CALPAIN AND LIPOPOLYSACCHARIDE MEDIATED HEPATITIS

A Thesis

by

ROBERT EDWARD ROSE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2006

Major Subject: Laboratory Animal Medicine

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Approved by:

Chair of Committee, Shashi K. Ramaiah
Committee Members, Ann B. Kier
W. Les Dees
Head of Department, Gerald R. Bratton

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ABSTRACT

Calpain and Lipopolysaccharide Mediated Hepatitis. (August 2006)

Robert Edward Rose, B.S., Texas A&M University;

D.V.M., Texas A&M University

Chair of Advisory Committee: Dr. Shashi K. Ramaiah

This study tested the role of the calcium dependent cytosolic protein calpain in neutrophilic hepatitis. We hypothesized that inhibition of calpain would protect against LPS-induced neutrophilic liver damage. To test our hypothesis, a reliable LPS-mediated hepatitis model to investigate the mechanisms of hepatic neutrophil infiltration following LPS administration was developed by repeat intravenous injection of LPS at a dose of 10 mg/kg to rats. Blood was collected for hematologic and biochemical analysis and multiple organs including liver were collected for evaluation of histopathologic changes. Flow cytometry was employed to investigate L-selectin (CD 62L) and MAC-1 (CD11b/18) expression on neutrophils both *in vivo* and *in vitro*. Significant hematologic changes included neutrophilia, elevation in neutrophil to lymphocyte ratio with toxic changes and left shift. Biochemical changes were observed in several liver (AST, GGT) and kidney (BUN) parameters generally at the earliest time points. Histopathology revealed a time-dependent neutrophil and mononuclear infiltration around the periportal areas in the single dose study and mid-zonal inflammation with multifocal coagulative necrosis in the repeated dose study. CD 11b was up-regulated both *in vitro* and *in vivo*. After development of a suitable model, the first goal was to investigate the role of the

intracellular enzyme calpain in the development of neutrophilic hepatitis and midzonal necrosis. A second goal was to compare the observed protective effects of calpain inhibition with a relatively selective inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine and an inhibitor of coagulation, heparin. When compared to rats administered LPS alone, administration of calpain 1 inhibitor prior to LPS significantly reduced hepatic iNOS expression, hepatic neutrophil infiltration and attenuated midzonal hepatic necrosis. Administration of heparin and aminoguanidine prior to LPS also decreased liver iNOS expression, hepatic neutrophil infiltration and liver pathology comparable to calpain inhibition. Blood neutrophil activation, as measured by the neutrophil adhesion molecule CD11b integrin, was upregulated in all the LPS treated groups regardless of inhibitor administration. We conclude that amelioration of liver pathology via calpain inhibition is likely dependent on the down-regulation of iNOS expression in the rat model of LPS mediated hepatitis.

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CHAPTER I

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) is a component of the outer membrane of the cell wall of gram negative bacteria, which are often normal inhabitants of the mammalian intestinal tract (Uesugi et al., 2002). Enteric bacterium often serve as a source for systemic LPS exposure and under certain conditions, such as those associated with chronic alcohol consumption, increased gut derived LPS is released into the portal circulation (Keshavarzian and Fields, 2003; Uesugi et al., 2002). LPS from lysed gram negative bacteria entering the blood stream is bound by lipopolysaccharide binding protein. Protein bound LPS interacts with CD14 receptors on monocytes and macrophages, including hepatic Kupffer cells, setting off a series of pro-inflammatory events (Bautista, 2002; Limaye et al., 2003). First, CD14 mediated activation of the toll-like receptor 4 results in NF-κB regulated production of cytokines**,** including IL-1β, IL-6, IL-8, TNF-α and platelet-activating factor along with upregulation of the inducible isoform of nitric oxide synthase (iNOS) (Kamanaka et al. 2003; Luyendyk et al., 2003; Nagy, 2003). These strong mediators of inflammation initiate a cascade, which can lead to neutrophil activation and generation of reactive oxygen species (ROS) (Enomoto et al. 2000).

This thesis follows the style of *Toxicologic Pathology*.

Activated neutrophils express surface adhesion molecules, such as L-selectin and Mac-1 (CD11b/18), which bind with complementary adhesion molecules, such as ICAM-1 and VCAM-1, on vascular endothelium promoting hepatic transmigration (Jilma et al. 1999; Picker, 1999). Concurrent with neutrophil activation, LPS mediated circulatory changes including reduced mean arterial pressure, formation of microthrombi and altered microcirculation, impair hepatic perfusion and promotes leukocyte adhesion with subsequent tissue transmigration (Asakura et al., 2005; Palmes et al., 2004). Transmigrated neutrophils can elicit tissue damage through release of ROS, proteolytic enzymes and additional chemotactic factors further driving neutrophil mediated hepatitis (Ikeda and Young, 2002).

Several animal models of endotoxemia with concurrent hepatic inflammatory lesions are reported in literature which include parenteral injections of LPS along with surgical preparations such as the cecal ligation and puncture technique (Parker and Watkins, 2001). However, there are no well accepted animal models of endotoxemia with hepatic neutrophil infiltration and necrosis in literature that is consistently reproducible. Furthermore, the exact dose and route of LPS administration has also not been standardized in endotoxemic hepatitis models. Generally speaking, endotoxemic hepatitis animal model usually should exhibit at least one characteristic of the human condition, be reproducible and be developed in a manner consistent with humane animal care and use. In addition, a reliable animal model should provide markers of organ injury that allow mechanisms of disease of interest to be thoroughly investigated. Historically, some of the markers of LPS mediated hepatic damage have included

increased serum activity of the hepatic intracellular enzymes alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) (Suliburk et al., 2005), hepatic neutrophil transmigration (Bautista, 2002; Vadjova et al., 2000.), apoptosis (Tsuchiya et al., 2004), and hepatocellular necrosis (Ou et al., 1997, Yee et al., 2003). However, systematic serum biochemical and histologic alterations following LPS-induced neutrophilic hepatitis in rats have not been investigated on a temporal basis. The second chapter of this thesis describes the development of a suitable animal model to test our hypothesis concerning calpain and lipopolysaccharide mediated hepatitis.

Recent literature suggests that calpain may play an important role in liver injury induced by hepatotoxicants such as carbon tetrachloride and acetaminophen (Limaye et al., 2003; Mehendale and Limaye, 2005). Limaye et al. (2003) reported that Calpain inhibitor substantially decreased the progression of liver injury and led to protection against carbon tetracholoride-induced lethality. Emerging evidence also suggests the role of calpain as a pro-inflammatory molecule by activation of nuclear factor kappa-B (NF-κB) (Picker, 1999; Riedemann et al., 2003) and the subsequent production of a variety of pro-inflammatory mediators including tumor necrosis factor alpha, interleukins such as IL-1β, IL-6, and IL-8, and cell adhesion molecules such as VCAM-1 and ICAM-1 (Eipel, et al., 2004, Czermak, et al., 1999). However, the role of calpain in endotoxin-mediated hepatitis is currently not known and is the basis of this investigation as described in Chapter III.

Because of the exclusive midzonal necrosis noted in our model and association of such lesions with LPS mediated hypotension and microvascular disturbance, the role

of iNOS and coagulation was also investigated in this study. Excessive production of nitric oxide (NO) as a consequence of macrophage release of iNOS contributes to the hypotension that is a feature of endotoxemia (Imai et al., 1994; Landry and Oliver, 2001). Recent reports have demonstrated the pathological role of iNOS in models of disseminated intravascular coagulation (DIC) (Asakura et al., 2005) and acetaminophen toxicity (Kamanaka et al., 2003). We thus compared the results of calpain inhibition with those of an iNOS inhibitor aminoguanidine and an inhibitor of coagulation and microthrombi formation, heparin. Finally we evaluated the effect that calpain inhibition has on expression of the cytochrome P450 enzyme, CYP2E1, in our model of LPS mediated hepatitis. CYP2E1 was selected as it is known to be down regulated in inflammatory conditions and it has been suggested that nitric oxide (NO) may play a role in reduced CYP expression consequent to LPS exposure which may have vascular function implications (Udosen et al., 2003). It is our hypothesis that amelioration of liver pathology via calpain inhibition is likely dependent on the down-regulation of iNOS expression in the rat model of LPS mediated hepatitis.

CHAPTER II

DEVELOPMENT OF THE NEUTROPHILIC HEPATITIS MODEL

MATERIALS AND METHODS

Animals and treatment

All animal procedures were approved by the Texas A&M University Laboratory Animal Care Committee (ULACC) and conformed to the standards set forth in the Guide for the Care and Use of Laboratory Animals (NRC 1996). Commercially obtained Sprague-Dawley (SD) rats (250-270 g body weight; Harlan, Indianapolis, IN) employed in this study were barrier raised and housed in an AAALAC accredited facility. Standard rodent chow (Teklad 8604, Madison WI) and reverse osmosis filtered water were supplied ad libitum.

Single LPS dose experiments: Male SD rats were divided into experimental and control groups (n = 4 in each group). The experimental groups were administered LPS (E Coli serotype 0111: B4, Sigma Chemical Co., St Louis, MO) in saline at a dose of 5 or 10 mg/kg compounded to yield a dose volume of 1 ml/kg intravenously via tail vein injection. Two control groups were employed. One control group received saline at a dose volume of 1 ml/kg with the second control group not subjected to saline or LPS injection. Following treatment, the rats were euthanized via CO_2 at 0 (untreated control), 12, 24 and 48 hrs post LPS or vehicle administration (Figure 1).

Repeated two dose experiments: Male SD rats were divided into three groups (n =5 in each group) and administered LPS in saline twice, 24 hours apart, via tail vein injection at a dose of 10 mg/kg. The rats were sacrificed via CO_2 at 6, 12, and 24 hours

post second injection of LPS (Figure 1). The selection of LPS dose and sacrifice time points was based on the results of the single dose study. A follow up repeat dose study (n=5 in each group) to confirm the results of the first repeat dose experiment was performed with LPS administration route, dose, and sample time points identical to the first study.

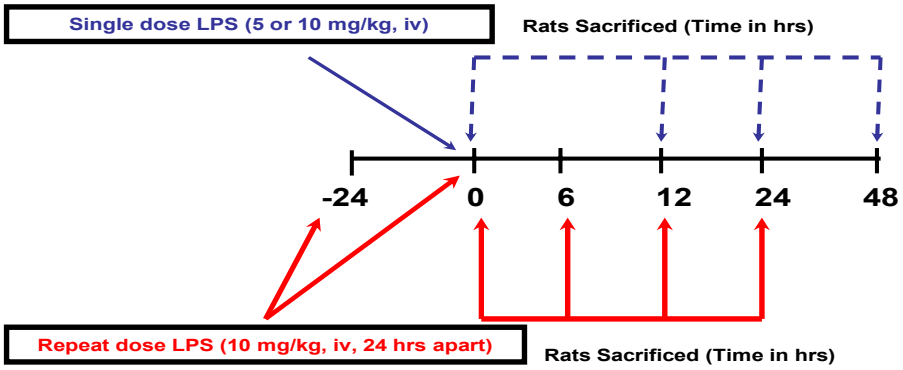

Figure 1- Experimental scheme to demonstrate single and repeated two dose LPS injections in relation to sample collections in Sprague Dawley rats.

Sample harvesting and processing

Blood from the single and repeated dose study was collected via the abdominal vena cava and divided into three separate tubes. An EDTA tube (Terumo Medical Co, Elkton, MO) was employed for determination of complete blood count (CBC) on a Cell-Dyn 3700 (Abbott Diagnostics, Abbott Park, IL) hematology analyzer. A serum tube (Monoject, Sherwood Medical, St Louis, MO) was used for clinical chemistry determinations using a Hitachi 911 (Roche, Indianapolis, IN) chemistry analyzer. The remainder of the blood was placed into a lithium heparin tube (Monoject, Sherwood Medical, St Louis, MO) for determination of neutrophil adhesion molecule expression. Blood smears were made from the EDTA blood and stained for manual hematology evaluation. Serum was separated from red blood cells by centrifugation at 4000 rpm for 10 minutes and plasma separated from lithium heparin tubes was stored at -70° C for later analysis. Representative liver sections were collected from different lobes of the liver and placed in liquid nitrogen and stored at -70° C until further analysis. The remaining liver and other organs (kidney, lungs, intestines, brain and spleen) were collected and placed in 10% neutral buffered formalin for histopathological evaluation and chloracetate esterase staining for neutrophils as previously described (Banerjee et al., 2006).

Flow cytometry studies

To further evaluate neutrophil stimulation, *in vitro* and *in vivo* experiments were designed to assess the neutrophil adhesion molecules, L-selectin (CD62-L) and Mac-1 (CD 11b/18). In these studies, blood was collected from rats via cardiac puncture directly into lithium heparinized tubes (Monoject, Sherwood Medical, St Louis, MO) and was stored on ice until processing. Blood was divided into 250 uL aliquots in

separate glass tubes and was kept on ice unless otherwise stated. Duplicate tubes of blood were designated to antibody stained or unstained groups.

For the *in vitro* experiments, neutrophils were stimulated *in vitro* with LPS prior to antibody staining using a dose of 0.04 mg of LPS dissolved in 10 uL of distilled water per sample to mimic an i.v. dose of 10 mg/kg for a 250g rat. After the addition of LPS, tubes were gently shaken in a warm water bath at 37° C for 30 minutes. All but unstained samples were incubated with 10µl of reagent grade mouse IgG (Sigma, St Louis, MO) mixed at a concentration of 1mg/ml, to reduce nonspecific binding of the conjugated antibodies. Neutrophils were labeled with fluorescein-conjugated HIS 48 (BD Biosciences, San Jose, CA) to allow for identification via flow cytometry. Mac 1 integrins were labeled with R-phycoerythrin conjugated CD11b (Serotec Inc., Raleigh, NC) and L-selectins were labeled using R-phycoerythrin CD62L (Serotec Inc., Raleigh, NC). Following incubation with antibodies (20 min), the samples were treated with RBC lysis buffer for 2 min. The tubes were then centrifuged at 1000 rpm for 10 min at 3° C and resuspended several times until erythrocytes were removed. After lysing steps, the cells were washed with 50 µL of PBS and centrifuged at 1000 rpm for 10 minutes. Cells were suspended in 300 µL of PBS prior to analysis.

For determination of neutrophil activation *in vivo*, flow cytometry was performed using blood collected from both single and repeat LPS dose studies. Briefly, heparinized blood collected from single 10 mg/kg LPS dose at the 12 hour time point (n=3) and blood collected from repeat LPS dose at the 6 hour time point (n=3) were used to assess levels of CD11b and CD62L expression on neutrophils and compare their levels with *in vitro* studies. Blood collected from naive rats (n=3 both single and repeat dose) were employed as controls. The blood was processed for flow cytometry identical to the *in vitro* samples.

The cells were analyzed using a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometry, equipped with a 15mW air-cooled argon laser. Green fluorescence from fluorescein was collected through a 530/30-nm bandpass filter and orange fluorescence from R-phycoerythrin through a 585/42-nm bandpass filter. List mode data were acquired on a minimum of 10,000 events defined by light scatter gates to include the neutrophil population. Data analysis including spectral compensation was performed using FlowJo (Treestar Inc., Ashland, OR). A neutrophil gate was established based on side scatter and positive HIS48 staining, and the median fluorescence channel was determined for CD11b staining.

Statistics

To identify significant differences among groups, analysis of variance (ANOVA) with a *post hoc* Tukey's HSD was performed using a computer software program (SPSS version 12.0, Claritas Inc.). Results were considered significant at the 0.05 confidence level.

RESULTS

There were no significant statistical differences in hematology, serum chemistry or hepatic neutrophil counts between the untreated and saline treated controls in the single dose study. For ease of reporting and to increase statistical power, both control groups were reported together in the results section. In order to reduce animal numbers, saline and untreated controls from the single dose study provided control group values for the repeat dose study. Since both repeat dose studies were identically performed, results presented reflect both studies.

Clinical signs

Single and repeat dose studies: Rats were lethargic as compared to controls within 1 hour of dosing and developed diarrhea within 3 hours of LPS administration.

Increased activity and a resolution of diarrhea was noted approximately 24 hours after LPS was discontinued.

Hematology findings

Single dose experiment: The most significant and consistent finding in the LPS group compared to the controls was the absolute increase in neutrophil numbers at 12 and 24 hours post LPS. The increase in N:L ratio peaked at 12 hours post LPS dose and started declining at 24 hours post LPS dose with almost control levels reached by 48 hours post LPS dosing at both dose rates. Changes in absolute counts for both neutrophils and lymphocytes contributed to the increased N:L ratio at 12 and 24 hours post dose. Absolute neutrophil numbers increased while lymphocyte numbers decreased at 12 and 24 hours (Table 1). The post dosing elevation in neutrophils and decline in lymphocytes was absent by 48 hrs post LPS dosing as reflected by a normal N:L ratio. Mild but infrequent monocytosis was present 48 hrs post LPS injection at 10 mg/kg LPS (data not shown). The platelet numbers were significantly decreased in the LPS treated group compared to controls with frequent clumping present throughout the blood smear. There were no alterations in the hematocrit noted between control and experimental groups.

Repeated dose study: Absolute neutrophil numbers were significantly higher at the 6 and 12 hr time point compared to the control with a trend of decline by 24 hr time point following the second LPS injection (Table 2). Absolute lymphocyte numbers were significantly reduced at the 6 hour time point as compared to the 12 and 24 hr time point. Consequent to increased neutrophils and decreased lymphocytes at the 6 hour time point, the neutrophil to lymphocyte ratio was significantly elevated at 6 hours as compared to the 12 and 24 hour time points. Monocytosis was significantly elevated at

the 24 hour time point post second LPS injection. Nucleated red blood cells were present at all three time points in this study (Table 2).

Table 1- Select hematology and serum biochemistry values from single intravenous 5 or 10 mg/kg dose LPS in male Sprague Dawley rats.

Dose	**Control**		**5 mg/kg**	
Time point		**12 hour**	**24 hour**	**48 hour**
WBC #/µl	13462 ± 542	10025 ± 792	10925 ± 668	12475 ± 776
Neutrophil #/µl	1591 ± 209	6511 ± 902*	5710 ± 724*	3228 ± 254
Lymphocyte #/µl	11658 ± 536	3315 ± 807*	5096 ± 573*	9126 ± 862
N:L ratio	.15 ± 0.3	2.3 ± .6*	1.2 ± .6	.4 ± .1
Albumin g/dl	4.4 ± .05	3.8 ± .04*	3.5 ± .17*	3.4 ± 0*
BUN mg/dl	20.2 ± .49	42.6 ± 3.8	37.7 ± 6.1	28.2 ± 1.0
AST U/l	77.6 ± 2.3	192.3 ± 32.6	134.8 ± 29.1	61.0 ± 5.8
ALT U/l	65.1 ± 1.9	66.2 ± 6.3	41.8 ± 9.8	27.8 ± 1.0
CK U/l	190.6 ± 13.1	209.3 ± 58.2	131.0 ± 17.1	134.5 ± 15.3

Dose	**Control**		**10 mg/kg**	
Time point		**12 hour**	**24 hour**	**48 hour**
WBC #/µl	13462 ± 542	9600 ± 962	9825 ± 1581	11950 ± 688
Neutrophil #/µl	1591 ± 209	6338 ± 437*	4550 ± 1314*	2637 ± 667
Lymphocyte #/µl	11658 ± 536	3215 ± 643*	5212 ± 1368*	8561 ± 422
N:L ratio	.15 ± 0.3	2.1 ± .3*	1.1 ± .5	.3 ± .1
Albumin g/dl	4.4 ± .05	3.7 ± .18 *	3.7 ± .17*	3.1 ± .15*
BUN mg/dl	20.2 ± .49	108.9 ± 51.5 #	43.4 ± 13.3	45.7 ± 3.4
AST U/l	77.6 ± 2.3	352.0 ± 166.1*	255.8 ± 123.9	83.0 ± 26.4
ALT U/l	65.1 ± 1.9	164.3 ± 94.1	154.8 ± 110.9	47.5 ± 19.9
CK U/l	190.6 ± 13.1	321.5 ± 96.0*	148.0 ± 11.7	136.3 ± 11.5

Serum chemistry was assayed using Hitachi 911 and complete blood count was analyzed on a Cell-Dyn 3700 analyzer followed by manual evaluation as described in the Materials and Methods section.

* Values significantly different form the controls, $p < 0.05$.

N=16 (control), N = 4 (LPS groups), Values are mean ± SE

Table 2- Select hematology and serum biochemistry values following repeat dose 10 mg/kg LPS administration in male Sprague Dawley rats.

Time point	Control	6 hour	12 hour	24 hour
WBC #/µl	13462 ± 542	8066 ± 2004	12450 ± 697	12600 ± 781
Neutrophil #/µl	1591 ± 209	4855 ± 1237*	4767 ± 1403*	3001 ± 287
Lymphocyte #/µl	11658 ± 536	2881 ± 369#	7278 ± 788*	8751 ± 733*
N:L ratio	.15 ± 0.3	1.8 ± .2#	.74 ± .1*	.37 ± .05
Nucleated RBC	0	1.5 ± .7	.7 ± .4	.3 ± .3
Albumin g/dl	4.4 ± .05	3.4 ± .13*	3.2 ± .19*	3.1 ± .1*
BUN mg/dl	20.2 ± .49	79.5 ± 17.5*	52.6 ± 6.3	48.5 ± 10.5
Creatinine mg/dl	.24 ± .01	.59 ± .13*	.33 ± .02	.34 ± .02
AST U/l	77.6 ± 2.3	281.7 ± 42.9*	138.3 ± 19.9	69.3 ± 6.9
ALT U/l	65.1 ± 1.9	90.6 ± 28.3	45.0 ± 7.9	36.4 ± 3.1
ALP U/l	202.3 ± 6.6	463.9 ± 118.7#	247.9 ± 21.4	175.6 ± 10.6
GGT U/l	< 3 ± 0	21.0 ± 9.6*	5.4 ± 1.6	< 3 ± 0
Total bilirubin	.13 ± .01	.26 ± .02*	.24 ± .02*	.17 ± .02
CK U/l	190.6 ± 13.1	344.1 ± 47.2*	366.9 ± 62.9*	250.1 ± 49.1

Serum chemistry was assayed using Hitachi 911 and complete blood count was analyzed on a Cell-Dyn 3700 analyzer followed by manual evaluation as described in the Materials and Methods section.

* Values significantly different form the controls, $p < 0.05$.

= Values significant as compared to controls and other dose groups, , $p \leq .05$

N=16 (control), N = 10 (LPS groups), Values are mean ± SE

GTT values below 3 U/l are reported as <3 by the laboratory

Serum chemistry findings

Single dose experiment: Biochemical changes were mostly noted in serum albumin, blood urea nitrogen (BUN), aspartate aminotransferase (AST) and creatinine kinase (CK) levels in the LPS treated groups. A mild but significant decrease in albumin levels was noted following LPS injection at all points following LPS injection at both LPS doses. Increased BUN levels were noted at early time points post LPS injection at both the doses although the elevation was much higher in the repeat dose study (Table 1). AST levels were consistently elevated in both the doses at 12 and 24 hr time point with the relative degree of elevation much higher at the 10 mg/kg dose (Table 1). Alanine aminotransferase (ALT) values were inconsistent with some extreme elevated outliers in several LPS dosed groups and multiple instances of lowered values compared to the control group. CK levels were minimally elevated as compared to controls in the 12 hour 10 mg/kg dose group (Table 1).

Repeated two dose study: Serum chemistry revealed elevations of AST, total bilirubin, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), BUN and creatinine generally at the 6 hour time point as compared to control ranges established in the single dose study (Table 2). Parameters used to evaluate hepatobiliary injury; AST, ALP and GGT had significantly increased levels at 6 hours but were no longer significantly elevated by the 12 hour time point (Table 2). BUN and creatinine were elevated at 6 hours as compared to the 12 and 24 hour time points. CK levels were increased compared to controls at the 6 hour time point. Similar to the single dose study, albumin levels were significantly decreased as compared to established controls at all three time points.

Microscopic examination of stained blood films

Single and repeated two dose experiment: Microscopic examination revealed significant immaturity in neutrophils as indicated by immature nuclear chromatin

pattern, band and donut neutrophils (Fig 2B). Toxic changes were present within neutrophils at all the doses of LPS, although the toxic changes were much higher in the repeat dose study. Toxic changes were evaluated based on the presence of one or more of the following as described in Veterinary literature (Lattimer et al., 2003); Dohle bodies, increase cytoplasmic basophilia, vacuolated cytoplasm and cytoplasmic granulation. Toxic changes such as Dohle bodies and occasional toxic granulation and were present in both low and high dose LPS dosing (Fig 2C & D). The presence of nucleated red blood cells was a consistent finding in the repeat dose experiment but was absent in the single dose study.

Organ pathology

Single dose experiment: Histopathology revealed minimal periportal infiltrate of neutrophils in the liver at 12 hours post dose. By 24 to 48 hours the periportal infiltrate was predominately mononuclear (Fig 3B). A slight increase in hepatocellular apoptosis and mitosis was identified to 48 hour post dose sections (Fig 3F). There were no histopathological alterations identified in other organs collected.

Repeated two dose study: Histopathology revealed marked multifocal midzonal neutrophilic hepatic necrosis/apoptosis accompanied by a predominately mononuclear periportal infiltrate (Fig 3C, 3D, 3E). Necrosis progressed from 6 to 12 hours post second dose. By the 24 hour time point, necrotic foci were being replaced by regenerating hepatocytes in some regions. Regeneration was marked by increased hepatocyte mitosis and megalocytosis as compared to control rats.

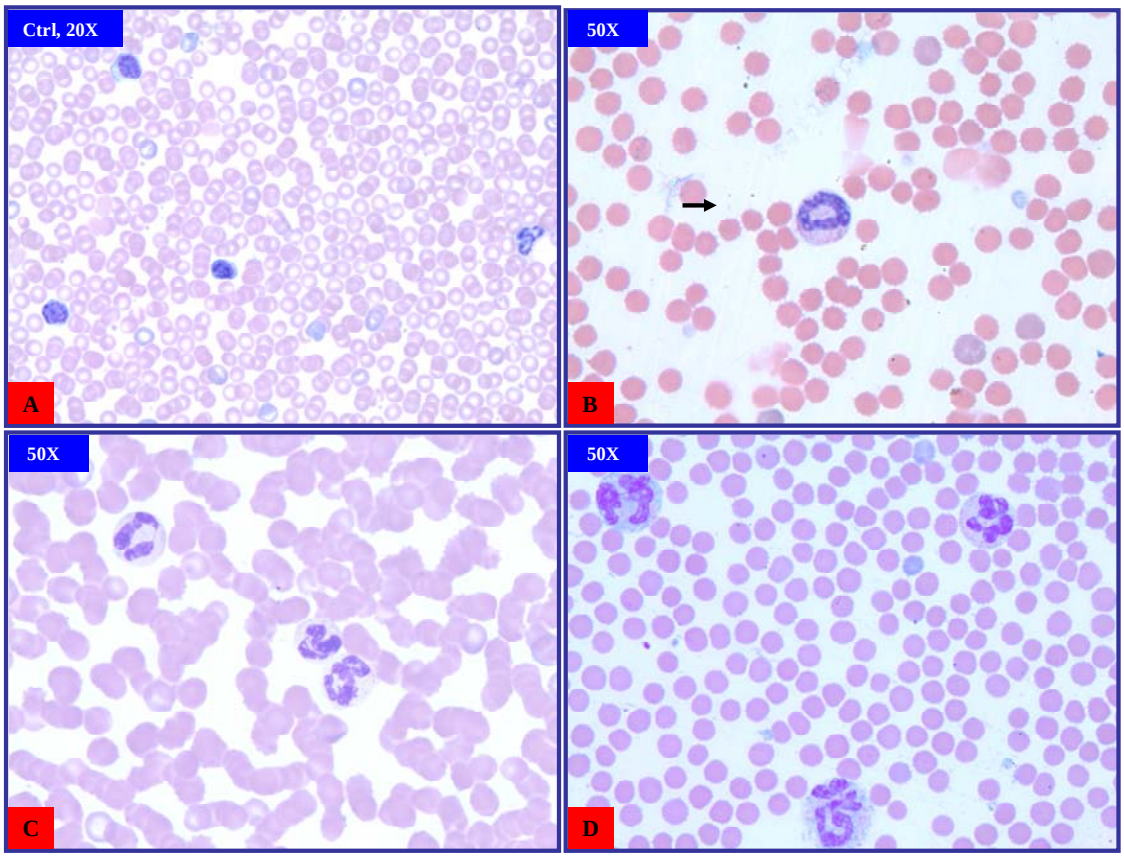

Figure 2- Microscopic examination of Wright-Giemsa stained blood film following single (5 or 10 mg/kg) and repeated two dose (10 mg/kg) LPS injections. 2A: control blood (20X); 2B: representative left shifted stab (donut) neutrophil single dose (50X); 2C & D: representative toxic neutrophils single and repeated two dose.

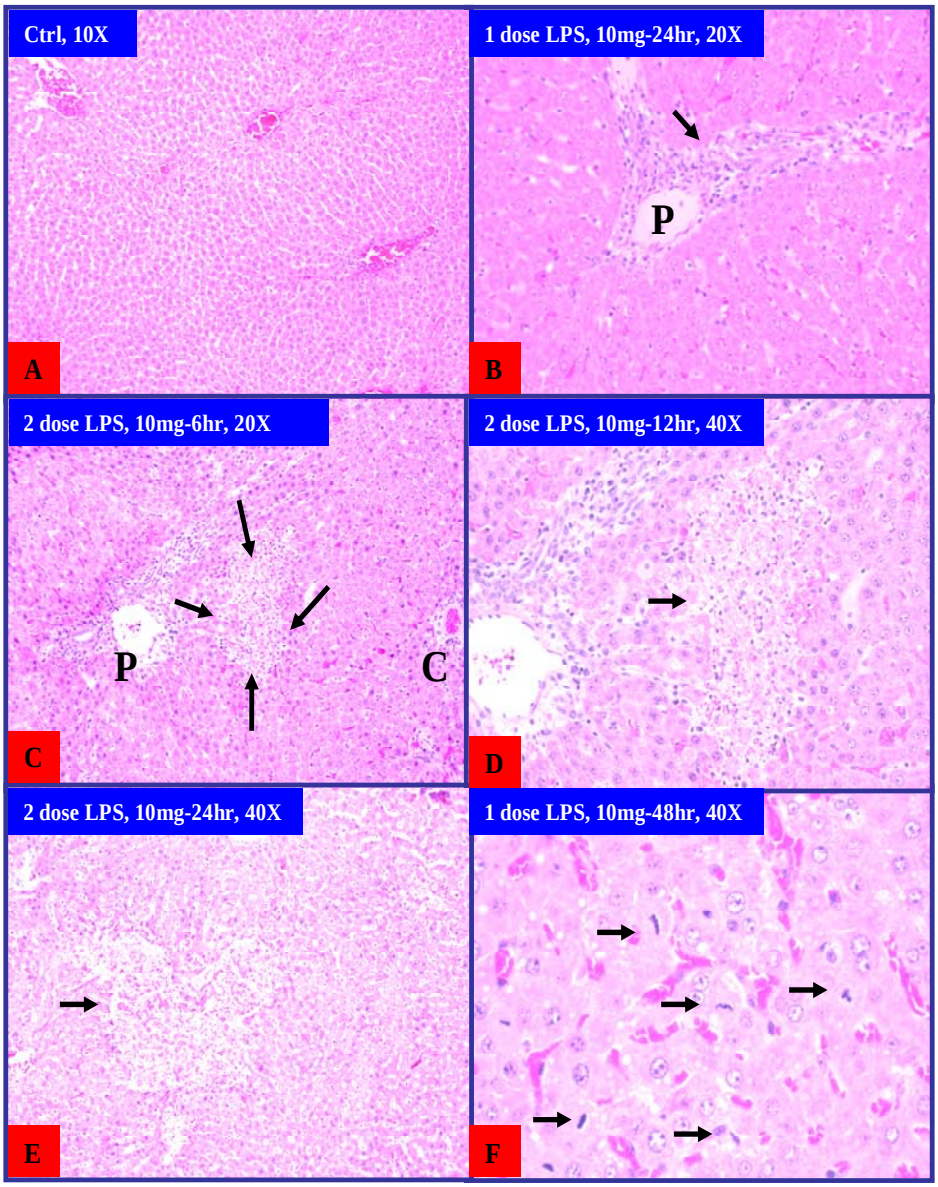

Figure 3- Microscopic examination of liver following single (5 or 10 mg/kg) and repeated two dose (10 mg/kg) LPS injections. 3A: control; 3B: single dose (10mg/kg 24 hr) showing representative periportal infiltrate (arrow), P- periportal region; 3C: repeated two dose at 6 hr showing representative midzonal necrosis (arrows). 3D: repeated two dose at 12 hr showing representative midzonal necrosis (arrows). 3E: repeated two dose at 24 hr showing representative midzonal necrosis (arrows). 3F: Single dose LPS 10mg/kg at 48 hr illustrating liver regeneration with increased mitotic figures (arrows) H&E stain.

Enumeration of hepatic neutrophils

Chloroacetate esterase staining of liver sections revealed increased numbers of extravasated neutrophils from the sinusoids. Hepatic neutrophil numbers were greatest at the early time points in both single and two dose experiments as compared to later time points. Extravasated neutrophil counts were highest at the 6 hour time point in the two dose study and coincided with the observed midzonal necrosis. In both single and two dose experiments, neutrophil counts diminished in a temporal fashion (Fig 4).

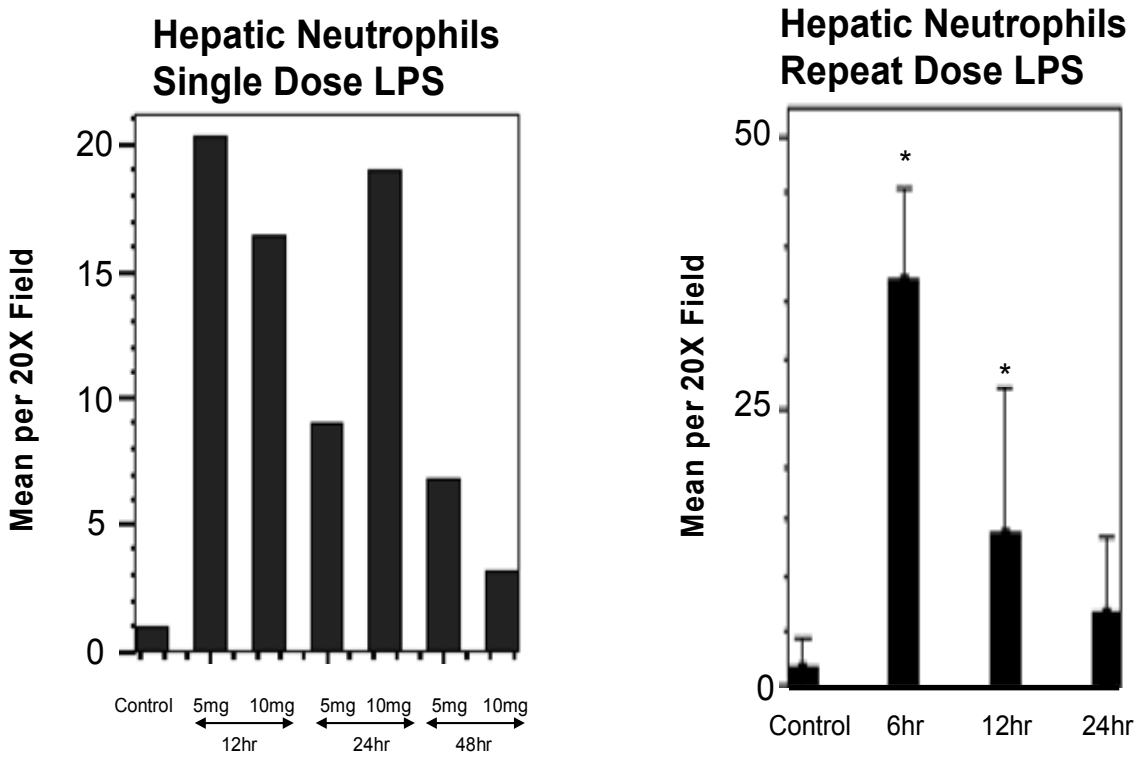

Figure 4- Graphs illustrating hepatic neutrophil counts using chloroacetate esterase staining of liver sections. Bars represent mean value of neutrophils counted in 10 high power fields (20X).

Flow cytometry studies

In single dose LPS study, analysis of neutrophil adhesion molecule expression revealed an up regulation of L-selectin (CD62-L) and Mac1 (CD11b) *in vitro*. However, in vivo, there was down regulation of L-selectin and up regulation of Mac1 (CD11b) in the LPS treated neutrophils as compared the controls (Fig 5). In the repeat dose study, upregulation of Mac 1 (CD11b) 6 hours post second LPS dose was the only significant finding. CD62L expression was not significantly different from control values (Fig. 5).

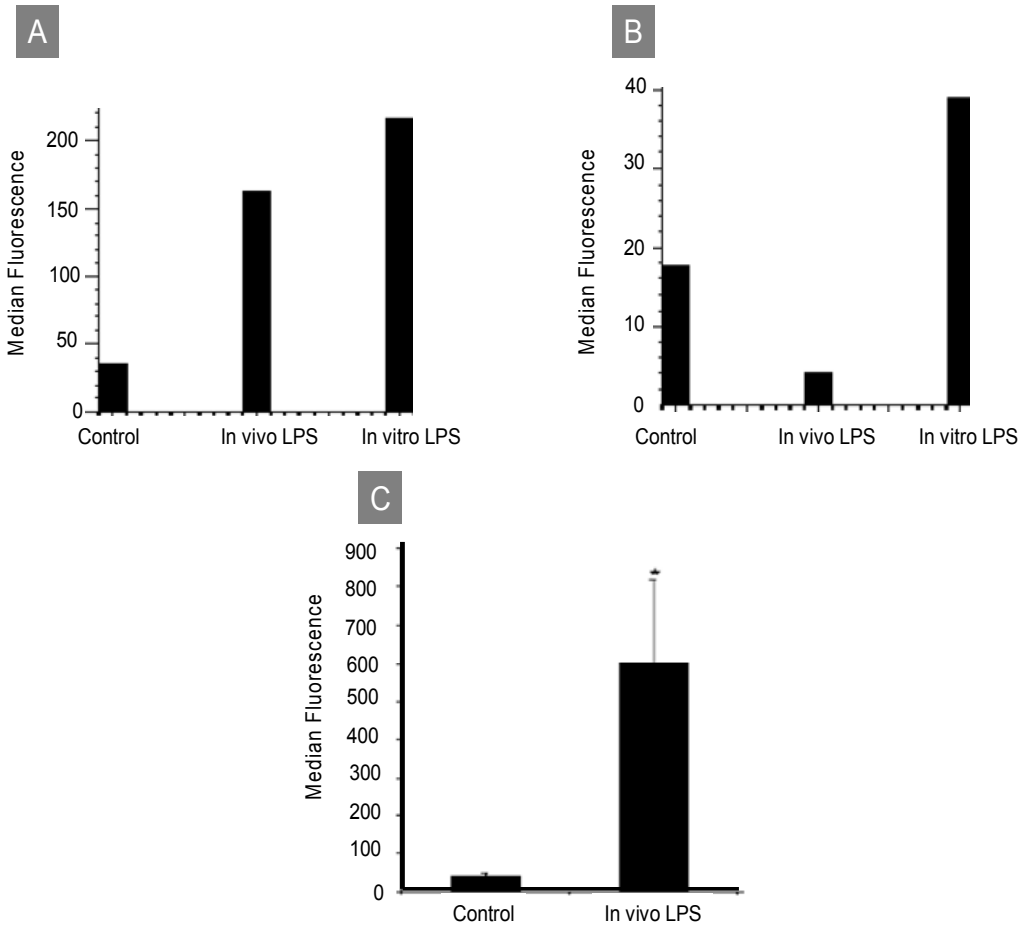

Figure 5- Flow cytometry results. Expression of 5A: Mac 1 and 5B: L-selectin: following in *vitro* and in vivo stimulation of neutrophils with single dose LPS at 10 mg/kg; 5C: Mac 1 following in vivo stimulation at 6 hr post repeat dose LPS administration.

DISCUSSION

We developed a reproducible endotoxemic necroinflammatory hepatitis rat model to study mechanisms of LPS mediated neutrophilic hepatitis and necrosis. A single dose of LPS elicited a profound peripheral neutrophilia with concurrent neutrophil activation (evidenced by upregulation of the neutrophil CD11b and downregulation of L-selectin) and increased neutrophil infiltration into hepatic parenchyma. In spite of adequate blood neutrophil activation and hepatic neutrophil infiltration, hepatic necrosis was minimal to none in the single dose study. Because our goal was LPS mediated hepatitis and necrosis, we decided to administer a second dose of LPS in an effort to induce histologically evident liver necrosis. Since, peripheral neutrophilia was most evident at the earliest time points when measured in a temporal fashion we selected 6 hr time point in the repeat dose study. Sample collection was terminated 24 hr after the second dose when neutrophilia began to wane in the repeat dose study.

The peripheral neutrophila noted both in the single and repeat dose study is not surprising given the well known inflammatory effect elicited by LPS. Mechanistically speaking, LPS binds to LPS binding protein (LBP) in the blood which interacts with the CD14 plasma membrane receptor on macrophages such as hepatic Kupffer cells (Bautista 2002; Muta and Takeshige, 2001). This complex then activates Toll-like receptor 4 (TLR-4) on the macrophage (Aderem and Ulevitch, 2000) resulting in the activation of NF-kB followed by transcriptional upregulation of pro-inflammatory cytokines (tumor necrosis factor alpha (TNF-a), IL-1β, IFN-g) and CXC chemokines (IL-8 (rodent equivalent cytokine induced neutrophil chemoattractant (CINC) (Cuzzocrea et al., 2000; Muta and Takeshige, 2001; Tsuji et al., 2005). TNFα and IL-1ß are known to stimulate leukocyte release from the bone marrow post maturation

storage pool (Picker, 1999) which is most likely responsible for the observed peripheral neutrophilia. The persistence of LPS-elicited inflammatory response is supported by the immature forms of neutrophils noted in this study. The lymphopenia noted may be attributed to the endogenous corticosteroid effect (Aster and Kumar, 1999).

The presence of nucleated red blood cells in the repeat dose LPS group most likely suggests their release from the marrow due to existing toxic environment in the bone marrow, although splenic release cannot be ruled out. As an adjunct to absolute neutrophil counts we assessed two CD11b and CD62L to determine peripheral neutrophil activation and possibly correlate neutrophil activation with hepatic neutrophil extravasation and infiltration in this model. Shedding of L-selectin and upregulation of CD11b is employed as a marker of peripheral neutrophil activation and usually precedes hepatic neutrophil extravasation (Bajt et al., 2002; Gujral et al., 2003)

Flow cytometry of neutrophils exposed to a single dose of LPS *in vivo* 12 hours prior to collection showed an up regulation of CD11b and a down regulation of CD62L which is consistent with neutrophil activation (Gujral et al., 2003). Neutrophil activation correlated with increased hepatic neutrophil extravasation and infiltration yet necrosis was not noted in the single dose study. An unexpected but interesting finding was noted with the L-selectin levels following exposure to LPS *in vitro*. There was upregulation of L-selectin *in vitro* compared to down regulation *in vivo*. This discrepancy of L-selectin expression *in vitro* versus *in vivo* (single dose) may be the result of duration of time between LPS exposure and flow cytometric analysis (30 minutes exposure *in vitro* versus 12 hours *in vivo)*. Similar findings are reported after intravenous administration of LPS to human volunteers where expression of L-selectin slightly increased from baseline values soon after dosing (90 minutes) before falling to

90% of baseline levels 6 hours post dosing (Jilma et al., 1999). Nonetheless, in the repeat dose study, CD11b expression was upregulated, hepatic neutrophil infiltration was increased and necrosis was also evident which was clearly absent in the single dose study. These results suggest that although neutrophil activation may predict hepatic neutrophil infiltration, hepatic necrosis may not be the sequale as was noted in this study.

Serum chemistry changes in both the single and repeat LPS study represents subtle but some interesting findings. BUN elevation noted is likely a secondary dehydration response to reduced water intake post LPS administration. The elevation in BUN is not likely a result of renal pathology based on the concurrent lack of clinically relevant persistent elevation in serum creatinine and no detectable renal histologic alterations. Reductions in serum albumin documented in LPS treated rats is likely secondary to GI loss associated with the LPS induced diarrhea. Elevations in GGT in repeat dose LPS treated rats at 6 hours is an interesting finding. Although GGT elevations can be identified in hepatic cholestasis (Latimer et al., 2003), cholestasis is less likely based on the absence of detectable cholestasis on histopathology. Based on the periportal mononuclear infiltration and bile duct proliferation noted, the elevation in GGT is possibly contributed from the proliferating biliary epithelium. In the absence of histologically evident cholestasis, the mild elevation in total bilirubin and ALP seen in the repeat dose study may be attributed to anorexia or endotoxemia (Crawford et al., 1998). Although AST elevations at the early time points in both single and repeat dose studies may suggest liver injury, the concurrent increase in CK suggests the elevation due to muscle injury. Muscle injury may have resulted from prolonged periods of LPS induced lethargy resulting in inactivity and subsequent pressure necrosis of muscles

bearing a recumbent animal's weight. Due to the fact that saline treated controls did not exhibit CK elevations, injections were not likely the cause of this increase.

The lack of consistent increase in ALT with marked hepatic necrosis in the repeat dose study is a surprising observation in this study. Two reasons can be attributed to this finding. First, it is known that pyridoxal 5' phosphate (P5P) an active metabolite of vitamin B6 is an essential cofactor for maximum serum transaminase activity (Stokol and Erb, 1998). Reduced vitamin B6 either due to decreased food intake or GI loss may be contributing to the lack of elevated serum transaminase values in this study. Due to limitations in the serum sample, we did not measure serum P5P or repeat the assay with the addition of P5P to confirm our findings. Another possibility is the half life of serum transaminases. Serum ALT half life is on the order of 40-60 hrs in several veterinary species (Lattimer et al., 2003) but no definitive studies have been carried out in the rat to accurately determine half life of this enzyme. The fact that there was demonstrated histologic evidence of hepatocellular damage after repeated doses of LPS but no associated ALT elevation may possibly indicate reduced half life of ALT in rats in the present study. Thus, to detect liver necrosis, caution should be exercised while interpreting the traditionally measured serum chemistry parameters in the LPS necrohepatitis model.

Clearly, a single dose of LPS was not sufficient to produce significant histopathological lesions in any of the tissues examined although there was mild mononuclear cell infiltrate in hepatic periportal regions. Similarly other investigators have shown single doses of LPS to be ineffective in producing significant histopathological lesions in the liver (Pirisi et al., 2000). Although not a feature on H&E stained tissue, chloracetate esterase staining of neutrophils revealed increased neutrophils near the periportal venule and within the sinusoids at all time points as

compared to controls in the single dose study. Based on this it can be surmised that the degree of neutrophil transmigration from the sinusoids was insufficient to incite significant hepatocyte apoptosis or additional neutrophil oxidative stress to cause hepatocellular damage. Due to the lack of hepatocellular necrosis in the single dose study we decided to develop the model further by administering a second dose of LPS using the higher dose level.

Histopathology in the repeat dose study revealed marked midzonal necrosis, apoptosis and hepatic regeneration. The mechanistic basis for such midzonal necrosis is recently shown to be due to alterations in hepatic microvasculature that is possibly iNOS dependent. What is apparent in the two dose LPS study is the neutrophil "priming" effect by the first LPS dose with significant histopathologic necrotic lesions following administration of the second LPS dose. It is reasonable to surmise that the initial exposure to physiologically stressful levels of LPS cause neutrophil activation and initiate production of ROS and inflammatory mediators and CXC chemokines which promote further hepatocellular oxidative stress (Gujral et al*.,* 2003). In the absence of adequate LPS clearance/recovery time, a second "hit" of LPS likely promotes a higher degree of oxidative stress, cytokines, chemokines and adhesion molecules such as ICAM1, VCAM1 resulting in marked neutrophil infiltration and necrosis. Others have also reported increased liver sensitivity and pathology to even innocuous levels of LPS after a "priming" event (Pirisi et al., 2000, Banerjee et al., 2006).

In conclusion, we have developed a reliable LPS-mediated neutrophilic necro-hepatitis model by administration of LPS in a repeat dose fashion which served as our test system as described in the next chapter. Midzonal hepatic necrosis, absolute peripheral neutrophilia, hepatic neutrophil infiltration and upregulation of the CD11b

neutrophil adhesion molecule were identified as the most significant and consistent markers of LPS mediated effects in this model. However, neither traditional serum biochemical parameters that detect liver injury nor neutrophil adhesion molecules reliably detected the observed liver injury and hepatic necrosis in this model.

CHAPTER III

CALPAIN AND LIPOPOLYSACCHARIDE MEDIATED HEPATITIS

MATERIALS AND METHODS

Animals and treatment

All animal procedures were approved by the Texas A&M University Laboratory Animal Care Committee (ULACC) and conformed to the standards set forth in the *Guide for the Care and Use of Laboratory Animals* (NRC 1996). All rats were barrier raised rodents obtained from a commercial vendor (Harlan, Indianapolis, IN) and were housed in an AAALAC accredited facility. Standard rodent chow (Teklad 8604, Madison WI) and reverse osmosis water were supplied *ad libitum*.

Description of the endotoxin neutrophilic hepatitis model: Two doses of LPS (10 mg/kg, i.v via the tail vein) were administered to male Sprague Dawley rats (250-270 g body weight) 24 hrs apart based on pilot studies performed in our laboratory. All sample collection times in this study occurred 6 hours after the second set of LPS injections. Samples from saline treated control animals were taken concurrent with treated animals. Inhibitors were administered one hour prior to each dose of LPS (Fig. 6).

Calpain inhibitor pilot study: Male Sprague Dawley rats (250-270 g body weight) were divided into three groups (N=5). One group served as a saline vehicle control. A second group received LPS (E Coli serotype 0111: B4, Sigma Chemical Co., St Louis, MO) at a dose of 10 mg/kg iv in saline twice at 24 hour intervals. The third group was administered calpain 1 inhibitor (Sigma Chemical Co., St. Louis, MO.) at 10

mg/kg i.p. followed one hour later by LPS in saline at a dose of 10 mg/kg via tail vein injection with a second dose of calpain inhibitor and LPS treatment 24 hours later. All rats were sacrificed via CO_2 inhalation 6 hours after the second LPS injection.

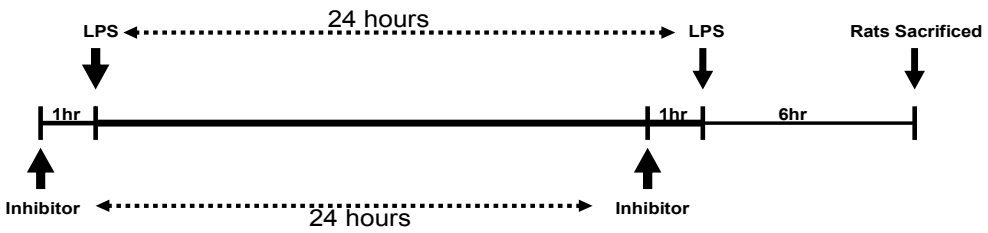

Figure 6- Experimental protocol to develop LPS mediated hepatitis model and strategy for calpain, iNOS and coagulation inhibition experiments. Male Sprague-Dawley rats (250-270 g) were injected two doses of LPS (10 mg/kg, i.v. via the tail vein 24 hrs apart to induce hepatitis. For inhibition experiments calpain 1 inhibitor (10 mg/kg, ip), iNOS inhibitor (aminoguanidine, 45 mg/kg, ip) and coagulation inhibitor (heparin, 1000 IV/kg, S.C) were administered in separate experiments one hour prior to first LPS injection. Appropriate controls were included for each treatment groups. Rats were sacrificed 6 hours after the second injection of LPS. Details of the experimental protocol have been described in Materials and methods.

Comparison of calpain inhibition with aminoguanidine (iNOS inhibitor) and heparin (coagulation inhibitor): Male Sprague Dawley rats (250-270 g body weight) were divided into five groups (N = 6). One group served as a saline vehicle control with the four other groups administered LPS in saline twice, 24 hours apart, via tail vein injection at a dose of 10 mg/kg. One hour prior to each dose of LPS, one group received calpain 1 inhibitor (Sigma, St Louis, MO) in DMSO at a dose of 10 mg/kg i.p. A second group received the relatively selective iNOS inhibitor aminoguanidine (Sigma, St Louis, MO) in saline at a dose of 45 mg/kg intraperitoneally one hour prior to each LPS dose. A third group received heparin sodium (America Pharmaceutical Partners, Schaumburg, IL) at a dose of 1000 units/kg subcutaneously one hour prior to each LPS dose. The rats were sacrificed via CO_2 inhalation 6 hours after the second injection of LPS.

Sample collection and processing

Whole blood was collected via the abdominal vena cava and immediately transferred to a lithium heparin, EDTA or sodium citrate tube (Monoject, Sherwood Medical, St Louis, MO). The samples in lithium heparin tubes were used for evaluation of neutrophil activation via flow cytometry. Samples in EDTA tubes were used for hematology evaluation and the samples in sodium citrate tubes were used to assess coagulation parameters. Representative liver sections were collected from different lobes of the liver and placed in liquid nitrogen and stored at -70° C for further analysis. The remaining liver along with sections of the lung and kidney were collected and placed in 10% neutral buffered formalin. The liver was processed and slides stained

with hematoxylin and eosin (H&E) for histopathological evaluation or stained with choloracetate esterase for evaluation of hepatic neutrophil infiltration. Kidney and lung sections were processed and stained with Mallory's phosphotungstic acid hematoxylin (PTAH) for evaluation of fibrin.

Flow cytometry studies

To investigate treatment effects on neutrophil activation, heparinized whole blood samples from three rats per group were used to assess the neutrophil adhesion molecules, L-selectin (CD62-L) and Mac-1 (CD11b/18). Blood was divided into 250 uL aliquots in separate glass tubes and was kept on ice unless otherwise stated. Duplicate tubes of blood were designated to antibody stained or unstained groups. Prior to staining with the appropriate conjugated antibody, 10µl of reagent grade mouse IgG (Sigma, St Louis, MO) mixed at a concentration of 1mg/ml was added to all but the unstained samples to reduce nonspecific binding of the conjugated antibodies. In the next step, neutrophils in designated tubes were labeled with fluorescein-conjugated HIS 48 (BD Biosciences, San Jose, CA), Mac 1 integrins were labeled with R-phycoerythrin conjugated CD11b and L-selectins were labeled using R-phycoerythrin CD62L (Serotec, Raleigh, NC). Following incubation with antibodies (20 min), the samples were treated with RBC lysis buffer for 10 min. The tubes were then centrifuged at 1000 rpm for 10 min at 3° C and resuspended several times with the lysis buffer until erythrocytes were removed. After lysing steps, the cells were washed with 250 µL of PBS and centrifuged at 1000 rpm for 10 minutes. Cells were re-suspended in 300 µL of PBS for analysis.

The cells were analyzed using a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometry, equipped with a 15mW air-cooled argon laser. Green fluorescence from fluorescein was collected through a 530/30-nm bandpass filter and orange fluorescence from R-phycoerythrin through a 585/42-nm bandpass filter. List mode data was acquired on a minimum of 10,000 events defined by light scatter gates to include the neutrophil population. Data analysis including spectral compensation was performed using FlowJo (Treestar Inc., Ashland, OR). A neutrophil gate was established based on side scatter and positive HIS48 staining, and the median fluorescence channel was determined for CD11b staining.

Hematology evaluation (calpain inhibitor pilot study only)

Complete blood counts for all animals in the calpain inhibitor pilot study were performed on a Cell-Dyn 3700 (Abbott Diagnostics, Abbott Park, IL) hematology analyzer. Wright-Geimsa stained blood smears were also evaluated microscopically to determine hematologic abnormalities.

Histopathology/hepatic neutrophil infiltration

Cross sections of liver collected from the left lateral and median lobes were fixed in 10% neutral buffered formalin, processed by standard histological methods, and embedded in paraffin. 5 micron thick sections were stained with hematoxylin and eosin and evaluated for oncotic necrosis and apoptosis. Two liver sections per rat were evaluated with the reviewer blinded to the treatment groups. Lesions were scored using a semiquantitative scale as follows:

Lesion score (mean of two sections):

0- no evidence of lesions

1- periportal infiltrate only

2- periportal infiltrate with ≤ 2 foci of necrosis

3- periportal infiltrate with 3-5 foci of necrosis

4- periportal infiltrate with ≥ 6 foci of necrosis

Distribution score

P- periportal predominate

M- midzonal predominate

C- centrilobular predominate

The H&E staining was also employed to identify the neutrophils, based on the segmented morphology of the nucleus. To quantitate the degree of neutrophilic inflammation, unstained paraffin embedded slides were deparaffinized then stained with a chloroactetate esterase kit (Sigma, St Louis, MO) according to the manufacturer's recommendations. The number of neutrophils in 10 randomly chosen high power fields (20X) were recorded and the results expressed as the mean number per 20X field as described previously (Banerjee et al., 2006). To evaluate the effect of circulating microthrombi in this model, cross sections of the left lung lobe and longitudinal cross sections of the left kidney were fixed in neutral buffered formalin, processed by standard histological methods and embedded in paraffin. 5 micron sections were stained with phosphotungstic acid hematoxylin solution and evaluated for the presence of fibrin. The number of fibrin foci in 10 randomly chosen low power fields (10X)

were recorded and the results expressed as the mean number per 10X field for kidney and lung sections.

Western blot analysis for iNOS and α-fodrin

α-Fodrin is a substrate for calpain and was used to confirm calpain inhibiton. Liver lysates were prepared by grinding 300 mg frozen liver suspended in liquid nitrogen with a mortar and pestle in a lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 2 mM Na vanadate, 0.2 ml PMSF (200 mM PMSF in isopropanol), 1 mM Hepes (pH 7.6), 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Sample protein concentrations were determined using the method of Bradford (Bradford, 1976). Polyacrylamide gel (8%) electrophoresis was employed for α-fodrin analysis and 10% gel was employed to assess iNOS expression. After transferring the proteins onto nitrocellulose membranes, the membranes were blocked for 3 hours with 6% milk made with 1X TBS/Tween 20. After the blocking step, the membranes were incubated with primary rabbit polyclonal antibody for α-fodrin at 1:2500 dilution (Abcam, Cambridge, MA) and rabbit polyclonal antibody for iNOS at 1:2000 dilution (Cayman chemical company, Ann Arbor, MI). Subsequently, the nitrocellulose membrane was incubated with secondary goat anti rabbit antibody (Kirkgaard and Perry Labs, Gaithersburg, MD) in 2% milk made with 1X TBS/Tween 20 at a 1:4000 dilution. The blots were visualized using enhanced chemiluminescence kit (Pierce, Rockford, IL).

Western blot analysis for cytochrome P450 (CYP) 2E1

Preparation of microsomes: Frozen liver tissue was homogenized (1:5 w/v) using an Ultra-Turrax 25 homogenizer (IKA, Wilmington, NC) in ice-cold Tris-acetate buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 10,000g for 30 min at 4°C. The supernatant was decanted and centrifuged at 100,000g for 60 min at 4°C. Microsomal pellets were resuspended, manually homogenized and again centrifuged at 100,000g for 60 min at 4°C. Microsomal pellets were again recovered, suspended in assay buffer (pH 7.4), manually homogenized then quick frozen and stored at -70°C for later use. Sample protein concentrations were determined using the method of Bradford (Bradford, 1976).

Western blot analysis for CYP2E1: Microsomal protein from each rat was separated by SDS polyacrylamide gel (10%) electrophoresis and transferred to nitrocellulose membrane. After the blocking step, the membrane was incubated with a rabbit anti-human polyclonal anti-CYP2E1 primary antibody, 1:1000 dilution (generously gifted by Dr. Jerry Lasker, Hackensack University Medical Center, New Jersey) for 2 hours and further probed with HRP-linked goat anti-rabbit secondary antibody (Kirkgaard and Perry Labs, Gaithersburg, MD; 1;10,000 dilution) for 1 hr. Primary Rabbit polyclonal antibody for cytochrome 4A was supplied by Abcam (Cambridge, MA). The blots were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

Coagulation parameters

To determine treatment effects and confirm efficacy of heparin administration, the values for prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined from citrated blood samples collected from 3 rats per selected group (control, LPS only and LPS plus heparin) using the Beckman Coulter ACL 9000 coagulation analyzer (Fullerton, CA).

Statistics

To identify significant differences among groups, analysis of variance (ANOVA) with a *post hoc* Tukey's HSD was performed using a computer software program (SPSS version 12.0, Claritas Inc.). Results were considered significant at the 0.05 confidence level.

RESULTS

Clinical signs

No mortalities were recorded. Rats administered LPS alone appeared more lethargic than rats in the other groups and developed diarrhea within 1-2 hours after LPS administration. Rats given LPS plus calpain1 inhibitor appeared more lethargic than untreated controls but were brighter subjectively than LPS only rats. Rats administered LPS plus calpain 1 inhibitor developed diarrhea but the time to onset was slower (4-6 hours) as compared to LPS only rats. Rats given LPS plus aminoguanidine or LPS plus heparin appeared similar to rats administered LPS plus calpain 1 inhibitor. Due to the short duration of these studies (a total of 30 hours from initial LPS injection),

post treatment body weights, food consumption and water consumption were not measured.

Flow cytometry

The neutrophil adhesion molecule CD11b was significantly upregulated in the LPS, and LPS plus calpain 1 inhibitor as compared to untreated controls confirming neutrophil activation in both the calpain 1 inhibitor and the comparison study. CD 11b was also significantly upregulated in the LPS plus aminoguanidine and LPS plus heparin groups as compared to untreated controls in the comparison study (Fig. 7). However, when comparison of CD11b levels was made between aminoguanidine and heparin with calpain inhibitor a mild decrease in neutrophil activation was noted.

Hematology (calpain inhibitor pilot study only)

Absolute neutrophil counts and neutrophil to lymphocyte ratios were elevated in LPS and LPS plus calpain 1 inhibitor groups as compared to untreated controls. There was no significant difference in absolute neutrophil numbers or neutrophil to lymphocyte ratio between LPS and LPS plus calpain 1 inhibitor groups (Table 3).

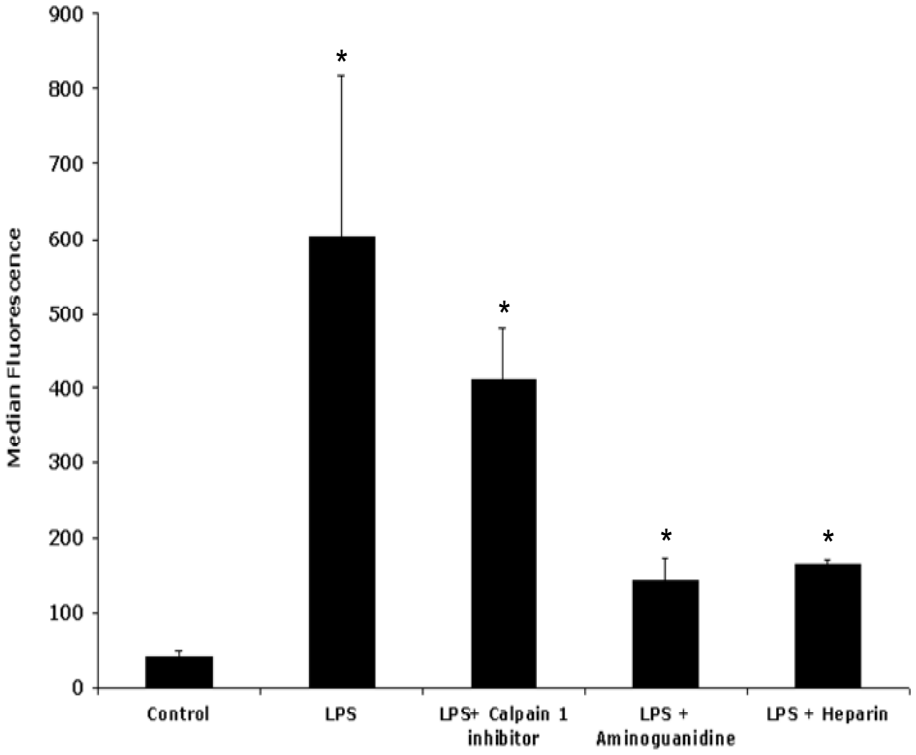

Figure 7- Flow cytometric analysis of CD11b expression. Neutrophils from different treatment groups were labeled with fluorescein-conjugated HIS48 and CD11b antibodies as described in the Materials and methods section. The scale represents median fluorescence. *Values significantly different from the controls. Data are expressed as mean±SE, $p \geq 0.05$.

Table 3. Absolute neutrophil count, neutrophil to lymphocyte ratio (N:L) , liver pathology score, and hepatic neutrophil scoring in the calpain inhibitor (CI) study evaluated in rat endotoxin (LPS) hepatitis model.

Parameter	Control	LPS	LPS + CI
Neutrophil (absolute number/µl)	1796 ± 882	4156 ± 878[a]	4710 ± 1377[a]
Neutrophil to lymphocyte ratio	.2 ± 0.2	1.8 ± 0.6[a]	1.9 ± .7[a]
Liver Pathology Score/ Lesion Distribution	0 ± 0/na	3.6 ± .5[c]/M	1.2 ± .4[a,b]/P
Liver Neutrophil Count (per 20X field)	1.2 ± .4	16.4 ± 2.3[a]	7.0 ± 1.0[a,b]

Absolute neutrophil count was performed on an automated Cell-Dyne counter and evaluated manually on blood smears. Hepatic neutrophils were counted as the number of neutrophils per five high power fields as described in the materials and methods section. Values are mean ± SD.
n = 5
na = not applicable
P = periportal
M = midzonal
[a] = significant as compared to control group
[b] = significant as compared to LPS only group
[c] = significant as compared to control and inhibitor group

Histopathology

Neutrophilic midzonal hepatocyte oncotic necrosis with apoptosis and periportal mononuclear inflammatory cell infiltrates were a major feature in the LPS only group as demonstrated by semiquantitative scoring (Tables 3 and 4) compared to the controls (Figs. 8 A-C). Significant decreases in necrosis and neutrophilic inflammation was identified in all of the inhibitor groups as demonstrated by semiquantitative scoring (Tables 3 and 4), although rare necrotic foci was identified in the iNOS inhibitor group (Figs. 8 D-F). There was reduction in fibrin deposition in the PTAH stained lung and kidney of only the heparin plus LPS group as compared to the LPS only group (Table 4). No significant difference in fibrin deposition was found in the lung or kidney among

the calpain 1 inhibitor or aminoguanidine groups as compared to the LPS only group (Table 4).

Neutrophil scoring

Calpain 1 inhibitor study: There were significant differences in liver neutrophil counts among the three groups (Table 3). Control rats had significantly lower neutrophil counts as compared to all the other groups (1.2 per 20X field). LPS only rats had the highest neutrophil counts with a mean of 16.4 neutrophils per 20X field. However, administration of calpain 1 inhibitor significantly decreased the hepatic neutrophils counts (7.0 per 20X field).

Comparison study: Control rats had significantly lower neutrophil counts as compared to all other groups in the comparison study (Table 4). LPS only rats had the highest hepatic neutrophil counts with a mean of 16.5 neutrophils per 20X field which was significantly higher than any other group. The LPS plus calpain 1 inhibitor group had a mean hepatic neutrophil count of 3.6 neutrophils per 20X field which was significantly reduced as compared to LPS alone group. Hepatic neutrophil counts for the heparin and aminoguanidine groups were significantly lower than the LPS only group but not different from the LPS plus calpain 1 inhibitor group.

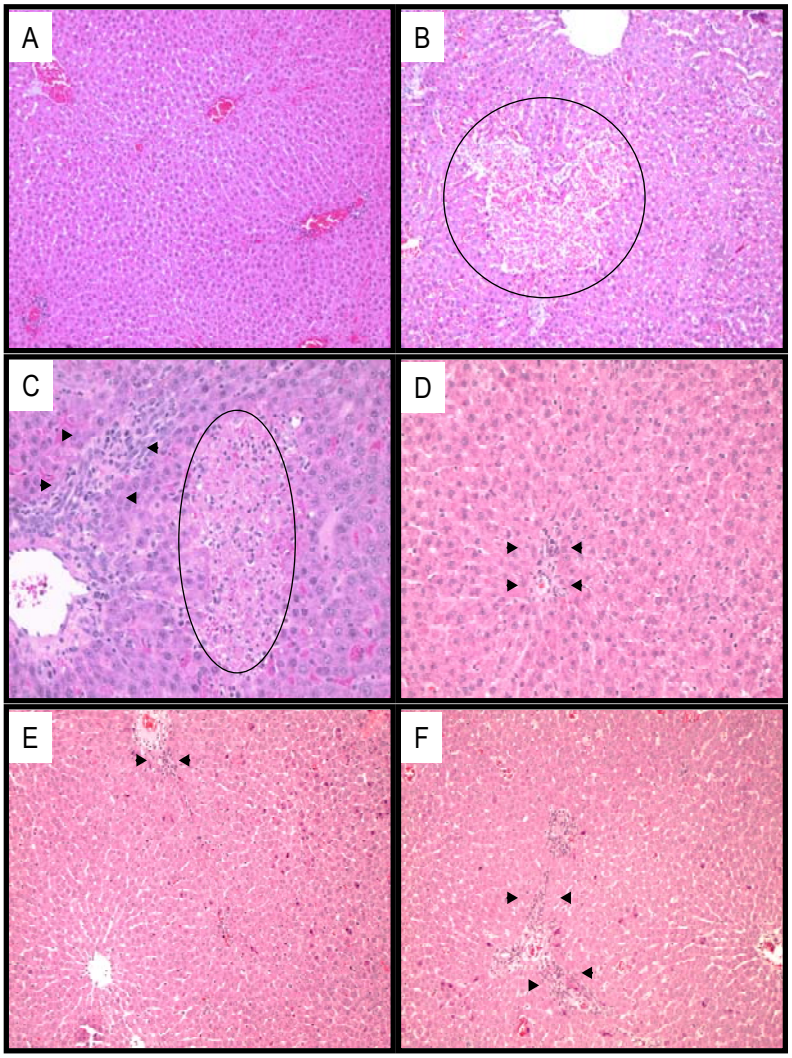

Figure 8- Representative photomicrographs of H&E stained liver sections. A: control 10X magnification; B: repeat dose LPS 10X magnification; C: repeat dose LPS 40X magnification; D: LPS plus calpain inhbitor 20X magnification; E: LPS plus aminoguanidine 20X magnification and F: LPS plus heparin 20X magnification. Rats were sacrificed six hours following second dose LPS injection in all groups. Circled areas indicate midzonal necrosis and arrows indicate periporal infiltrates.

Table 4- Summary results of semiquantitative liver pathology score, lung and kidney thrombi count, hepatic neutrophil count and coagulation times (PT and PTT) in the calpain inhibitor (CI), iNOS inhibitor (aminoguanidine, AG) and coagulation inhibitor (heparin, Hep) groups in the endotoxin (LPS) mediated hepatitis model.

Parameter	**Control**	**LPS**	**LPS + CI**	**LPS + AG**	**LPS + Hep**
Liver Pathology Score/ Lesion Distribution	0 ± 0/na	3.7 ± .5[c]/M	0.7 ± .5/P	1.3 ± .4[a]/P	0.8 ±.4[a]/P
Liver Neutrophil Count (per 20X field)	0.9 ± 1.0	16.5 ± 2.1[a]	3.6 ± 1.5[a,b]	6.5 ± 0.5[a,b]	4.8 ± 0.4[a,b]
Lung Thrombi Count (per 10X field)	0.1 ± .2	2.8 ± .6[a]	3.0 ± .5[a]	2.9 ± .7[a]	.9 ± .4
Kidney Thrombi Count (per 10X field)	.1 ± .2	6.1 ± 1.1[a]	5.4 ± 1.2[a]	6.5 ± 1.2[a]	0.3 ± 0.1
PT (sec)	15.4 ± 0.8	14.7 ± .6	N/A	N/A	20.1 ± 1.2[a,b]
APTT (sec)	11.0 ± 3.7	18.8 ± 3.3[a]	N/A	N/A	>248[a,b]

Lung and kidney thrombi count and hepatic neutrophil count were evaluated in ten high power fields as described in the materials and methods section. Coagulation analysis was evaluated by measuring prothrombin time (PT in secs) and activated partial thromboplastin time (APTT in secs) of the extrinsic and intrinsic coagulation pathways respectively. Values are mean ± SD, n = 6.

na = not applicable

P = periportal

M = midzonal

[a] = significant as compared to control group

[b] = significant as compared to LPS only group

[c] = significant as compared to control and inhibitor groups

Coagulation parameters

The mean PT and APTT values were employed to detect inhibition of major pathways of coagulation. PT and APTT values for the LPS plus heparin group were elevated as compared to the control and LPS only groups confirming inhibition of coagulation by heparin (Table 4). APTT values were elevated by LPS alone compared to the controls with further prolongation of APTT values by heparin.

Western blotting

α-fodrin: To evaluate the degradative effect of released calpain on its substrate α-fodrin, a Western blotting technique was used. Calpain is known to degrade the 240-kDa α-subunit of fodrin at several sites to produce breakdown products including a 150 kDa, 120 kDa and 35 kDa product (Abcam α-fodrin antibody data sheet). In normal control rat livers almost all α-fodrin was in intact form. The native form of α-fodrin was significantly higher in the LPS plus calpain 1 inhibitor groups as compared to the LPS only group confirming calpain inhibition (Fig.9). Comparing relative densitometry results, there was no significant difference in α-fodrin degradation between LPS only and aminoguanidine and heparin groups (p- 0.96) indicating that these two inhibitors do not inhibit calpain in our model.

Western blotting for iNOS: iNOS expression was significantly lower in the calpain 1 inhibitor group as compared to the LPS only group and the levels were comparable to the untreated control group (Fig. 10). iNOS expression in the aminoguanidine and heparin groups was also significantly lower than the LPS only group and was comparable to the calpain 1 inhibitor group (Fig. 10). To complement

measurement of iNOS expression via immunoblotting, iNOS activity can also be measured by in vitro assay of L-arginine conversion to L-citrulline. As reported by many investigators including Jinnou-Oue and colleagues (2003), immunoblotting results are generally strongly correlated to iNOS activity. Because of this correlation we chose to employ western blotting techniques as indicators of iNOS effects in this model.

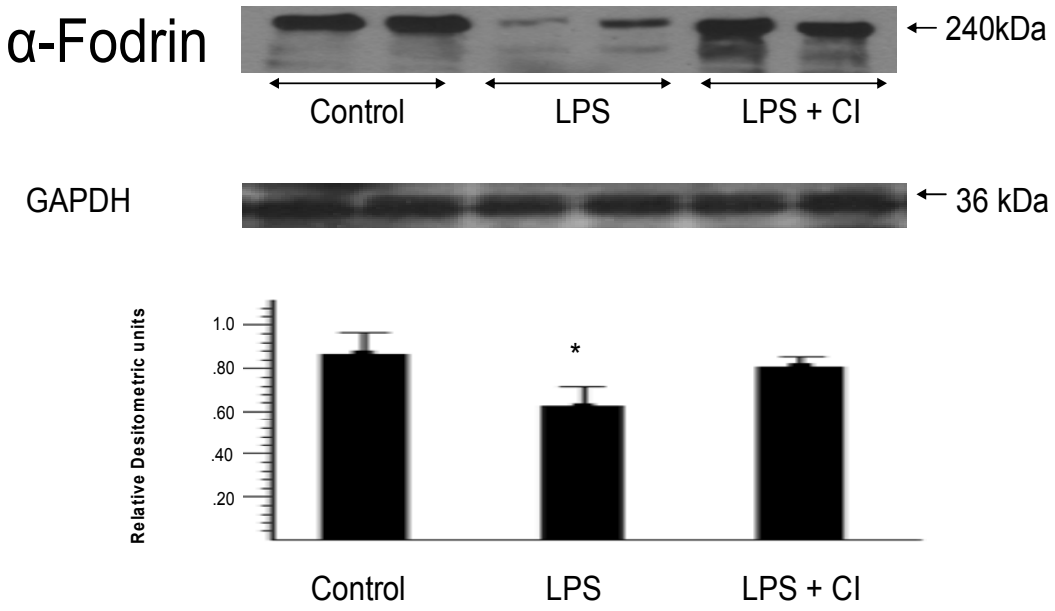

Figure 9- Representative western blotting showing α- fodrin degradation in LPS mediated hepatitis and the calpain inhibitor group. GAPDH was employed as internal loading control to ensure equal loading of proteins. * Values significantly different from the controls and inhibitor treatment groups. # values significantly different from controls at $p \leq 0.05$.

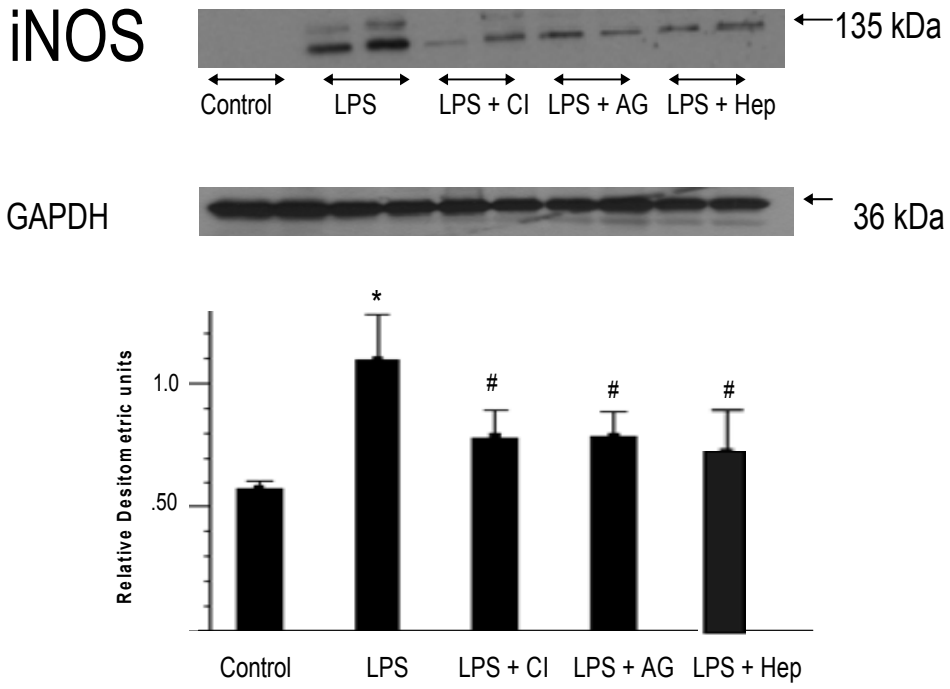

Figure 10- Representative western blotting showing iNOS expression in LPS mediated hepatitis and various inhibitor groups. GAPDH was employed as internal loading control to ensure equal loading of proteins. * Values significantly different from the controls and inhibitor treatment groups. # values significantly different from controls at $p \leq 0.05$.

Western blotting for CYP2E1: CYP2E1 expression was down-regulated in the LPS only group as compared to calpain 1 inhibitor and untreated controls. There was no significant difference between CYP2E1 expression among the aminoguanidine and heparin groups as compared to calpain 1 inhibitor and untreated controls (Fig. 11). To complement measurement of CYP expression via immunoblotting, CYP enzyme activity can also be measured by chlorazone 6-hydroxylase and p-nitrophenol hydroxylase assay. Results obtained in our lab and as reported by others indicate CYP activity is strongly correlated to CYP expression when employing an appropriate primary antibody that minimizes cross reaction (Amato et al., 1998; Turner et al., 1988). Because of this strong correlation, we chose to use western blotting techniques as an indicator of cytochrome effects in this model.

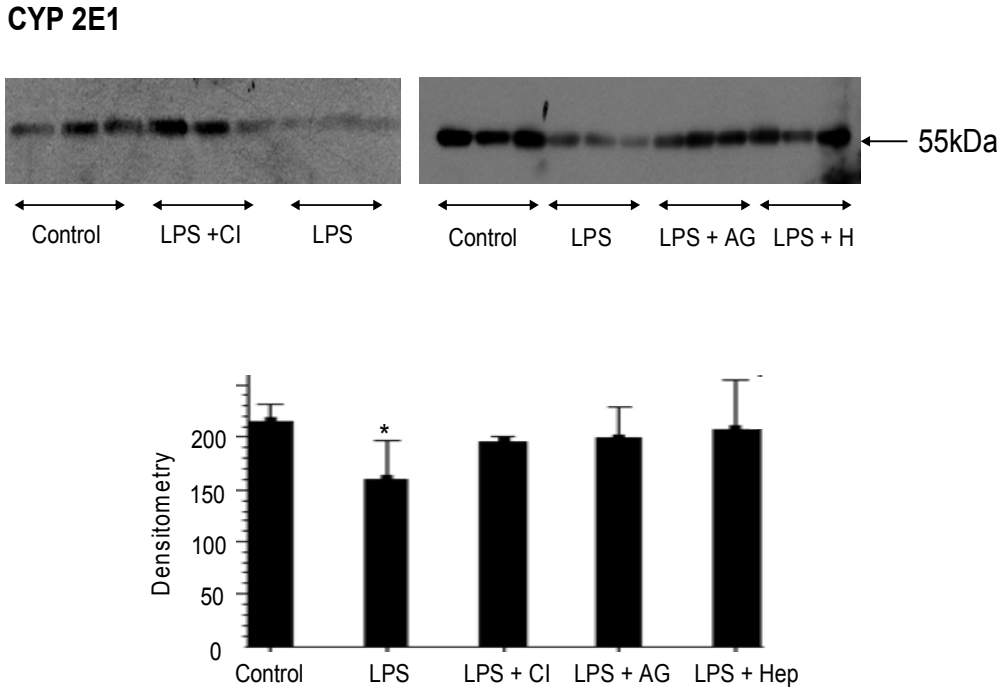

Figure 11- Representative western blotting showing CYP2E1 proteins in LPS mediated hepatitis and various inhibitor groups. * Values significantly different from the controls and inhibitor treatment groups.

CHAPTER IV

DISCUSSION AND CONCLUSION

DISCUSSION

The above experiments investigated the protective role of calpain in a LPS mediated neutrophilic hepatitis model in the rat. The interesting observation noted in this model was the exclusive midzonal distribution of necrosis. Although, midzonal lesions have been described by others during states of endotoxemia, these lesions were generally accompanied by periportal necrosis (Katsushige et al., 2005; Pirisi et al., 2000). However, the necrosis in the present study originated primarily in the midzonal region following increased neutrophil infiltration as noted during the model development phase performed in our lab (Rose et al., manuscript submitted). Primary midzonal hepatic lesions are uncommon but have been associated with viral yellow fever and shock associated hypotension (de la Monte et al., 1984). Yee and coworkers have also described midzonal necrosis following large doses of LPS in rats (Yee et al., 2003). It is reported that excessive production of nitric oxide (NO) as a consequence of macrophage release of iNOS contributes to the hypotension which is a feature of endotoxemia (Imai et al., 1994; Landry and Oliver, 2001). NO is known to play a dual role in the liver during inflammatory states with low levels of NO, produced consequent to activation of constitutive eNOS, shown to be protective and higher levels of NO, as a result of iNOS release, demonstrated to be cytotoxic (Rocky and Shah, 2004). Based on these reports, the midzonal distribution of hepatic lesions seen in this study is proposed

to be the result of a combination of nitric oxide mediated hypotension by iNOS and subsequent oxidative stress secondary to circulatory impairment, and parenchymal migration and proteolytic enzyme release by neutrophils resulting in hepatocyte injury (Jaeschke and Smith, 1997, Lawson et al., 2000).

The occurrence of midzonal necrosis combined with the fact that calpain inhibition decreased liver injury and neutrophil infiltration led us to investigate the relationship between calpain inhibition and iNOS. One explanation for decreased hepatic neutrophil infiltration in the calpain 1 inhibitor group may simply be the result of decreased neutrophil numbers or decreased transmigration consequent to decreased neutrophil activation in the vasculature. However, hematology and flow cytometry results revealed that neutrophilia and activation of neutrophils as measured by CD11b was not different between the LPS and LPS plus calpain 1 inhibitor groups. Based on these results, it can be interpreted that the afforded protection by calpain inhibition is not solely due to decrease in peripheral neutrophil count or decreased neutrophil activation. Although the neutrophil activation was decreased in the aminoguanidine and heparin group compared to the calpain 1 inhibitor group, there was enough neutrophil/leukocyte activation to account for hepatic neutrophil infiltration as evidenced by hepatic neutrophil counts and periportal infiltrates on histology. In light of the presence of adequate neutrophil activation and periportal neutrophil infiltrate in all treated groups, mechanisms other than neutrophil and vascular endothelial cell activation seem to be playing a role in the observed protection.

Noteworthy in our studies is the reduced expression of hepatic iNOS in the calpain 1 inhibitor group which coincided with reduced hepatic pathology. Inhibition of calpain is reported to block production of iNOS through an NF-κB dependent mechanism (Constantin et al., 2004; Kabori, 2006; Lancaster et al., 2001). Asakura et al., (2005) also reported attenuation of liver and kidney pathology after administration of the iNOS inhibitor L-NIL in an LPS mediated rat model of DIC. Similarly Guler et al., (2004) have demonstrated protection from liver damage, as measured by histopathology, in iNOS deficient mice infected with mycobacteria after challenge with LPS.

Similar to inhibition of iNOS by calpain inhibition, iNOS inhibition by aminoguanidine also resulted in significantly reduced neutrophils and liver pathology. These results are consistent with reports of Suliburk et al., (2005) who noted improvement in LPS mediated liver injury in rats, as measured by ALT and AST levels, after administration of aminoguanidine 1 hour prior to administration of LPS. Although we do not have cause and effect evidence (for example using iNOS knock out mice), the association of decreased iNOS with decreased neutrophil infiltration and necrosis suggests that iNOS inhibition is the possible reason for the observed protection of calpain inhibition noted in our model.

Interestingly, heparin administration prior to LPS also reduced microscopic liver pathology and iNOS expression comparable to calpain 1 inhibition and aminoguanidine. Reduced microthrombi deposition was demonstrated in the lung and kidney of heparin administered rats in this study. Moulin et al., (1996) reported that heparin

administration prior to LPS attenuates hepatocellular injury in rats as measured by ALT and histopathology in a thrombin dependent manner. Also, in a model of liver transplantation, Vajdova et al., (2000) reported co-administration of heparin minimized endotoxin mediated preservation-reperfusion injury by prolonging coagulation and limiting microthrombi deposition. Microthrombi inhibition and preservation of microcirculation by heparin is reported to result in reduced hepatocyte oxidative stress which subsequently decreases iNOS expression in LPS mediated injury (Guler et al., 2004; Suliburk et al., 2005). Based on our findings and reports in the literature, it appears that the protective effect of heparin noted in the present study may partly be the result of decreased microthrombi deposition resulting in improved microperfusion. We suggest that the reduction in hepatocyte damage resulted in reduced hepatocyte release of iNOS.

Although the precise mechanism behind decreased iNOS and protection noted in the current study is not investigated, the following mechanism may have some underlying mechanistic influence. Attenuating NO dependent hypotension via iNOS inhibition in the present study is likely resulting in preserved liver microperfusion and reduced oxidative stress secondary to reduced tissue oxygenation (Fig. 12). Indeed, using intravital microscopy, Horie and co-workers (2000) reported reduced sinusoidal blood flow and increased endothelial-leukocyte adhesion predominately in the midzonal region after LPS administration in rats.

Since CYP2E1 is known to be altered with endotoxin injection, we measured CYP2E1 protein to investigate if the observed protection mediated by calpain 1

inhibition is because of CYP2E1 inhibition. Based on our results, it appears that it is less likely involved. Finally emerging evidence suggests NO plays a role in epoxygenase dependent vascular responses via inhibition of CYP P450 enzymes (Udosen et al., 2003). Inhibition of NO production subsequent to LPS exposure might preserve epoxygenase dependent vasodilation that plays a role in maintaining microcirculation (Fleming, 2004). We employed CYP2E1 as a model for LPS mediated CYP downregulation as this cytochrome's response to LPS has been well documented (Morgan, 2001. Renton et al., 2000). Administration of calpain 1 inhibitor preserved CYP2E1 expression which may suggest a role of iNOS inhibition and cytochrome expression in this model. There is debate in the literature concerning NO downregulation of cytochromes and further mechanistic work is needed (Sewer et al., 1997; Sewer and Morgan, 1998; Vappugalla and Mehvar, 2004).

CONCLUSION

In summary, inhibition of calpain attenuated LPS induced midzonal hepatic necrosis in our two dose model of endotoxemia. Lowered hepatic neutrophil migration along with attenuated midzonal necrosis consequent to calpain inhibition accompanied reduced iNOS expression as compared to LPS only administered rats. Based on these findings and comparisons of the effect of calpain inhibition with those of aminoguanidine and heparin, which also decreased hepatic iNOS and neutrophil infiltration, we suggest that calpain inhibition reduces liver pathology via maintenance of hepatic microcirculation most likely in an iNOS dependent fashion.

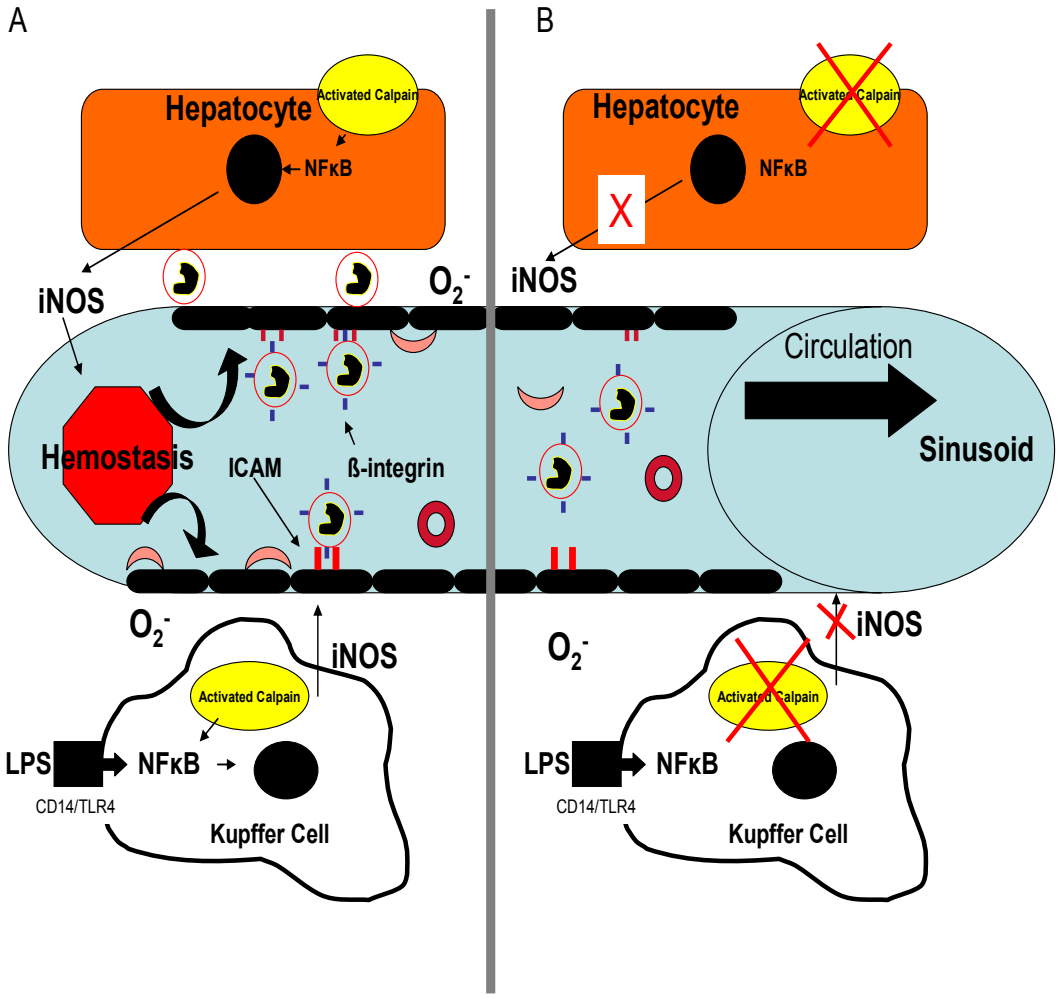

Figure 12- The hypothesized role of iNOS in calpain inhibition mediated protection in a repeated dose endotoxin model of hepatitis. Figure 7a demonstrates upregulated calpain mediated iNOS expression post LPS exposure. Due to reduced circulation, activated neutrophils and microthrombi adhere to the sinusoidal endothelial wall promoting neutrophil transmigration and release of proteolytic enzymes. Figure 7b demonstrates calpain inhibition with resulting downregulation of iNOS. Improved microcirculation afforded by iNOS inhibition hinders activated neutrophil and microthrombi adhesion to the sinusoidal endothelium reducing neutrophil transmigration, proteolytic enzyme release and consequently decreased liver injury.

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VITA

Name: Robert Edward Rose

Address: Building 972 Agronomy Road, TAMU, College Station, TX 77843-4473

Email : rrose@cvm.tamu.edu

Education: B.S., Veterinary Science, Texas A&M University, 1983
D.V.M., Texas A&M University, 1985
M.S., Laboratory Animal Science, Texas A&M University, 2006

Publications:

Rose, R. E., Smith, R., Banerjee, A., Weeks, B., Ramaiah, S. K., Effect of Single and Repeat Dose LPS Administration on Hematology, Serum Biochemistry and Neutrophil Adhesion Molecules in Sprague-Dawley Rats. *American College of Toxicology, Annual Meeting*, November, 2005.

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