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Neutrophil activation during acetaminophen hepatotoxicity and repair in mice and humans

C. David Williams [a], Mary Lynn Bajt [a], Matthew R. Sharpe [b], Mitchell R. McGill [a], Anwar Farhood [c], Hartmut Jaeschke [a,*]

[a] Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, Kansas City, KS, USA
[b] Department of Internal Medicine, University of Kansas Hospital, Kansas City, KS, USA
[c] Department of Pathology, St. David's North Austin Medical Center, Austin, TX 78756, USA

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ABSTRACT

Following acetaminophen (APAP) overdose there is an inflammatory response triggered by the release of cellular contents from necrotic hepatocytes into the systemic circulation which initiates the recruitment of neutrophils into the liver. It has been demonstrated that neutrophils do not contribute to APAP-induced liver injury, but their role and the role of NADPH oxidase in injury resolution are controversial. C57BL/6 mice were subjected to APAP overdose and neutrophil activation status was determined during liver injury and liver regeneration. Additionally, human APAP overdose patients (ALT: >800 U/L) had serial blood draws during the injury and recovery phases for the determination of neutrophil activation. Neutrophils in the peripheral blood of mice showed an increasing activation status (CD11b expression and ROS priming) during and after the peak of injury but returned to baseline levels prior to complete injury resolution. Hepatic sequestered neutrophils showed an increased and sustained CD11b expression, but no ROS priming was observed. Confirming that NADPH oxidase is not critical to injury resolution, $gp91^{phox}-/-$ mice following APAP overdose displayed no alteration in injury resolution. Peripheral blood from APAP overdose patients also showed increased neutrophil activation status after the peak of liver injury and remained elevated until discharge from the hospital. In mice and humans, markers of activation, like ROS priming, were increased and sustained well after active liver injury had subsided. The similar findings between surviving patients and mice indicate that neutrophil activation may be a critical event for host defense or injury resolution following APAP overdose, but not a contributing factor to APAP-induced injury.

Introduction

Acetaminophen (APAP) overdose can result in severe liver injury, acute liver failure and potentially, death (Larson, 2007; McGill et al., 2012). The hepatotoxicity of APAP begins with metabolic conversion of APAP to a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI). The reactive metabolite causes glutathione (GSH) depletion and binds to cellular proteins, which is the initiating event in toxicity (McGill and Jaeschke, 2013). Downstream of protein binding are mitochondrial dysfunction, increased oxidant stress, mitochondrial permeability transition pore (MPT) opening, DNA damage by mitochondrial endonucleases, and hepatocellular necrosis (Jaeschke et al., 2012a). The massive cellular necrosis results in the release of damage associated molecular patterns (DAMPs) into systemic circulation. These DAMPs (including HMGB-1, nuclear DNA, and numerous others) then trigger a sterile inflammatory response resulting in increased cytokine/chemokine formation and recruitment of innate immune cells into the liver (Jaeschke et al., 2012b). In the mouse model, neutrophils are recruited to the site of injury first, followed by monocytes/macrophages (Dambach et al., 2002; Holt et al., 2008; Lawson et al., 2000). However, it is controversial whether or not neutrophils actually contribute to the injury (Jaeschke, 2008). A few studies that have used neutropenia-inducing antibodies have provided evidence for the involvement of neutrophils in the late-stage injury process (Ishida et al., 2006; Liu et al., 2006; Marques et al., 2012). However, this particular approach has been repeatedly criticized for potential off target effects (Jaeschke and Liu, 2007; Jaeschke et al., 2013). On the other hand, a substantial number of other studies using a wide variety of approaches to inhibit neutrophil function have not found any evidence for a neutrophil-induced injury phase (Connolly et al., 2011; Cover et al., 2006; Hou et al., 2012; James et al., 2003; Lawson et al., 2000; Williams et al., 2010a, 2010b). In addition, there is no support for activation of neutrophils in circulation or in the liver, a hallmark of any model where neutrophils are critical (Gujral et al., 2003; Jaeschke et al.,

* Corresponding author at: Department of Pharmacology, Toxicology & Therapeutic, University of Kansas Medical Center, 3901 Rainbow Blvd, MS1018, Kansas City, KS 66160, USA. Fax: +1 913 588 7501.
E-mail address: hjaeschke@kumc.edu (H. Jaeschke).

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1992), during the early phase of APAP-induced liver injury at 6 h (Williams et al., 2010a).

Neutrophils are a first-line defense against pathogens and are essential for microbial phagocytosis and killing (Nathan, 2006). During APAP hepatotoxicity, this innate immune function is critical for host defense, especially if the patient progresses toward fulminant hepatic failure (FHF) (Antoniades et al., 2008; Taylor et al., 2013). It was reported that bacteremia occurs in up to one third of FHF patients, and bacterial infection may be the cause of death for up to 20% of FHF patients (Wyke, 1987). This link between progression of liver injury toward liver failure and innate immune function is of considerable interest. This loss of host defense could be the result of impaired synthesis of complement components, impaired phagocytosis of gut-derived bacteria by Kupffer cells, or multiple immune deficits which may include neutrophils (Taylor et al., 2013; Wyke et al., 1980, 1982).

Characterization of resident and infiltrating macrophages/monocytes has been performed in the mouse model of APAP overdose following the peak of injury, and it was demonstrated that the infiltrating monocyte/macrophage population is inherently anti-inflammatory and needed for injury resolution (Dambach et al., 2002; Holt et al., 2008; You et al., 2013). No characterization of neutrophils following the injury phase of APAP has been performed, however, and for this reason the activation status of peripheral blood and liver-sequestered neutrophils beyond the early injury phase of APAP remains unclear. In this study, we determined the progression of neutrophil activation and hepatic accumulation in the mouse model and utilized mice deficient for NADPH oxidase activity to investigate the importance of phagocytic oxidative burst. Additionally, we evaluated the activation status of human granulocytes in several APAP overdose patients during the course of their hospital stay and compared the activation status to what we observed in the mouse model, in which the mechanisms of APAP toxicity are better understood.

Methods

Animals. Male gp91^{phox} -deficient (aka: Nox-2 −/−, Cybb −/−, Phox −/−) mice (B6.129S-Cybb^{tm1Din}/J; Stock number: 002365) and C57BL/6J control mice (Stock number: 000664) with an average weight of 18 to 20 g were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were housed in an environmentally controlled room with 12 h light/dark cycle and allowed free access to food (# 8604 Teklad Rodent, Harlan, Indianapolis, IN) and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center and followed the criteria of the National Research Council for the care and use of laboratory animals in research.

Experimental design. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Mice were intraperitoneally (i.p.) injected with 300 mg/kg APAP (dissolved in warm saline) or saline vehicle after overnight fasting. The animals were euthanized 6, 12, 24, 48 or 72 h after APAP treatment, blood was withdrawn from the vena cava into a heparinized syringe for measurement of alanine aminotransferase (ALT) activities (Pointe Scientific, Canton, MI) and flow cytometric analysis. The liver was removed and rinsed in saline; liver sections were fixed in 10% phosphate buffered formalin for histological analyses. The remaining liver was used for isolation of non-parenchymal cells (as described below). Additionally, as a positive control, some mice were treated (i.p.) with 100 μg/kg *Salmonella abortus equi* endotoxin (ET) for 90 min and then euthanized (approximately at 10 am).

Patient selection and consent. Blood samples were obtained from APAP overdose patients (n = 14; 9 females and 5 males) admitted to the University of Kansas Hospital in Kansas City, Kansas. The study protocol and design were approved by the institutional review board (IRB). Acute and chronic APAP overdose patients were from the emergency department or admitted to the intensive care unit with evidence of overdose. The diagnosis was made by a physician on site and all study subjects were required to sign a consent form. The inclusion criteria were two or more of the following: (1) patient-reported APAP overdose, (2) high serum APAP levels, and (3) abnormal liver function tests (based on ALT, AST, PT, bilirubin) (Table 1). Patients were excluded if there was reasonable evidence for liver injury due to another cause (viral hepatitis, ischemic liver, etc.). All overdose patients received standard of care *N*-acetylcysteine (NAC) treatment.

Histology. Formalin-fixed mouse tissue samples were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin and eosin (H&E) for blinded evaluation of the areas of necrosis by the pathologist. The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross sectional area. Additional liver sections were stained for neutrophils with anti-mouse neutrophil allotypic marker antibody (AbD Serotec, Raleigh, NC) as described in detail (Williams et al., 2010a). Quantification was performed by staining tissue sections, randomly selecting 10 high power fields (HPF, ×400) and counting positively stained cells consistent with neutrophil morphology. The sum of these 10 HPFs for each animal is then used to determine the group mean and standard error. Additional sections were stained for proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology, Santa Cruz, CA) as described in detail in Chosay et al. (1998). Positively stained hepatocytes (careful determination was made to not count binucleated cells twice) were quantified in ten randomly selected high power fields (HPF, ×400) in a manner consistent with the neutrophil quantification. Additionally, some sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche, Indianapolis, IN) as described in detail in Lawson et al. (1999)).

Isolation of hepatic non-parenchymal cells. The procedure was adapted from a method described by Watanabe et al. (1992). Under isoflurane anesthesia mice were exsanguinated from the caudal vena cava into heparinized tubes and the blood was placed on ice. The liver was immediately excised, placed in ice-cold PBS and minced with scissors. The tissue was then pressed through a 200-gauge stainless steel mesh into a 50 mL conical tube. The cell suspension was centrifuged at 50 ×*g* for 2 min to remove hepatocytes and large debris. The supernatant containing non-parenchymal cells was then centrifuged at 350 ×*g* for 5 min and cells were passed through a 40 μm nylon screen and washed twice in a 15 mL conical bottom tube. Viable, nucleated cells were counted by trypan blue exclusion and brought to a uniform cell density.

Flow cytometric analysis of neutrophil function

Neutrophil CD11b staining (Bajt et al., 2001; Daley et al., 2008). 5 μg Fc receptor (FcR) blocking antibody (BioLegend, San Diego, CA) diluted in 100 μL 0.1% BSA in PBS was added to 100 μL non-parenchymal

Table 1
Patient information.

	n = 14		
	Median (range)	Sex (% female)	Survival (%)
Age	44 (19–65)	64	100
Peak ALT (U/L)	2851 (842–5365)		
Peak PT (s)	41.3 (14.0–121.8)		
Peak bilirubin (mg/dL)[a]	5.3 (1.6–9.1)		

ALT = alanine aminotransferase. PT = prothrombin time.
[a] When available.