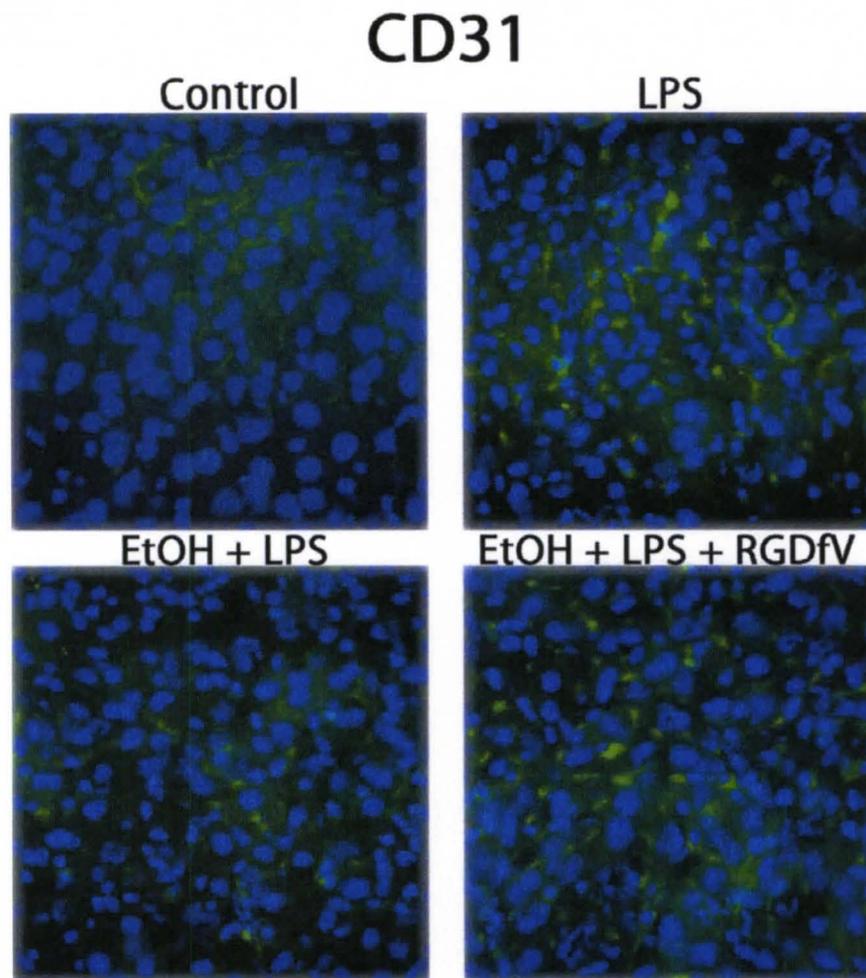


Figure 6: Effect of cycloRGDFV on CD31 expression.

Hepatic CD31 expression was determined by immunofluorescent detection as described in Methods. Representative photomicrographs (400x) of the 8 h time point are shown.

Figure 7

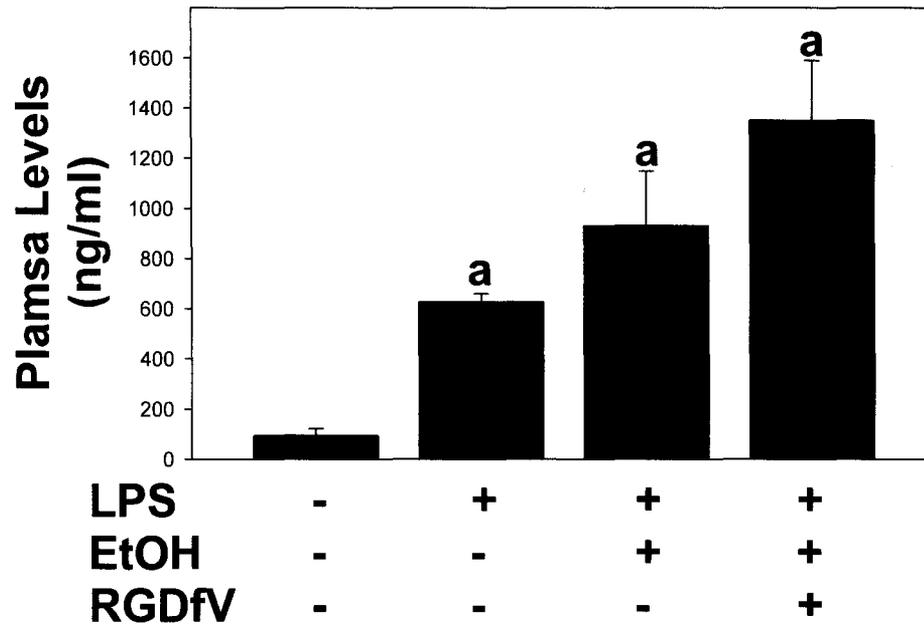


Figure 7:

Effect of RGDFV on plasma levels of hyaluronic acid (HA).

Plasma levels of HA were determined for the 8 hour time point as described in Materials and Methods. Data are means \pm SEM (n=4-6). ^a p <0.05 compared to MD controls

Figure 8

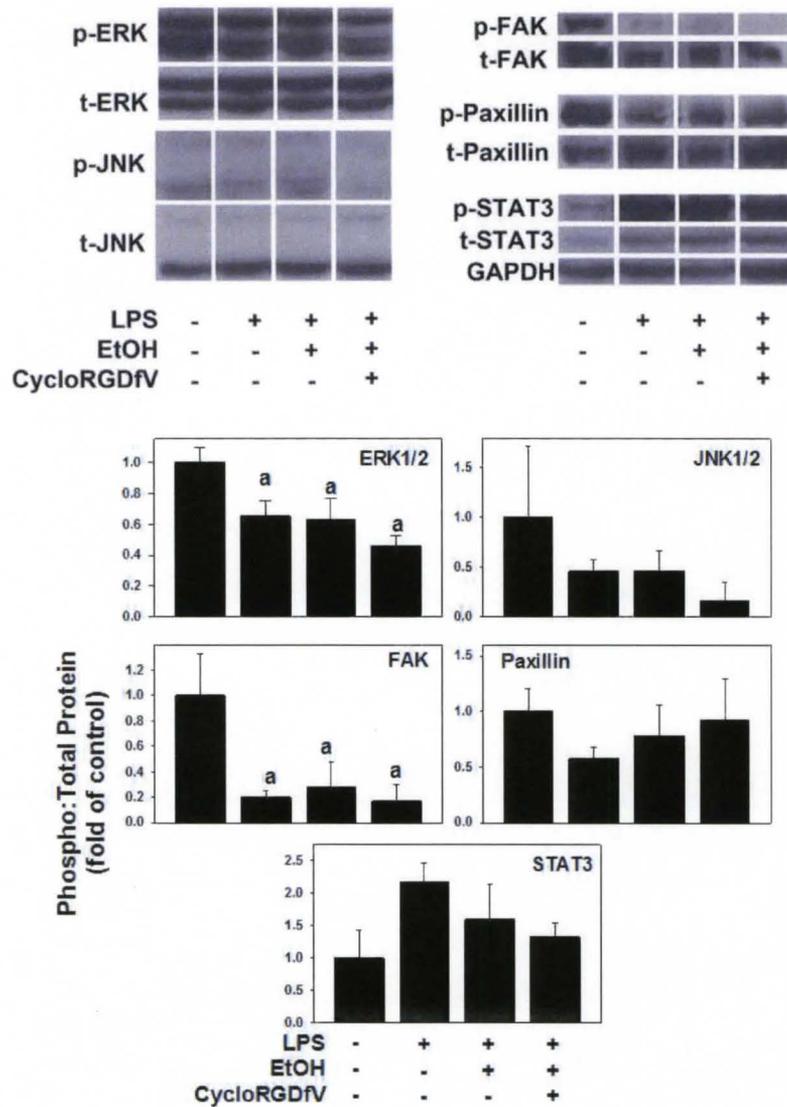


Figure 8: Phosphorylation of signaling kinases and adaptor proteins.

Immunoblots were performed as described in Methods. Representative immunoblots for phosphorylated and total ERK, JNK, FAK, paxillin, and STAT3 for the 8 h time point are shown (top panel). Phosphorylated protein levels were normalized to levels of total protein and quantified by densitometry. Data are means \pm SEM (n = 4-6). ^a $p < 0.05$ compared to MD controls.

JAK/STAT signaling (i.e. IL-6; Table 1), expression of JAK/STAT responsive genes was differentially affected by ethanol preexposure and cycloRGDFV. Eight hours after LPS administration, no significant changes were seen in mRNA expression of c-Myc or c-Fos, however suppressor of cytokine signaling 3 (SOCS3) mRNA expression was significantly increased in animals pre-exposed to ethanol compared to those administered LPS alone (Table 1). CycloRGDFV administration blunted the increase in expression of SOCS3 caused by ethanol preexposure followed by LPS. Whereas mRNA expression of SOCS3 and c-Myc were similar across all groups 24 hours after LPS, expression of c-Fos was significantly increased by ethanol pre-exposure compared to LPS alone. CycloRGDFV attenuated this increase in c-Fos caused by ethanol and LPS. The ratio of phosphorylated signal transducer and activator of transcription 3 (STAT3) to total STAT3 was significantly increased by LPS administration compared to maltose-dextrin fed controls (Figure 8). Ethanol preexposure blunted this increase in STAT3 phosphorylation; cycloRGDFV had no effect on phosphorylation of STAT3 compared to controls or ethanol preexposed animals.

DISCUSSION

Previous studies from using this 2-hit model of liver injury have shown that hepatic fibrin accumulation is correlated to liver injury and inflammation (2). Furthermore, enhanced LPS-induced liver injury and inflammation caused by alcohol was prevented by blocking fibrin accumulation. Such studies suggested that fibrin plays a pro-inflammatory role in alcoholic liver injury, however the mechanism by which fibrin mediates these effects remain unclear. The experiments described here were performed to test the hypothesis that fibrin mediates pro-inflammatory effects in the liver via interaction with integrins, specifically integrin $\alpha\text{v}\beta\text{3}$, in a 2-hit model of acute alcohol exposure.

While nearly all individuals who drink alcohol develop some degree of steatosis, only a small fraction of alcoholics ever progress to steatohepatitis, a disease state characterized by persistent inflammation and continued fat accumulation. One explanation for this phenomenon is the 2-hit hypothesis proposed by Day and James (28). The 2-hit hypothesis suggests that ethanol exposure is not sufficient for alcoholic liver disease, but rather that a second hit is required in order for later stages of disease to develop. Thus, 2-hit models of liver injury, where ethanol serves as the 'first hit' that sensitizes the liver to a 'second hit' such as LPS are commonly used to study alcoholic liver disease. The acute 2-hit model (Figure 1) of alcohol exposure was chosen for this study because the response to LPS with and without alcohol preexposure appears similar mechanistically to certain aspects of alcohol-induced liver injury, specifically the inflammatory phase of steatohepatitis. Indeed, although ethanol alone causes no gross morphological changes in this model, it nevertheless robustly enhances liver injury caused by LPS (2).

The experiments described in this thesis were designed to study the role of the integrin $\alpha\text{v}\beta\text{3}$ in enhanced LPS-induced liver injury after acute alcohol exposure. The integrin $\alpha\text{v}\beta\text{3}$ was specifically chosen for this study because of its proximity to fibrin ECM molecules and ability to bind RGD-containing ECM components including fibrin. Indeed, integrin $\alpha\text{v}\beta\text{3}$ is expressed by

sinusoidal endothelial cells which line the sinusoids where fibrin accumulates in the acute 2-hit model of alcohol exposure used for this study. Additionally, others have shown the integrin $\alpha\beta3$ binds fibrin (5). The interaction between RGD-containing ECM components, such as fibrin, and the integrin $\alpha\beta3$ can be studied using commercially available peptide antagonists such as cycloRGDFV. CycloRGDFV is a small cyclic peptide that binds the $\alpha\beta3$ integrin, preventing the integrin's interaction with other molecules. CycloRGDFV was chosen for use in this study because of its relative specificity for integrin $\alpha\beta3$.

As expected, LPS administration significantly increased fibrin deposition (Figure 3), liver damage (Figure 4) and liver inflammation (Figure 5), all of which were enhanced by alcohol preexposure. Animals that received cycloRGDFV were protected against enhanced liver injury and inflammation, but had fibrin deposition similar to animals that received only ethanol and LPS. These observations support our hypothesis and show that the protection conferred by cycloRGDFV was independent of the magnitude of fibrin deposition. Protection was also independent of integrin expression (Figure 2), suggesting that the protective mechanism(s) at work in this model are 'downstream' of the ligand and receptor required for integrin activation, *per se*.

Dysregulated immune response after alcohol exposure is known to contribute to the pathogenesis of ALD. Indeed, alcohol exposure can cause changes in the expression of inflammatory mediators favoring a pro-inflammatory profile that allows for normal tissue damage. For example, alcohol increases circulating levels of endotoxin (29) and sensitizes Kupffer cells, the resident macrophages of the liver, to stimulation by LPS (30). Moreover, sensitization of Kupffer cells by ethanol can result in enhanced release of pro-inflammatory mediators including TNF α (31). The release of TNF α can result in recruitment and activation of other cells of the innate immune system, promoting necrotic cell death, such as that observed in animals that received a combination of ethanol and LPS in this study. Indeed mRNA expression of TNF α , a pro-inflammatory cytokine released from activated Kupffer cells, was significantly increased by more than 40-fold in all groups compared to controls. Additionally, neutrophil count correlated to severity of liver injury with alcohol preexposure enhancing both injury and neutrophil count.

CycloRGDFV attenuated these detrimental effects of alcohol preexposure. These results suggest that the integrin antagonist may protect against liver injury by blunting the enhanced LPS-induced inflammatory response that occurs after ethanol preexposure.

One mechanism by which cycloRGDFV may protect against inflammatory injury is by attenuating the increase in inflammatory mediators caused by alcohol preexposure. Indeed, protecting against increased levels of inflammatory mediators can protect against hepatic injury after alcohol exposure. However, cycloRGDFV did not differentially affect hepatic levels of the proinflammatory mediators TNF- α , IL-6, PAI-1 or the antiinflammatory cytokine IL-10. In fact, all groups had increased hepatic mRNA expression of TNF- α , IL-6, PAI-1, and IL-10 compared to controls (Table 1). These results demonstrate that the protective effects of cycloRGDFV were independent of changes in the mRNA expression of inflammatory mediators investigated in this study.

Protection against enhanced inflammation in this model was indicated by significantly fewer neutrophils in the liver tissue of animals that received cycloRGDFV in addition to ethanol and LPS compared to those that received the combination of ethanol and LPS without the integrin antagonist. Infiltration of neutrophils and other inflammatory cells into tissue is mediated by cell-surface receptors. For example, ICAM-1, a cell surface adhesion receptor expressed by endothelial cells, interacts with neutrophil-specific receptors to allow for neutrophil chemotaxis. The mRNA expression of ICAM-1 was significantly increased in all groups compared to controls and was not differentially affected by cycloRGDFV administration. CD31, also known as platelet-endothelial cell adhesion factor (PECAM), is a cell surface receptor expressed by sinusoidal endothelial cells (SECSs) and neutrophils (32) which has been linked to integrin activation (33). CD31 receptors expressed on SECs can facilitate neutrophil transmigration via homophilic interactions with CD31 receptors on immune cells such as neutrophils (12, 34). The mRNA expression of CD31 was significantly decreased in all groups compared to controls. In contrast, protein expression of CD31 in hepatic tissue was increased by LPS administration (Figure 6). Ethanol preexposure caused a robust decrease in CD31 expression compared to LPS alone. This decrease in CD31 expression caused by ethanol preexposure suggests that the enhanced

neutrophil migration caused by ethanol after an inflammatory stimulus was not a result of increased CD31-mediated neutrophil migration. CycloRGDFV increased CD31 expression compared to ethanol + LPS. Considering the proinflammatory role of CD31 in neutrophil migration, increased CD31 expression caused by cycloRGDFV administration would be expected to correlate with increased neutrophil migration into tissue, the opposite of what was observed in this study. In fact, cycloRGDFV attenuated the increase in neutrophil migration caused by ethanol preexposure (Figure 5). Recent studies have shown that CD31 can facilitate neutrophil migration via heterophilic interactions with other cell surface receptors, including integrin $\alpha\beta3$ (33) (Figure 9, top panel). Indeed, blocking heterophilic interactions between CD31 and integrins can prevent neutrophil migration mediated by these interactions. In this study, the integrin antagonist cycloRGDFV was used to block ECM-integrin $\alpha\beta3$ interactions. However, cycloRGDFV may have additionally blocked CD31-integrin $\alpha\beta3$ interactions, preventing neutrophil migration mediated by such heterophilic interactions (Figure 9, bottom panel). This mechanism could explain the decrease in neutrophils in the liver tissue due to cycloRGDFV despite increased hepatic CD31 expression in these animals.

CD31 also has potent anti-inflammatory roles; its expression has been shown to be protective against LPS-induced endotoxemia (35, 36). It comprises the majority of intracellular junctions of endothelial cells (37) and can mediate anti-inflammatory effects by maintaining vascular integrity (26). Additionally, increased CD31 expression on SECs indicates angiogenesis, which is important for recovery after liver injury. In this study, hepatic expression of CD31 was decreased by ethanol exposure and rescued by cycloRGDFV administration. Reduction in CD31 expression in ethanol preexposed mice may indicate reduced vascular integrity and/or angiogenesis, two factors necessary for restoration of damaged hepatic tissue. CycloRGDFV rescued CD31 expression. Thus, enhanced liver injury caused by alcohol exposure in this model may be due, at least in part, to an inability to recover after inflammatory insult. Furthermore, protection conferred by cycloRGDFV may be a result of increased vascular integrity and angiogenesis which allowed for hepatic recovery.

Figure 9

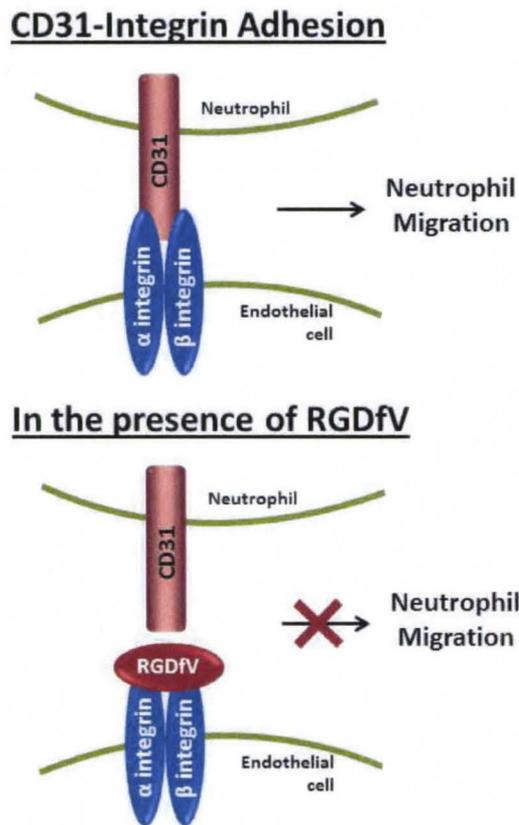


Figure 9. Heterodimeric interactions of CD31 with integrin $\alpha\beta 3$. CD31 expressed on neutrophils has been shown to interact with the integrin $\alpha\beta 3$ expressed on endothelial cells. Interaction between CD31 and integrin $\alpha\beta 3$ allows for transmigration of neutrophils into tissue. One mechanism by which cycloRGDfV may attenuate neutrophil migration into liver tissue is by blocking heterodimeric interactions between CD31 and integrin $\alpha\beta 3$.

Previously published data using this model of alcohol exposure showed that increased activation of ERK1/2 4 h after LPS administration occurs downstream of TNF α and mediates increased PAI-1 expression and subsequent fibrin accumulation in the liver (2). Interestingly, integrin activation can also activate ERK signaling via changes in the phosphorylation status of various adaptor proteins (11). Whereas TNF α and PAI-1 mRNA expression were still significantly increased 8 hours after LPS administration in this study (Table 1), ERK1/2 phosphorylation was reduced in all groups compared to controls at this time point (Figure 8). Because activation of ERK1/2 can occur downstream of adaptor proteins that associate with the cytosolic domains of activated integrins, the phosphorylation status of FAK was determined. FAK is an integrin-linked adaptor protein that can contribute to activation of ERK signaling downstream of integrin activation (12). Phosphorylation of FAK was significantly decreased in all groups compared to controls; however neither ethanol preexposure nor cycloRGDFV administration had additional effects on FAK phosphorylation (Fig. 8). Decreased FAK phosphorylation could account for the decrease in ERK1/2 signaling seen in our model, but not for the protective effects of cycloRGDFV. Reduced FAK phosphorylation may also account for the decrease in phosphorylation of JNK which occurs downstream of FAK activation (27). However, changes in the phosphorylation status of these adaptor proteins and kinases cannot account for the protective effects of cycloRGDFV in this model.

Previous studies from this group have suggested a possibility for cross-talk between IL-6 receptor signaling and integrin signaling via STAT3 (Figure 10). JAK-STAT signaling pathways are activated by a myriad of extracellular signaling molecules, including IL-6. The JAK-STAT pathway has also been shown to be activated by other integrins (38). Upon binding of IL-6 to the IL-6 receptor (IL-6R), JAK undergoes autophosphorylation, allowing for the binding of and subsequent phosphorylation of STAT3. Although hepatic IL-6 mRNA expression was significantly increased in all groups, STAT3 phosphorylation was not significantly affected by LPS, ethanol, or cycloRGDFV compared to controls. The mRNA expression of the STAT3-responsive genes c-Fos, c-Myc, and SOCS3 was also determined. Hepatic c-Fos expression was significantly increased by ethanol pre-exposure, but blunted by cycloRGDFV. C-Fos has been shown to

Figure 10

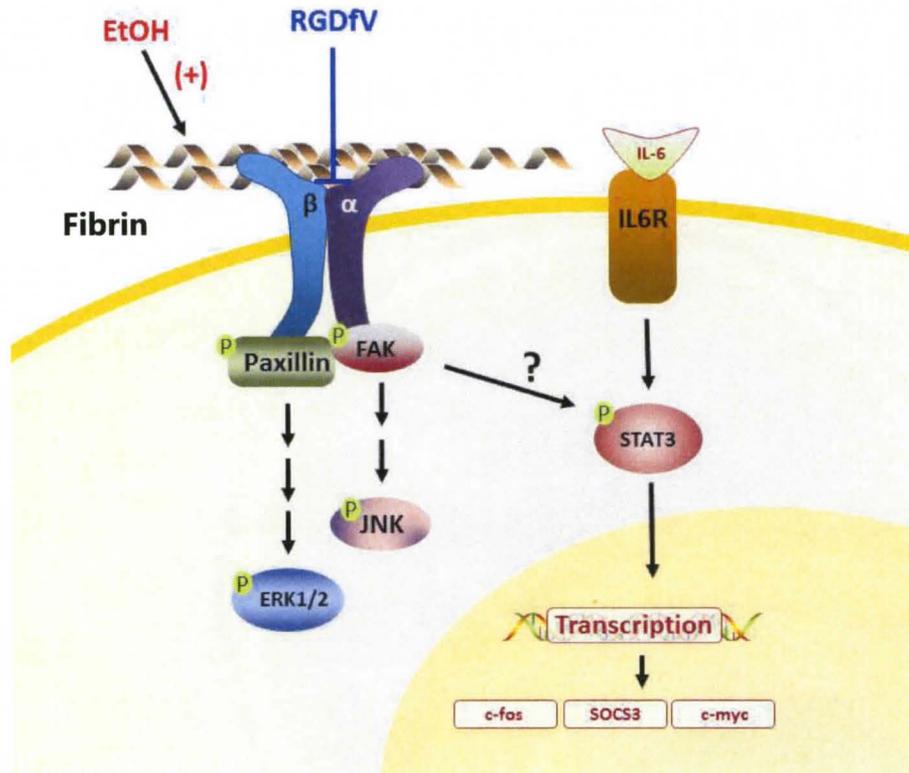


Figure 10. Intracellular signaling downstream of integrin activation. Ethanol enhances increased fibrin deposition due to LPS. Fibrin is associated with pro-inflammatory effects, possibly by activating integrins via its RGD sequence. Integrin activation triggers a myriad of intracellular signaling events including the phosphorylation of adaptor proteins (e.g. FAK, and paxillin). Adaptor proteins facilitate activation of signaling cascades including MAPK and JNK and possibly crosstalk with IL-6 signaling via STAT3 activation.

mediate LPS-induced liver injury by increasing hepatic levels of proinflammatory chemokines (i.e. MIP-1 α and MCP-1) (39). Therefore, blocking enhanced c-Fos expression could provide protection in this model. SOCS3 expression was differentially affected by ethanol pre-exposure and cycloRGDfV with integrin inhibition protecting against enhanced mRNA expression of SOCS3 due to ethanol preexposure.

SUMMARY AND CONCLUSIONS

STRENGTHS OF THIS WORK

This work has many strengths. First, it identifies a new player, integrin $\alpha\beta3$, in alcoholic liver disease. Currently, no FDA approved therapy exists to halt or reverse alcoholic liver disease. In order for new therapies to be developed, new key players must be identified as potential therapeutic targets. This work suggests that integrin inhibitors, some of which have already been studied in clinical trials for asthma and chronic obstructive pulmonary disease (16) as well as cancer therapies (17), may also be beneficial in treating ALD. More specifically, inhibitors of the integrin $\alpha\beta3$ may be particularly useful in the inflammatory phases of liver injury such as steatohepatitis.

All the work described here was performed in the whole animal. Use of the whole animal is particularly important when studying diseases that involve multiple organs, such as ALD. Indeed, although in vitro studies using single cell culture or co-culture are useful, whole animal experiments allow better translation to the human disease. Additionally, this study used a specific peptide inhibitor of integrin $\alpha\beta3$, cycloRGDFV. The use of a pharmacological inhibitor provides advantages over a whole mouse deletion of the $\alpha\beta$ integrin. Whole mouse deletions can result in non-specific effects due to compensatory mechanisms that are selected for early in development. Deletion of the $\beta3$ integrin subunit is highly lethal, and while some animals do survive to adulthood, the adult population of this strain of mouse may have compensatory mechanisms at work.

For these studies, an acute model of alcohol-induced liver injury was used. While no model fully recapitulates the human disease, this model does provide a number of advantages over other alcohol feeding models. For example, in this model, the experimenter can control how much and when the animals ingest ethanol to ensure all animals receive similar concentrations of ethanol and reach similar states of inebriation.

CAVEATS AND WEAKNESSES

The main purpose of this work was to investigate the role of integrin $\alpha\beta3$ in a model of enhanced LPS-induced liver injury caused by alcohol. In order to study the effects of inhibiting integrin $\alpha\beta3$, a commercially available peptide inhibitor, cycloRGDFV, was used. While use of this inhibitor conveyed some advantages to this study, cycloRGDFV may interact with other integrins that recognize RGD sequences. Furthermore, a variety of integrins recognize RGD sequences that could also interact with the RGD sequences of fibrin. Therefore, we cannot conclusively state that the specific interaction between $\alpha\beta3$ and fibrin is the mechanism at work under these conditions. This study also did not offer insight into which cells are being primarily affected by cycloRGDFV administration. Nearly all cells express a number of different integrin heterodimers and a single integrin can be expressed by a variety of cell types, for instance the integrin of primary interest in this study, $\alpha\beta3$, is known to be expressed on SECS, Kupffer cells, neutrophils, and platelets. Additional studies would be required to examine more specifically the ECM-integrin interactions that are key to protection in this model.

This study was conducted in an acute alcohol exposure model that enhances liver injury due to LPS. While this model has advantages (see Strengths), it does not fully recapitulate alcohol exposure in humans. This model requires forced feeding of alcohol to overcome rodents' natural aversion to ethanol, which prevents them from voluntarily consuming enough toxin to cause liver damage. Although this acute model of exposure has advantages, most liver damage is associated with chronic alcohol exposure. Therefore, effects found in this model do not necessarily recapitulate the chronic disease. However, recent studies suggest that a critical component of ALD may be 'acute on chronic' drinking behavior in which chronic alcoholics binge periodically. Additionally, acute models have proven to be a useful screening to complement chronic studies. Indeed, there is great mechanistic overlap of protective agents used in both acute and chronic models and, to date, there have been no 'false positives' observed when studying protective agents in acute models of alcohol exposure (40-42). This model recapitulates

the inflammatory aspects of the pre-fibrotic stages of alcoholic liver disease, mainly chronic inflammation. While the mechanisms involved in various stages of alcohol-induced liver injury have been extensively investigated, the mechanism behind progression from earlier stages of ALD (i.e. steatosis) to later stages, such as steatohepatitis, remain unclear. Accordingly, a major goal of this work was to identify key players in earlier, highly reversible stages of the disease with an emphasis on the inflammatory response characteristic of steatohepatitis and is the justification for using the model for this study.

FUTURE DIRECTIONS

The future directions described here are to address the issues discussed in Caveats and Weaknesses and the questions that remain unanswered with regard to the role of integrins in alcohol-induced organ injury. The purpose of the experiments described herein would be to build on the theme of this thesis, but are not necessary to complete this project.

Role of integrins in animal models of chronic alcohol exposure.

This study provides compelling evidence indicating that blocking integrin activation protects against enhanced LPS-induced liver injury caused by acute alcohol exposure. However, the role of $\beta 3$ -containing integrins after chronic alcohol exposure remains unknown. While the model used in this study recapitulates the chronic inflammation characteristic of pre-fibrotic stages of liver injury, steatosis does not persist in this model. Acute models of alcohol exposure are convenient and allow for more precise control over timing and extent of inebriation, however chronic models of alcohol exposure better recapitulate the human condition. The role of $\beta 3$ -containing integrins in alcohol-induced organ injury could be investigated further using a chronic model of alcohol exposure and $\beta 3^{-/-}$ mice. A whole animal knockout would be advantageous in a chronic model of alcohol exposure in order to ensure that activity of β subunit-containing integrins is inhibited throughout the duration of the study.

Do integrins mediate susceptibility to injury in the alcoholic lung?

Integrins have also been shown to play a role in enhanced susceptibility to lung injury caused by alcohol exposure. Alcohol is known to sensitize to acute lung injury (43, 44). These effects may be due to a myriad of changes that occur after ethanol exposure and are similar to changes seen in alcohol-induced liver injury and include altered inflammatory response, oxidative stress, and tissue remodeling. Several key players that this group has identified in liver injury also appear to play important roles in the alcoholic lung. For example, LPS enhances injury due to ethanol in both organs (45). Additionally, fibrin mediates pro-inflammatory effects in lung (46). These observations further support the possibility of mechanistic overlap of alcohol-induced injury in these two organs. Therefore, it would be pertinent to determine if β 3 containing integrins may mediate inflammation downstream of tissue remodeling in the lung. The role of integrins in enhanced susceptibility to pulmonary injury due to alcohol could be studied in a chronic alcohol 2-hit model of organ injury using whole mouse knockouts of the β 3 integrin subunit.

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ABBREVIATIONS

ALD	Alcoholic Liver disease
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CAE	Chloroacetate esterase
CD31	Platelet-endothelial cell adhesion molecule (PECAM)
ERK	Extracellular signal-related kinases 1/2
EtOH	Ethanol
FAK	Focal adhesion kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin & eosin
HA	Hyaluronic acid
ICAM	Intracellular adhesion molecule
IL-10	Interlukin-10
IL-6	Interlukin-10
ILK	Integrin-linked kinase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MD	Maltose-dextrin
PAI-1	Plasminogen activator inhibitor-1
SEC	Sinusoidal endothelial cell
SOCS3	Suppressor of cytokine signaling-1
STAT3	Signal transducers and activators of transcription 3
TNF α	Tumor necrosis factor-alpha

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1. Massey VL, Poole LG, Schmidt RH, Arteel GE (2012) Integrin $\beta 3^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY
2. Massey VL, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Ethanol sensitization to LPS-induced liver injury: protection by the integrin inhibitor, cycloRGDfV. OVSOT Dayton, Ohio
3. Massey VL, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Integrin $\alpha v\beta 3$ mediates ethanol-enhanced liver damage caused by LPS. Research!Louisville Louisville, KY

Abstracts – National/International

1. Massey VL, Beier JI, Schmidt RH, Zhong H, Arteel GE (2011) Integrin inhibitor Cyclo-RGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice. *Hepatology* 54(S1):970A
2. Tan M, Massey VL, Ding X, Zhong H, States JC, and Arteel GE (2010) Arsenic pre-exposure delays cell cycle entry and progression in mouse liver after partial hepatectomy. *Hepatology* 52:462A

PRESENTATIONS

1. Research Seminar, 11/12, Parallel and interdependent mechanism of alcohol-induced organ injury in the liver and lung. University of Louisville Alcohol Research Center, Louisville, KY
2. Poster Presentation, 10/12, Integrin $\beta 3^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY
3. Poster Presentation, 11/11, Integrin inhibitor Cyclo-RGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice. American Association for the Study of Liver Disease, San Francisco, CA.
4. Poster Presentation, 10/11, Integrin $\alpha v\beta 3$ mediates ethanol-enhanced liver damage caused by LPS. Research!Louisville, Louisville, KY.
5. Poster Presentation, 10/11, Ethanol sensitization to LPS-induced liver injury: protection by the integrin inhibitor, cycloRGDfV. Ohio Valley Society of Toxicology Regional Meeting, Dayton, OH.
6. Research Seminar, 03/11, Integrin inhibitor Cyclo-RGDfV blunts enhanced LPS-induced liver injury caused by ethanol in mice. University of Louisville, Seminar in Pharmacology and Toxicology, Louisville, KY.