CONTRIBUTION OF THROMBIN AND PLATELETS TO ACETAMINOPHEN HEPATOTOXICITY IN MICE

By

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ABSTRACT

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Acetaminophen (APAP) is one of the most widely used antipyretic and analgesic drugs in both children and adults. Although APAP is relatively safe when it is used within its recommended dosing, overdosing with APAP is the most common cause of acute liver failure in the U.S. Because treatment options for APAP hepatotoxicity are limited, many studies have been conducted to understand the mechanism of APAP hepatotoxicity. It is hoped that a greater understanding of mechanisms will lead to supplemental or alternative treatments.

APAP-induced liver injury in humans is associated with robust coagulation cascade activation and thrombocytopenia. However, it is not known whether coagulation-driven platelet activation and thrombin generation participates causally in acetaminophen hepatotoxicity. Here, we hypothesized that platelets and thrombin contribute to APAP hepatotoxicity. Our results in a mouse model suggest that thrombin, the thrombin receptor, protease activated receptor-4 (PAR-4), and platelets contribute to the progression of APAP-induced liver injury. Also, our results suggest that platelets and thrombin contribute to the release of nitric oxide (NO) needed for generation of peroxynitrite. Results in isolated murine hepatocytes suggest that thrombin does not enhance hepatocellular injury directly but more likely plays a role by stimulating NPCs in the liver. Treatment of mice with lepirudin after APAP significantly decreased hepatic

injury, however, potential hemorrhagic complications may limit use of thrombin inhibitors or other direct coagulation inhibitors in human patients. Further evaluation of the mechanisms of platelet and thrombin enhancement of APAP-induced liver injury is therefore warranted.

Interestingly, during the development of the in vitro experiments using primary mouse hepatocytes (HPCs), an issue arose regarding the appropriate APAP concentration to use, since other investigators have used many different APAP concentrations (0.1-50 mM) in studies of APAP cytotoxicity in vitro. It is widely accepted that APAP toxicity requires bioactivation by cytochromes P450 (CYPs), yet our concentration-response study in murine HPCs suggested contribution of a CYP-independent mechanism. This led to another hypothesis that APAP toxicity in vitro includes CYP-independent mechanism(s). Our results suggest that at least two mechanisms contribute to APAP cytotoxicity. One is a CYP-dependent mechanism that operates at small, cytotoxic APAP concentrations and that occurs rapidly and is limited both in degree and duration. This mechanism is related to CYP-dependent NAPQI production. The second is a CYP-independent mechanism that predominates at larger APAP concentrations and that is slower to develop and highly lethal. Deacetylation of APAP to Para-aminophenol (PAP) appears to be a contributor to the CYP-independent mechanism and might enhance cell death by its own mechanism or by acting on cells already stressed by NAPQI. Whether the CYP-dependent and CYP-independent initiating mechanisms activate similar or different cell death signaling pathways remains to be determined.

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KEY TO ABBREVIATIONS

ABT	1-aminobenzotriazole
ABAM	Antibiotic-antimycotic
ADP	adenosine diphosphate
ALF	Acute liver failure
ALT	Alanine Aminotransferase
APAP	Acetaminophen
BNPP	bis(4-nitrophenyl) phosphate
COX	Cyclooxygenase
CYP	Cytochrome P-450
DILI	Drug induced liver injury
FBS	Fetal bovine serum
FSC	Forward scatter
GSH	Glutathione
GSSG	Glutathione disulfide
HIF-1α	Hypoxia-inducible factor 1- α
HO-1	Hemoxygenase-1
HPCs	Hepatocytes
i.p.	Intraperitoneal
MdFI	Median fluorescence intensity
MMP	Metalloproteinase
MT-1	Metallothionein-1

NAC	N-acetyl-L-cysteine
NAPQI	N-acetyl-p-benzoquinone imine
NATs	N-acetyltransferases
NO	Nitric oxide
NOS	Nitric oxide synthase
NPCs	Non-parenchymal cells
NSAIDs	Non-steroidal anti-inflammatory drugs
$ONOO^-$	Peroxynitrite
PAI-1	Plasminogen activating inhibitor-1
PAP	p-aminophenol
PAR	Protease activated receptor
PBS	Phosphate-buffered saline
PgE1	Prostaglandin E1
PS	Phosphatidylserine
RBCs	Red blood cells
ROS	Reactive oxygen species
SEM	Standard error of the mean
SSC	Side scatter
TAT	Thrombin-antithrombin
TEM	Transmission electron microscopy
TF	Tissue factor
TXA_2	Thromboxane A2
vWf	von Willebrand factor

WME	Williams’ Medium E
WT	Wild type

CHAPTER 1:

General Introduction

1.1 Overview of liver physiology

1.1.1 Functions of liver

The liver is one of the critical organs in an animal's body to sustain life. Liver contains different types of cells, and the predominant cell type (60% of the total cells) is the hepatocyte that carries out most of the functions related to liver. Some of these main roles are:

A) Plasma protein synthesis which is critical for homeostasis (i.e., albumin, transferrin, prothrombin, fibrinogen, coagulation factors, lipoproteins and complement proteins)
B) Glucose homeostasis: maintaining the blood glucose concentration by glycogenolysis (breaks down glycogen and releases glucose) and gluconeogenesis (synthesizing glucose from other sugars like fructose and amino acids)
C) Bile synthesis: Produces bile acids that are the main component of bile. Bile functions 1) to excrete cholesterol and phospholipids from the liver, 2) to keep fat-soluble organic compounds (i.e. fat-soluble vitamins) in solution and 3) to promote the dissolution and hydrolysis of triglycerides by pancreatic enzymes
D) Metabolism of drugs and toxins: hepatocyte enzymes (i.e., CYP) metabolize xenobiotics delivered from the gut and portal circulation (McCuskey, 2006; Peter and Faber, 2007; Jaeschke, 2008)

1.1.2 Non-parenchymal cells in liver

There are several different types of cells recognized as NPCs in the liver. They include sinusoidal endothelial cells, Kupffer cells, stellate cells and granular lymphocytes (previously called pit cells) (Wisse et al., 1996).

A) Kupffer cells phagocytize toxicants and particles in the blood like bacteria, secrete inflammatory mediators and produce various substances that can be protective or harmful to the liver.
B) Sinusoidal endothelial cells form a fenestrated lining between hepatocytes and blood flowing in the sinusoid. This fenestrated lining works as a sieve and prevents red blood cells (RBCs) and other cellular components from easily contacting hepatocytes, but allows access to different substances in the blood. Endothelial cells can change their shape and also secrete and release various cytokines, NO and other agents to work in host defense and regulate sinusoidal blood flow in the liver.
C) Stellate cells store fat-soluble vitamins and synthesize collagen when activated.
D) Granular lymphocytes in the liver now are recognized as NK cells and NKT cells. They have the capability of releasing cytokines and causing cytotoxicity (Godfrey et al., 2000).

1.1.3 Heterogeneity of hepatocytes

One thing that is quite unique about liver is that even though hepatocytes are indistinguishable histologically, hepatocytes function differently depending on their position along the porto-central axis of the liver cell plate (Colonot and Perret, 2011). Liver lobules are divided largely into three zones. Zone 1 is around the periportal area that is exposed to most oxygenated blood, zone 2 is the midlobular population of hepatocytes and zone 3 is around the central vein that is exposed to most poorly oxygenated blood. Not all the liver functions are zonal, but glucose metabolism, ammonia detoxification CYP distribution and metabolism of drugs are the main liver functions that have differences based on these zones (Colonot and Perret 2011).

1.1.4 Liver circulation

Another unique feature of liver is its blood circulation. The blood flow that comes into the liver is brought from 2 different sources, one is the hepatic artery that accounts for 1/3 of the blood flow in humans and 50% of the oxygen, and another is the portal vein that supplies 2/3 of the blood and 50% of the oxygen supply. The portal vein carries blood from spleen and omentum and also carries most substances absorbed from the gut to the liver (Bureau et al., 2007).

1.2 Hemostatic system (primary and secondary hemostasis)

The key components of hemostasis are based on the interactions of vascular endothelium, platelets, coagulation pathway and fibrinolysis. The hemostatic system normally maintains the fluidity of blood and responds to vascular injury and rapidly forms a clot. Briefly, hemostasis can largely be divided into 3 parts: primary hemostasis that is defined as formation of platelet plugs, secondary hemostasis defined as formation of fibrin by the coagulation cascade and last, tertiary hemostasis defined as plasmin formation and fibrinolysis. The focus of this dissertation is toward platelets and thrombin which both play critical roles in primary and secondary hemostasis.

1.2.1 Platelets

Platelets are small, anucleate cells in mammalian vertebrates, originating as fragments of megakaryocytes that mostly work as the first step of hemostasis at vessel injury sites to prevent hemorrhage. However, it seems that the role of the platelet is not limited to primary hemostasis, as they play important roles in coagulation, inflammation and wound healing by releasing substances from granules.

1.2.1.1 Basic role of platelets in primary hemostasis

In normal blood circulation, platelets do not undergo significant interaction with endothelial surfaces. However, once severe enough vascular injury occurs, platelets will start to interact with the injury site to form a platelet plug. As soon as the subendothelial matrix is exposed, the blood is exposed to various adhesive molecules that adhere

platelets to the injury site. These include collagen, von Willebrand factor (vWf), laminin, fibronectin and thrombospondin. (Broos et al., 2011; Varga-Szabo et al., 2008).

When subendothelial extracellular matrix is exposed, vWf starts binding to the exposed collagen (Figure. 1). Following to this, GP1bα on the platelet starts binding to vWf and mediates a tethering of the platelets. This binding of GP1bα and vWf is not sufficient to mediate stable adhesion but is enough for the platelets to start rolling and to become activated. During this period, platelet receptor GPVI binds to collagen and further activates the platelets. This series of events shifts the platelet integrins to a high affinity state and causes prompt release of secondary mediators like adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2), and with locally produced thrombin, platelets will be fully activated (Varga-Szabo et al., 2008). The final step of adhesion is mediated by high affinity β1 integrins including αIIbβ3 (known as platelet aggregating receptor, that also mediates firm adhesion by binding to vWf) and α2β1 (directly binds to collagen). There are other β integrins like αVβ3 (binds to victronectin), α5β1 (binds to fibronectin) and α6β1 (binds to laminin) that also contribute to platelet adhesion, however their importance is still not clear (Broos et al., 2011). Platelet aggregation occurs following activation and adhesion of the platelets, and this will form a fibrinogen-rich thrombus. Aggregation involves various ligands (fibrinogen, fibronectin and vWf) and receptors (GP1bα and αIIbβ3). Another interesting thing about these adhesive molecules is that, based on the difference in the shear rates in venules the effective adhesive molecules change. In a low shear rate (larger venous), platelet adhesion depends more on α2β1 binding to collagen, fibronectin and laminin; however, in high

shear rate (microvasculature and stenotic arteries), GP1bα and vWf become more important for the adhesion (Jurk and Kehral, 2005). Aggregation of platelets shows a similar difference: with low shear rate the aggregation is mainly mediated by αIIbβ3 and fibrinogen. However, at higher shear rates, GP1bα and vWf are more important for platelet aggregation (Broos et al., 2011). A diagram of platelet adhesion and aggregation is provided as Figure1.

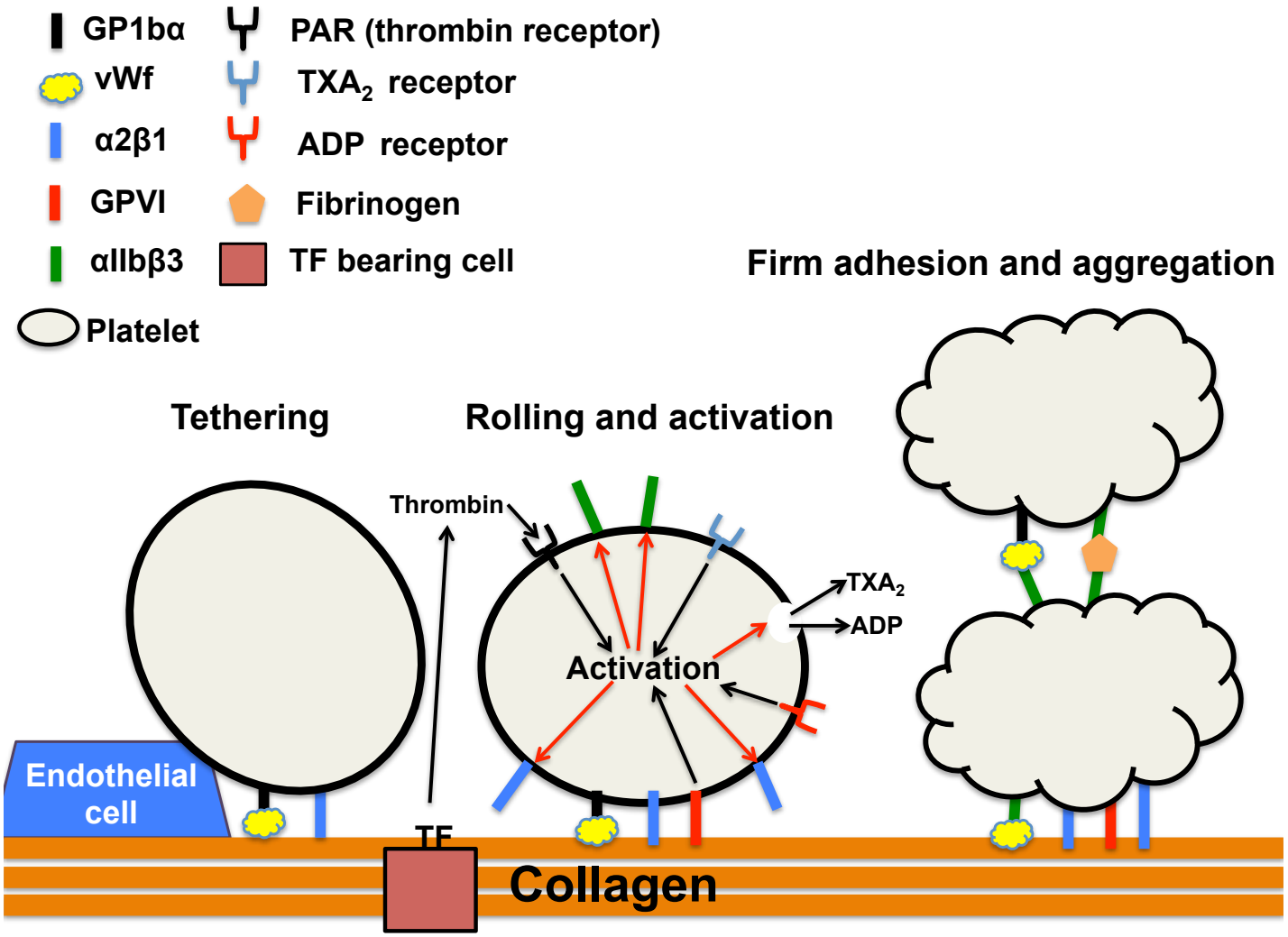

Figure 1: Platelet adhesion and aggregation. See section 1.2.1.1 for a detailed explanation of the pathway.

1.2.1.2 Platelet role in coagulation

Coagulation is largely divided into intrinsic and extrinsic pathways. In vivo, extrinsic pathway activation from injury to the vessel wall and exposure of collagen and tissue factor (TF) is proposed to be the main activator of the coagulation cascade. Platelets play a critical role in primary hemostasis, but they also have an important role in secondary hemostasis. One of the important roles of platelets in coagulation is to promote thrombin generation. Platelet adhesion increases from 2% of phosphatidylserine (PS) to 12% on the platelet surface (Jurk and Kehral, 2005). This exposure of PS provides a catalytic surface to bind coagulation factors and cofactors via Ca^{2+} and by specific receptors to form a prothrombinase complex (factor Xa, factor Va, Ca^{2+} and PS) that converts prothrombin to thrombin. Another important role is release of the coagulation factors (factor V, factor XI and prothrombin) from the α-granules when platelets are activated. Factor V in platelets accounts for 1/5 of the entire factor V pool in humans (Camire et al., 1998). All of these would lead to a robust increase in thrombin generation.

1.2.1.3 Platelet roles besides hemostasis

Platelets not only play a role in hemostasis, but they are also known to play important roles in wound healing, inflammation, infection, vascular contraction and communication with different cell types to enhance their activity. Platelets contribute to wound healing by generating thrombin (angiogenesis independent of fibrin (Tsopanoglou et al., 1993)) and release of many growth factors, cytokines that stimulate

tissue regeneration (Nurden, 2007). Mammalian platelets do not have nuclei, but they can produce proteins like TNFα, IL-1β, CD40L, RANTES, NF-κB and others that promote inflammation (Leslie, 2010; Ferroni et al., 2012). Platelets have been discussed as contributors to peroxynitrite formation (one of the reactive nitrogen and oxygen species) by production of NO as well (van Gils et al. 2009; Gkaliagkousi et al.,2007). Also, platelets can phagocytize and kill organisms like trypanosomes and malaria parasites (McMorran et al., 2009; Shaw et al., 1991).

Platelets can also adhere to endothelial cells and monocytes and activate these cells. Activation of endothelial cells can lead to NO, IL-1 β, IL-6, and IL-8 release, reactive oxygen species (ROS) production and increase in metalloproteinase (MMP)-2 and -9 that are likely pro-inflammatory (van Gils et al., 2009). Monocytes activated by platelets also produce many pro-inflammatory mediators, like TNFα, IL-1 β, IL-8, NF-κB and O_2^- (van Gils et al., 2009; Nagata et al., 1993).

1.2.2 Thrombin

Thrombin is a serine protease that plays a central role in coagulation. Its main role in coagulation is to convert fibrinogen to fibrin that forms a more secure hemostatic plug with platelets, but it also activates other coagulation factors. In mice, prevention of thrombin formation and regulation is lethal, suggesting that thrombin is essential for life (Mann et al., 2003). Besides its role in the coagulation system, thrombin plays roles in inflammation, vasomotor regulation and cellular proliferation.

1.2.2.1 Thrombin (Factor IIa) generation

Thrombin generation can largely be divided into three overlapping phases called initiation, priming and propagation (Monroe et al., 2002). In the initiation phase, the coagulation cascade is triggered by injury in the vessel wall leading to exposure of TF-bearing cells to blood (Figure 2). This leads to the forming of factor VIIa-TF complex with a blood circulating factor VII and that activates factor X and IX to Xa and IXa. This factor Xa produced on the TF-bearing cells activates and combines with factor V on these TF-bearing cells and produces small amount of thrombin that further activates the platelets adhered to an injury site. The factor VIIa/TF complex is shut down through the action of the TF pathway inhibitor in complex with factor Xa (Monroe et al., 2002).

In the priming phase, the small amount of initial thrombin produced in the initiation phase moves to platelets. Factor IXa can be produced from TF-bearing cells, but factor IX can also be activated by factor XIa on a platelet surface activated by

thrombin. This factor IXa forms a factor IXa-VIIIa complex on the platelet surface. Factor VIII is normally bound to vWf in an inactive form. Thrombin cleaves factor VIII from vWf and activates it to factor VIIIa.

In the propagation phase, the formation of the factor IXa-VIIIa complex activates Factor X to Xa, and also the activation of factor V by thrombin further enhances the production of factor Va-Xa complexes on the platelet surface to generate a thrombin burst that forms a fibrin clot. A diagram of thrombin generation is provided as Figure 2.

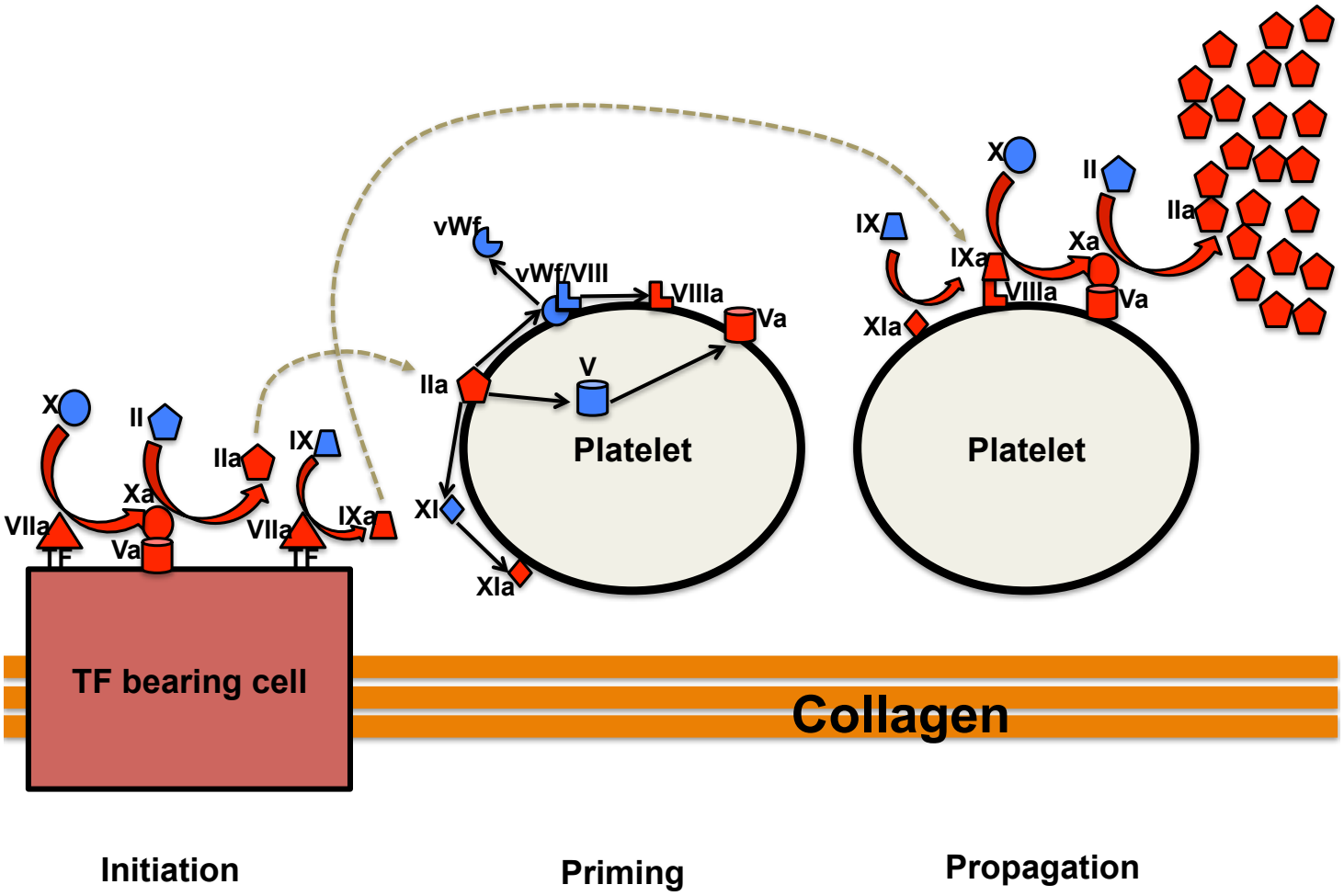

Figure 2: Thrombin (Factor IIa) generation. See section 1.2.2.1 for a detailed explanation of the pathway.

1.2.2.2 Thrombin and thrombin receptors

Besides the activity of thrombin as a serine protease for zymogen conversion in hemostasis, thrombin can activate platelets and other cells through a group of receptors called PARs (Coughlin 2000; Coughlin 2005). These PARs are G protein-coupled receptors and are activated when thrombin cleaves its N-terminal exodomain at a specific site, exposing a new N terminus that binds intramolecularly to the body of the receptor. This binding initiates transmembrane signaling (Coughlin 2000; Coughlin 2005). There are currently 4 recognized PARs (PAR-1, PAR-2, PAR-3 and PAR-4). Except for PAR-2, the PARs can be activated by thrombin. Additionally, other serine proteases can activate PARs (Borissoff et al., 2009; Coughlin 2005). Well known activators for each receptor are summarized in table 1.

Table 1: Known serine protease activators for each PAR receptor.

	Thrombin	VIIa	Xa	APC	Plasmin	Trypsin	Tryptase
PAR-1	+	+	+	+	+		
PAR-2		+	+	+		+	+
PAR-3	+						
PAR-4	+				+		

Activated protein C (APC)

The concentration of thrombin needed for receptor activation differs among the PARs. For example, PAR-4 requires more thrombin to be activated than PAR-1 (Xu et al., 1998; Kahn et al., 1999; Coughlin 2005); however the activation of PAR-4 can be enhanced by activation of PAR-3 working as a cofactor.

Platelets are well known to express PARs. Interestingly, there are some differences in the occurrence of PARs on platelets between humans and mice (Coughlin 2000; Coughlin 2005). Human platelets express PAR-1 and PAR-4, and activation of either of them can sufficiently activate platelets (Coughlin 2005). However, it seems that PAR-1 is more important at small thrombin concentrations than PAR-4, since PAR-1 blocking decreased platelet activation at a low concentration of thrombin but not at high thrombin concentration (Kahn et al., 1999).

Unlike humans, mice have PAR-3 and PAR-4 on their platelet surfaces. Interestingly, PAR-3 on the mouse platelet surface itself is not sufficient to activate the platelet, and it seems that PAR-3 works as an accessory protein with thrombin to further activate PAR-4 for platelet activation (Nakanishi-Matsui et al., 2000). These differences need to be addressed when we compare PARs on platelets between humans and mice, but at least it seems as though PAR-4-deficient mouse platelets can be considered to be similar to PAR-1 and PAR-4 deficient human platelets (Coughlin 2000).

Importantly, many other cells besides platelets express PARs on their cell surfaces. Some of those are endothelial cells, vascular smooth muscle cells and

leukocytes (Borissoff et al., 2009). Some of the roles of thrombin within these cells are summarized in Table 2.

Table 2: Cell types that have PARs, and thrombin effects on these cells.

Cell type	Role
Platelets	Shape change (Huang et al. 2007) Secretion from granules (Couglin 2005; Macfarlane et al. 2001) TXA_2 synthesis and release (Macfarlane et al., 2001) Mobilization of P-selectin and CD40L to membrane surface (Couglin 2005; Macfarlane et al., 2001)
Endothelial cells	P-selectin display (Couglin 2005) vWf release (Couglin 2005) Change shape (Macfarlane et al., 2001) Increase permeability (Macfarlane et al., 2001) Endothelial cell dependent vasodilation (Kataoka et al., 2003) Chemokines production (Johnson et al., 1998) COX2 (Houliston et al., 2002), PGs (Weksler et al., 1978), PAF (Zimmerman et al., 1996) and NO (Momota et al., 2006) production
Vascular smooth muscle cells fibroblasts	Relaxation/contraction (Macfarlane et al., 2001) Cytokine production (Loppnow et al., 1998) Mitogenic effect (Trejo et al., 1996)
Leukocytes	Ca signaling in T-cells (Mari et al. 1994) Cytokine release from monocytes and macrophages (Li et al. 2006)

1.3 **Acetaminophen (APAP)**

Currently, APAP is one of the most widely used antipyretic and analgesic drugs in both children and adults. In 1886, while studying the effect of naphthalene on intestinal parasites, Drs. Cahn and Hepp requested naphthalene from the local pharmacy, but were incorrectly sent acetanilide. They noted that acetanilide behaved differently than expected and had an antipyretic effect (Meredith and Goulding, 1980; Toussaint et al., 2010). Acetanilide was soon marketed as an antipyretic but was discovered to be toxic, causing methemoglobinemia (Toussaint et al., 2010). In the 1940s, it was recognized that APAP, a metabolite of acetanilide, was mainly responsible for the antipyretic and analgesic effects of acetanilide (Toussaint et al., 2010). APAP was first marketed in the US in 1950 as a combination product with aspirin and caffeine, but the preparation was removed due to agranulocytosis (not from APAP). APAP was reintroduced into the US market in 1955 as prescription-only Tylenol by McNeil, Inc.. Since then, APAP was found to be a good alternative to aspirin, since APAP did not promote the gastrointestinal bleeding that can be caused by aspirin. APAP gained further popularity in the 1980s when aspirin was associated with Reye's syndrome in children with viral illness (Toussaint et al., 2010).

Despite being used for many years as an analgesic and antipyretic, the mode of action of APAP is still unclear (Hinz et al., 2008). It is widely considered that the analgesic and antipyretic effects of APAP resemble the mode of action of nonsteroidal anti-inflammatory drugs (NSAIDs), i.e., inhibition of the cyclooxygenase (COX) pathway. However, APAP has only weaker anti-inflammatory effects than NSAIDs do. There are

many other mechanisms of action that have been postulated, including inhibition of the L-arginine– NO pathway, central effects by inhibiting the serotonergic pain pathways, COX-3 inhibition, and active metabolite effects on cannabinoid receptors (Toussaint et al., 2010; Anderson 2008; Kis et al., 2005). However, none of these pathways has yet been widely accepted as the definitive mechanism of action of APAP.

1.3.1 **ALF and APAP hepatotoxicity (clinical findings in humans)**

ALF is an uncommon, liver-related disease (6% of liver-related deaths in the US) characterized by loss of normal liver function, leading to jaundice, coagulopathy and mental status change (Ostapowicz et al., 2002; Sass 2005; Khashab et al., 2007; Fontana 2008). Many affected patients deteriorate rapidly, develop multi-organ failure and die. Some recover with supportive treatment; however, most of the continuously deteriorating patients require liver transplantation. Before the development of liver transplantation, the mortality rate of ALF was more than 80% (Ostapowicz et al., 2002; Sass 2005; Khashab et al., 2007; Fontana 2008; Karvellas et al., 2010). Until the early 1980s, most common cause of ALF was related to infectious diseases, especially virus related hepatitis (hepatitis B and A). However in recent decades, the incidence of ALF (especially in the UK and US) related to drug induced liver injury (DILI) has dramatically increased. Recently, it has been reported that more than 50% of the cases of ALF are now due to DILI and 80% of the DILI is due to APAP hepatotoxicity in the US, UK and in other western countries (i.e., Sweden) (Ostapowicz et al., 2002; Sass 2005; Khashab et al., 2007; Fontana 2008; Karvellas et al., 2010). Although APAP is relatively safe when

it is used within its recommended dosing, overdosing of APAP leading to severe ALF is relatively common. One of the first reports of APAP hepatotoxicity, published in 1966, showed two cases of APAP overdose with severe, fulminant, centrilobular necrosis of the liver (Davidson and Eastman, 1966). Since then, the numbers have increased, and it was recently reported that in the US there are approximately 56,000 cases of emergency visits and 458 deaths due to APAP overdose per year (Nourjah et al., 2006; Holubek et al., 2006).

Early studies in the 1970s revealed that APAP metabolism by CYPs is critical to hepatotoxicity and results in a reactive metabolite that binds to proteins when glutathione (GSH) stores are depleted. This leads to formation of protein adducts and hepatotoxicity (Mitchell et al., 1971A; Jollow et al., 1973; Potter et al., 1973). In the late 1970s and early 1980s, it was found that *N*-acetyl-*L*-cysteine (NAC) is somewhat effective for treating APAP toxicity (Piperno and Berssenbruegge 1976; Peterson and Rumack 1977; Lauterburg et al., 1983), probably at least in part by promoting hepatic GSH synthesis. Since then, NAC has been the most effective antidote to decrease mortality when given early after ingestion of APAP. However, in many cases, patients present to the hospital too late in the course of liver injury for NAC to help, and, if liver injury is severe, liver transplantation can be the only choice. For patients who develop ALF but do not receive a liver transplant, mortality is around 30% (Larson et al., 2005).

1.3.2 **Initiation of APAP hepatotoxicity**

Many studies have been performed to evaluate how APAP causes toxicity. It is now generally accepted that the most critical events that occur are GSH depletion and NAPQI production (James et al., 2003; Larson et al., 2005; Gunawan and Kaplowitz 2007; Hinson et al., 2010; Jaeschke et al., 2011). In the early 1970s, after an overdose of APAP in mice, Mitchell and coworkers found that 80-90% of GSH is depleted (Mitchell et al., 1973B; Jollow et al., 1973). Following that, it was shown that the reactive metabolite causing this GSH depletion was NAPQI (Dahlin et al., 1984).

Within the therapeutic range, most APAP is conjugated by sulfation and glucuronidation. A small amount of NAPQI is formed by oxidation of APAP by CYPs, especially CYP2E1, 1A2, 3A4 and 2D6. At therapeutic APAP doses, NAPQI is conjugated with GSH and excreted. However, marked consumption of APAP leads to GSH depletion, then NAPQI starts to react with cellular proteins to form APAP adducts (Potter et al., 1973; Larson et al., 2007; Gunawan et al., 2007; Hinson et al., 2010; Jaeschke et al., 2011; Dahlin et al., 1984). A diagram for APAP metabolism and initiation of APAP hepatotoxicity is provided in Figure 3. It is now well accepted that this covalent binding of APAP to proteins is a critical initiating event for liver damage, and this initiating event of adduct formation triggers secondary mechanisms (progression events) within and outside of hepatocytes that lead to hepatocellular necrosis (Roth and Ganey 2010).

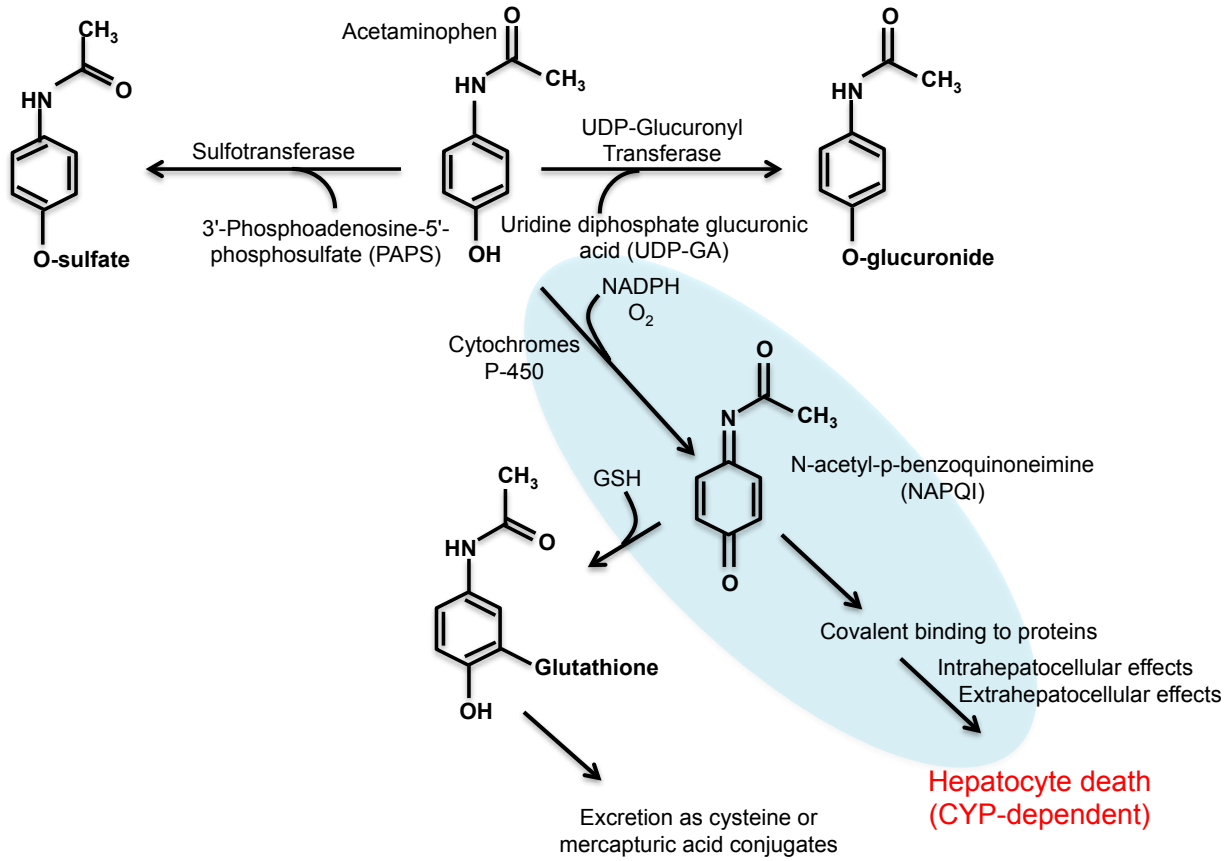

Figure 3: APAP metabolism and NAPQI production. See section 1.3.2. for a detailed explanation of the pathway.

1.3.3 **Progression events in APAP hepatotoxicity**

APAP hepatotoxicity has been recognized for more than 50 years, yet the treatment options for APAP overdose have been limited to only two: NAC (effective in the early stage) and liver transplantation (in more severe cases). Because of this, many studies have been conducted to understand the mechanism of APAP hepatotoxicity. It is hoped that a greater understanding of mechanisms will lead to supplemental or alternative treatments. There are many studies that have evaluated the progression events in APAP hepatotoxicity; the following discussion is limited to the studies that are important relative to this thesis.

1.3.3.1 **Role of oxidative stress in APAP hepatotoxicity**

ROS formation in hepatocytes seems to occur after the initial metabolisim of APAP (Jaeschke 1990; Bajt et al., 2004). Also, peroxynitrite formation plays a role in APAP hepatotoxicity (Hinson et al., 1998; Cover et al., 2005). This peroxynitrite is a reactive species formed by a rapid reaction of NO and superoxide. ROS and peroxynitrite damage mitochondrial DNA (Cover et al., 2005). ROS is also related to early activation of c-jun-N-terminal kinase (Gunawan et al., 2006; Hanawa et al., 2008). Damage to mitochondria DNA and activation of c-jun-N-terminal kinase lead to mitochondrial permeability transition (Cover et al., 2005; Kon et al., 2004). Numerous studies in this area suggest that peroxynitrite formation occurs in mitochondria. The most likely source of the superoxide has been reported to be hepatocyte mitochondria (Jaeschke et al., 2011; Murphy 2009). However, the cellular source(s) and the

isoform(s) of NO (inducible, endothelial and neuronal) that contribute to peroxynitrite formation are unclear (Jaeschke et al., 2011, 2012).

1.3.3.2 **Role of the coagulation system in APAP hepatotoxicity**

Coagulopathy is a common finding in APAP hepatotoxicity and is even one of the criteria for ALF. Using 400 mg/kg APAP in mice, a significant increase in procoagulant activity (a measure of functional TF activity) was observed at 30 min, and plasma thrombin-antithrombin (TAT: marker of thrombin generation/activation) complexes at 2 h. Following this, plasminogen activating inhibitor-1 (PAI-1: a major endogenous inhibitor of fibrinolysis) was significantly increased by 6 h. Histologically, fibrin deposition was observed around the pericentral areas of liver lobules by 2 h. All of these findings suggested that the coagulation system is activated within 2 h of APAP administration, i.e., before the progression of liver injury. Also, anticoagulation with heparin significantly decreased injury at 6 h but not at 24 h. The contribution of PAR-1 and TF were evaluated using PAR-$1^{-/-}$ mice and low TF-expressing mice, and both of these strains had less liver injury from APAP treatment compared to wild-type mice at 6 h but not at 24 h. These results suggested that the coagulation system is activated and that PAR-1 signaling contributes to early APAP hepatotoxicity (Ganey et al., 2007). Others have also evaluated the role of PAI-1 and fibrin/fibrinogen, and the results suggested that there is a protective role of PAI-1 (Bajt et al., 2008; Sullivan et al., 2012) but no contribution of fibrin/fibrinogen to APAP hepatotoxicity (Sullivan et al., 2012).

1.3.3.3 **Congestion and hemorrhage in APAP hepatotoxicity**

In multiple species, it is known that congestion and hemorrhage occur in liver with APAP overdose (Rose 1969; Walker et al., 1980, 1981, 1983, 1985; Francavilla et al., 1989; Ito et al., 2003). Some investigators suggested that RBC accumulation occurs before the necrosis develops and is associated with a transient change in liver weight and hemoglobin content (Walker et al., 1981, 1983, 1985). The importance of this congestion and hemorrhage is not clear, however, some investigators proposed that blocking of the liver sinusoids might lead to hypoxic damage (Walker et al., 1985). Also, it has been shown that other drugs and chemicals have hypoxia as a component of their toxicities (Lee et al., 2007).

Hypoxia-inducible factor 1- α (HIF-1α) is transcription factor that influences organ homeostasis and cell survival in response to hypoxia, inflammation and oxidative stress. HIF-1α contributes to APAP hepatotoxicity as early as 6 h by HIF-1α-dependent Bax translocation to mitochondrial membranes, but this early involvement was not apparent at later times (24h), suggesting a dual role for this transcription factor (Chaudhuri et al., 2011). Interestingly, in our previous study, there was a less early increase in plasma TAT complexes in HIF-1α depleted mice suggesting that thrombin might be one of the contributors to this early protection of APAP hepatotoxicity afforded by HIF-1α depletion (Sparkenbaugh et al., 2011).

It has been reported by others that HIF-1α protein increases in liver as early as 1 h after APAP administration (James et al., 2006; Chaudhuri et al., 2011) and that pimonidazole labeling, a marker of tissue hypoxia, was elevated by 4 h (Chaudhuri et al.,

2011). These results suggest that the very early HIF-1α protein increase might not be due to hypoxia, but at later times hypoxia might contribute.

1.4 **Summary**

Hemostasis plays an important role to maintain blood circulation in the body. However, several components of the hemostatic system are not limited to hemostasis but also contribute to inflammation, regeneration and other processes. In many cases of ALF from APAP hepatotoxicity, coagulopathy is one of the main clinical signs of the disease. However, detailed evaluation of its contribution to acetaminophen hepatotoxicity is limited. Previously, we explored the involvement of the coagulation system and PAR-1 in APAP hepatotoxicity in mice. Within that study, activation of the coagulation system and contributions of PAR-1 signaling to liver injury were observed. Platelets are known to be the main amplifier of thrombin and both are known to play a critical role in primary and secondary hemostasis. Interestingly, some of the roles of platelets and thrombin enhance release of factors also suggested to contribute to APAP hepatotoxicity (i.e. NO, ROS, TNF-α). Understanding the possible contribution of platelets and thrombin could provide new insight into the mechanism by which APAP-induced liver injury progresses and might lead to new therapeutic targets to treat APAP poisoning as an adjunct to NAC therapy.

1.5 Hypothesis and specific aims

The main hypothesis for this study was that platelets and thrombin contribute to APAP hepatotoxicity. In order to investigate this main hypothesis, the following aims were investigated in the chapters to follow.

Aim 1 Hypothesis: Platelets accumulate in liver after APAP hepatotoxicity (Chapter 2 and Chapter 3).

Aim 2: Hypothesis: Thrombin contributes to APAP hepatotoxicity (Chapter 2).

Aim 3: Hypothesis: PAR-4 contributes to APAP hepatotoxicity (Chapter 2).

Aim 4: Hypothesis: Platelets contribute to APAP hepatotoxicity (Chapter 2).

Aim 5: Hypothesis: vWf contributes to APAP hepatotoxicity (Chapter 3).

Aim 6: Hypothesis: Thrombin contributes to APAP hepatotoxicity indirectly through NPCs (Chapter 3).

Interestingly, during the development of the in vitro experimental design using primary mouse HPCs and NPCs for Aim 6, an issue arose regarding the appropriate APAP concentration to use, since other investigators have used many different APAP concentrations (0.1-50 mM) to treat primary mouse HPCs. This led to another hypothesis for this thesis that APAP hepatotoxicity in vitro includes CYP-independent mechanisms. To investigate this hypothesis further, the following aims were investigated.

Aim 7: Hypothesis: APAP hepatocellular injury at high APAP concentrations in vitro involves CYP-independent mechanisms (Chapter 4).

Aim 8: Hypothesis: A CYP-independent mechanism is related to a minor APAP metabolite, p-aminophenol (PAP) (Chapter 4).

CHAPTER 2:

Platelets contribute to acetaminophen-induced liver injury in mice by amplifying thrombin generation

2.1 Abstract

APAP-induced liver injury in humans is associated with robust coagulation cascade activation and thrombocytopenia. The procoagulant response is recapitulated in mice given a hepatotoxic dose of APAP, as indicated by a rapid rise in plasma TAT concentration, a stable biomarker of thrombin generation. However, it is not known whether coagulation-driven platelet activation participates in acetaminophen hepatotoxicity. Here, we found that APAP overdose in mice caused significant thrombocytopenia associated with accumulation of platelets in the injured liver, and these changes were attenuated by administration of the direct thrombin inhibitor lepirudin. To determine whether thrombin activation of platelet signaling contributed to hepatic platelet accumulation, studies were performed using mice deficient in PAR-4, which mediates thrombin signaling in mouse platelets. Compared to APAP-treated, wild type (WT) mice, TAT concentration and hepatic platelet accumulation were reduced in PAR-4$^{-/-}$ mice. Moreover, lepirudin administration or PAR-4 deficiency were each associated with a reduction in APAP-induced liver injury, as indicated by reduced activity of alanine aminotransferase in serum and reduced hepatocellular necrosis. Platelet depletion with an anti-CD41 antibody also significantly reduced liver injury and the rise in TAT concentration in mice after APAP overdose. Moreover, RBCs accumulation and protein nitration in the liver were reduced by lepirudin, PAR-4 deficiency, and platelet depletion, suggesting that platelets contribute to accumulation of RBCs in liver sinusoids and to the generation of peroxynitrite in mice overdosed with APAP.

Conclusion: These results suggest that thrombin-mediated activation of platelets contributes to amplification of the procoagulant response and to the progression of APAP-induced liver injury in mice.

2.2 Introduction

APAP hepatotoxicity is the leading cause of drug-induced liver injury and acute liver failure in the US and other developed countries (Ostapwicz et al., 2002; Kahshab et al., 2007; Fontana et al., 2008; Karvellas et al., 2010). It is commonly understood from early studies in murine models that APAP overdose leads to excessive production of the reactive metabolite, NAPQI, and to consequent depletion of hepatic GSH, which is responsible for detoxifying NAPQI. This depletion of GSH permits NAPQI binding to cellular proteins, which initiates events leading to oncotic necrosis of the liver (Mitchell et al., 1973; Jollow et al., 1973; Potter et al., 1973). APAP hepatotoxicity has been recognized for more than 50 years, yet the main treatment options for APAP overdose have been limited to: NAC (effective in the early stage) and liver transplantation (in more severe cases). Because of this, many studies have been conducted to understand the mechanisms of APAP hepatotoxicity and to seek alternative therapies. The binding of NAPQI that serves as the initiating event is followed by numerous intrahepatocellular and extrahepatocellular events that result in the progression of liver injury (Jaeschke et al., 2012, 2011; Hinson et al., 2010). Evidence has been presented for numerous contributors to the pathogenesis of APAP-induced liver injury, including NO and peroxynitrite formation, oxidative stress, mitochondrial injury, alteration in hepatic blood flow, and the innate immune response. The contributions of these numerous factors are still under debate (Jaeschke et al., 2012, 2011; Hinson et al., 2010; James et al., 2003).

APAP overdose is associated with activation of the coagulation cascade in mice and in humans (Lee et al., 1993; Larson et al., 2005; Ganey et al., 2007). Indeed, consumptive coagulopathy and decreased blood platelet concentration (Lee et al.,

1993; Larson et al., 2005) are among the major clinical signs in acute liver failure from APAP poisoning (Lee et al., 1993; Larson et al., 2005). Generation of the coagulation protease thrombin occurs rapidly after administration of a hepatotoxic dose of APAP in mice, and this procoagulant response is tissue factor dependent (Ganey et al., 2007; Sullivan et al., 2013). Also, in rats, a 30% decrease in platelet concentration in blood with repeated dosing of APAP has been reported (O'brien et al., 2000). Prostacyclin, an eicosanoid that inhibits platelet activation, was significantly decreased by APAP consumption in humans (Grèen et al., 1989), and prostacyclin administration reduced APAP hepatotoxicity in mice (Guarner et al., 1988). Additionally, the plasma concentration of platelet activating factor, which can cause platelet aggregation and degranulation, was increased in APAP hepatotoxicity in rats (Grypioti et al., 2005), and a platelet activating factor receptor antagonist (BN52021) attenuated liver injury (Grypioti et al., 2006). Collectively these findings suggest that platelets might contribute to APAP hepatotoxicity.

We have shown previously that the thrombin receptor, PAR-1, contributes to early APAP hepatotoxicity in mice (Ganey et al., 2007). Whereas PAR-1 is the primary thrombin receptor mediating platelet activation in humans (Coughlin 2005), mouse platelets respond to thrombin primarily through PAR-3/PAR-4 (Coughlin 2005). To date, the role of thrombin-mediated platelet activation in APAP-induced thrombocytopenia and hepatotoxicity has not been explored. In this study, we used a combination of pharmacologic, genetic, and cell-specific depletion strategies to define the role of thrombin-mediated platelet activation in the procoagulant and hepatotoxic responses accompanying APAP overdose in mice.

2.3 Materials and Methods

Materials

APAP (A7302) was purchased from Sigma- Aldrich Co. (St. Louis, MO). Low-endotoxin and azide-free anti-CD41 antibody (Rat IgG1 (133910)) and control rat IgG1 (400427) for platelet depletion, and anti-CD41 antibody (Rat IgG1, 133902) for platelet immunofluorescence were purchased from Biolegend Inc. (San Diego, CA). Infinity Alanine Aminotransferase (ALT) Reagent (TR71121) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). GSH Assay Kit (703002) was obtained from Cayman Chemical Co. (Ann Arbor, MI). TAT ELISA kit (Enzygnost TAT Micro Kit) was obtained from Siemens Healthcare Diagnostics Inc. (OWMG15 Deerfield, IL). Lepirudin was purchased from the Kansas University Medical Center Pharmacy. BMP leukochek test kit (BMP-LUKCHK-50) was purchased from Biomedical Polymers Inc. (Gardner MA). Nitrotyrosine rabbit polyclonal antibody (A-21285), Alexa-594 conjugated antibody and Slow Fade Gold Antifade reagent with DAPI (S-36939) were purchased from Life Technologies Co. (Grand Island, NY). TRI reagent was purchased from Molecular Research Center (Cincinnati, OH). iScript cDNA synthesis kit was purchased from Bio-Rad Laboratories (Hercules, CA). Universal Dako LSAB+ kit, Peroxidase (K0679), Serum-Free Protein Block (X0909) and Antibody Diluent (S0809) were purchased from Dako North America, Inc. (Carpinteria, CA).

Animals

All mice were male between 8 and 16 weeks of age. Wild-type C57Bl/6J mice (9 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME) and were

used for thrombin inhibition studies using lepirudin and for platelet depletion studies using anti-CD41 antibody. They were acclimated for at least one week at 22°C with alternating 12 h light/dark cycle and access to standard chow diet (Teklad 22/5 Rodent Diet 8940: Harlan laboratories) and spring water ad libitum. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee. PAR-4$^{-/-}$ (Sambrano et al., 2001) and WT mice of an identical genetic background (N8 C57Bl/6J) were maintained by homozygous breeding, and aged-matched males were used for experiments after confirming genotype by PCR analysis. These mice were maintained in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International at the University of Kansas Medical Center or Michigan State University.

Experimental Protocols

Mice were fasted for approximately 15 h before being treated with APAP (300 mg/kg) or saline by intraperitoneal (i.p.) injection. The thrombin inhibition pretreatment study was performed by injecting lepirudin (1 mg/kg, dissolved in saline, i.p.) or saline vehicle, i.p. at -0.5 h and +0.5 h, +2.5, +4.5 h relative to administration of APAP. The lepirudin post-treatment study was performed by giving lepirudin (1.5 mg/kg in saline, i.p.) or saline vehicle at +1.5, +2.5 and +4.5 h after APAP injection. Platelet depletion was accomplished according to the method of Luyendyk et al. (Sullivan et al., 2010) with some modification. Briefly, low-endotoxin and azide-free anti-CD41 antibody (2 mg/kg, mixed in phosphate-buffered saline (PBS)), isotype control rat IgG1 κ (2 mg/kg, mixed with PBS), or PBS was injected i.p. 12 h and 2 h before administration of APAP.

At termination of experiments, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) or isoflurane, and blood and liver tissues were collected immediately. Blood was drawn from the caudal vena cava into a syringe containing 3.2% sodium citrate (final concentration 0.32%) for blood platelet concentration determination and plasma preparation. The left lateral liver lobe was collected and fixed with 10% neutral-buffered formalin for 24 h, then the formalin was replaced with 70% ethanol for histopathology. The median lobe was collected and flash-frozen in liquid nitrogen for glutathione measurement and RNA analysis. The right lobe was collected and embedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) medium and frozen in liquid nitrogen-chilled isopentane for immunofluorescence studies.

Blood Platelet Concentration

Platelet concentration in blood samples anticoagulated with sodium citrate was determined using BMP leukochek test kit and a hemocytometer. Briefly, whole blood (2 µL) was added to 998 µL of 1% buffered ammonium oxalate solution, gently mixed and allowed to rest for 10 min. 10 µL of the mixture was loaded into each side of the hemocytometer, and platelet counting was performed under 400 x magnification, counting cells in all 25 squares of the central large square. Platelet counts were multiplied by 5000 to calculate the platelet concentration (platelets/µL). Presence of platelet aggregates were evaluated during counting, and, if present, samples with platelet aggregation were not included in the study.

APAP Bioactivation

Reduction in hepatic glutathione concentration was used to evaluate whether treatments affected bioactivation of APAP in mice. Frozen liver tissue (50 mg) was homogenized in 1 mL of cold buffer (0.2 M 2-(N-morpholino)ethanesulphonic acid, 0.05 M phosphate and 1 mM EDTA [pH 6.0]). Homogenates were centrifuged at 10,000 g for 15 minutes at 4℃, and the supernatants were collected to determine the hepatic total glutathione (GSH+ glutathione disulfide (GSSG)) concentration (expressed as μmol GSH+GSSG/g of liver tissue) using a modification of the Teitz assay (Jaeschke et al., 1990).

Liver Injury

Liver injury was estimated from increases in plasma ALT activity, determined spectrophotometrically, and by morphometric evaluation of left lateral liver lobes. Formalin-fixed livers were embedded in paraffin, sectioned 5 μm thick, and stained with hematoxylin-eosin. Slides were scanned and digitized using the Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20× objective. An automated, random sub-sampling of 60 images was further performed on the digitized slides with NewCast software (Visiopharm, Hoersholm, Denmark) at 200× magnification. Calculation of the percentage of area with hepatocellular necrosis/degeneration (i.e., moderate to severe irregular vacuolar degeneration adjacent to necrosis) was carried out with the STEPanizer software (http://www.stepanizer.com) (Tschanz et al., 2011). Briefly, a point grid was superimposed over the images, and points intersecting normal liver

parenchyma and hepatocellular necrosis/degeneration were counted. The percentage of liver tissue with hepatocellular necrosis/degeneration was estimated by dividing the number of points intersecting hepatocellular necrosis/degeneration by the number of total points on liver tissue (i.e., the sum of the points of normal liver parenchyma and hepatocellular necrosis/ degeneration). The accumulation of RBCs in liver tissue was evaluated similarly.

Platelet Accumulation in the Liver

Frozen liver sections cut 8 μm thick were fixed with 4% neutral buffered formalin for 30 min at room temperature, washed 3 times with PBS for 5 min each, and blocked with 10% goat serum in PBS for 1 h. Sections were incubated with anti-CD41 antibody (1:1000 diluted with 10% goat serum in PBS) overnight at 4 ℃. Sections were washed 3 times with PBS for 5 min each and incubated with goat anti-rat Alexa-594-conjugated antibody (1:1000 diluted in 10% goat serum and 2% mouse serum) for 3 h at room temperature. Sections were then washed 3 times with PBS for 5 min each and treated overnight with Slow Fade Gold Antifade reagent with DAPI. Slides were photographed using an Olympus IX71 inverted fluorescent microscope, with the following fluorescence filter sets: CD41-antibody, excitation 535 nm, emission 645 nm; DAPI: excitation 350 nm, emission 460 nm. Quantification of fluorescence was performed using Image J software. The integrated intensity of CD41 fluorescence from at least 3 representative microscopic fields per liver section was measured using a 10 × objective, and the average was calculated as one replicate.

Immunohistochemical Detection of Nitrotyrosine-protein Adducts in Liver

Tissue fixed in formalin for 24 h and then immersed in 70% ethanol was embedded in paraffin. Each section was cut 5 μm thick. Sections were rehydrated by first placing slides in a container in a water bath at 60 ℃ for 30 min, and then they were incubated successively with 100% xylazine, 95% ethanol, 80% ethanol, H_2O, and PBS for 3 min each. The sections were labeled with anti-nitrotyrosine antibody (1:400 diluted with antibody diluent), using universal LSAB+kit, peroxidase, and protein blocking solution. First the control slides were evaluated (without APAP) for background labeling. Following that, slides were evaluated without knowledge of treatment using a grading system based on the area of labeling and, to a lesser degree, on the labeling intensity relative to controls. Scoring of the area of labeling was as follows: 0=no to rare hepatocyte labeling; 1=labeling of 1-2 layers of hepatocytes around few to many central venules; 2=labeling of 2-4 layers of hepatocytes around most central venules with no bridging of labeled areas; 3=labeling of 2-4 layers of hepatocytes around most central venules, with infrequent and narrow bridging of labeled areas; 4=labeling of > 5 layers of hepatocytes around most central venules, with moderate to extensive bridging. Intensity of labeling was graded as follows: 0=no labeling; 1.2=mild; 1.5=moderate; 1.8=marked. Scores from the area and intensity of the labeling were multiplied together to obtain a summary score; intensity-weighting factors were chosen to be evenly distributed between 1 and 2 such that intensity would augment but not dominate area scores at each grade (Figure 4).

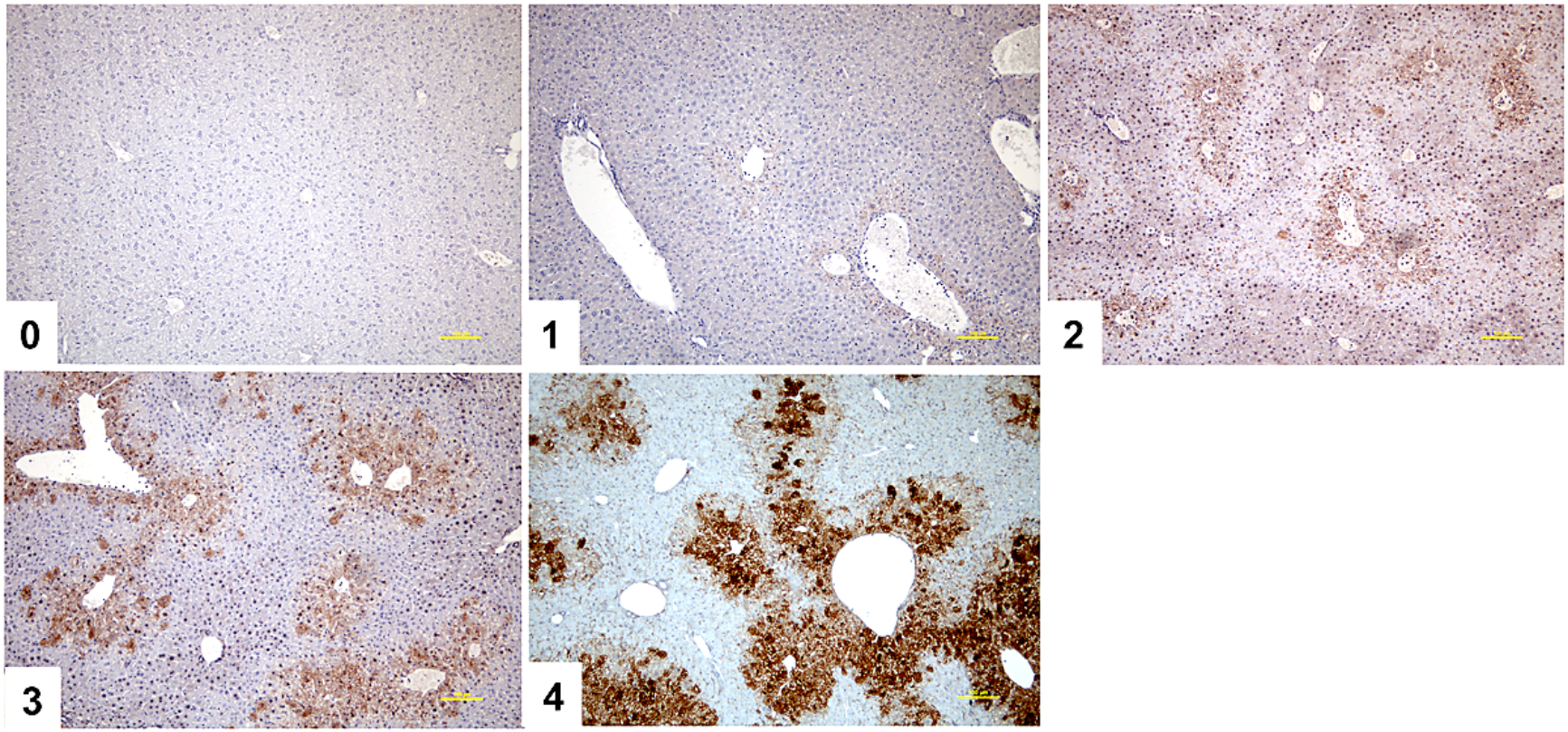

Figure 4: Examples of scores assigned for degree of labeling with anti-nitrotyrosine antibody in liver sections.

Formalin fixed tissues were labeled with anti-nitrotyrosine antibody. Slides were evaluated based on the area and intensity of labeling.

Figure 4 (cont'd)

Examples of each score are presented in the figure:

0=no to rare hepatocyte labeling

1=labeling of 1-2 layers of hepatocytes around few-to-many central venules

2=labeling of 2-4 layers of hepatocytes around most central venules with no bridging of labeled areas

3=labeling of 2-4 layers of hepatocytes around most central venules, narrow bridging of labeled areas

4=labeling of > 5 layers of hepatocytes around most central venules, moderate to severe bridging.

Intensity of the labeling was graded as follows:

0=no; 1.2=mild; 1.5=moderate; 1.8=marked

Scores from the areas and intensity of labeling were multiplied together to obtain a summary score. In supplement figure 1, the corresponding intensity scores were 0, 1.2, 1.5, 1.5, 1.8, respectively. Corresponding summary score were 0, 1.2, 4.5, 6, 7.2.

RNA Isolation and Real Time PCR

Liver tissue (50 mg) was homogenized in 1 mL of TRI reagent using a Precellys 24 Tissue Homogenizer (Cayman Chemical, Ann Arbor, MI), and RNA was extracted. Total RNA was quantified spectrophotometrically using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Total RNA (1 µg) was reverse-transcribed to prepare cDNA using an iScript cDNA synthesis kit. Messages for metallothionein-1 (MT-1) (primer sequences MT-1: Forward (F)- AATGTGCCCAGGGCTGTGT, Reverse (R)- GCTGGGTTGGTCCGATACTATT) and hemoxygenase-1 (HO-1) (primer sequences Hox-1: Forward (F)-CCTCACTGGCAGGAAATCATC, Reverse (R)- CCTCGTGGAGACGCTTTACATA) genes were determined using specific DNA oligonucleotides and SYBR green PCR master mix (Applied Biosystems, Foster City CA) on a Step-One real-time PCR system (Applied Biosystems). Copy number was determined by comparison to standard curves for the respective genes generated from pooled cDNA. Target gene expression levels were standardized to β-actin (primer sequences β-actin: Forward (F)- TGTGATGGTGGGAATGGGTCAGAA, Reverse (R)- TGTGGTGCCAGATCTTCTCCATGT).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Data that were not normally distributed were subjected to appropriate transformation (i.e., logarithmic, square root, arcsin squared). Student's t-test was used to compare two means, and analysis of variance was used when more than two means were compared. If a difference was detected with analysis of variance, appropriate post-hoc testing (Holm-

Sidak) was performed. If data transformation failed to generate results with a normal distribution, nonparametric tests were performed (Mann-Whitney U test for comparison of two means, and Kruskal-Wallis one-way analysis of variance for multiple comparisons). $P < 0.05$ was set as the criterion for statistical significance.

2.4 Results

APAP overdose causes blood platelet depletion and hepatic platelet accumulation in mice

In agreement with previous studies, serum ALT activity increased within 3 hours of APAP administration and continued to increase thereafter (Figure 5A). APAP administration caused a decrease in blood platelet concentration within 6 h (Figure 5B). Hepatic accumulation of platelets (i.e., CD41 detected in liver by immunohistochemistry (IHC)) in APAP-treated mice began between 0.5 and 2 h and continued through 6 h (Figure 5C). The accumulation began as a diffuse increase in signal (Figure 5D, 2 h) and progressed to more concentrated signals that appeared to radiate from the central venules (Figure 5D, 3 h and 6 h).

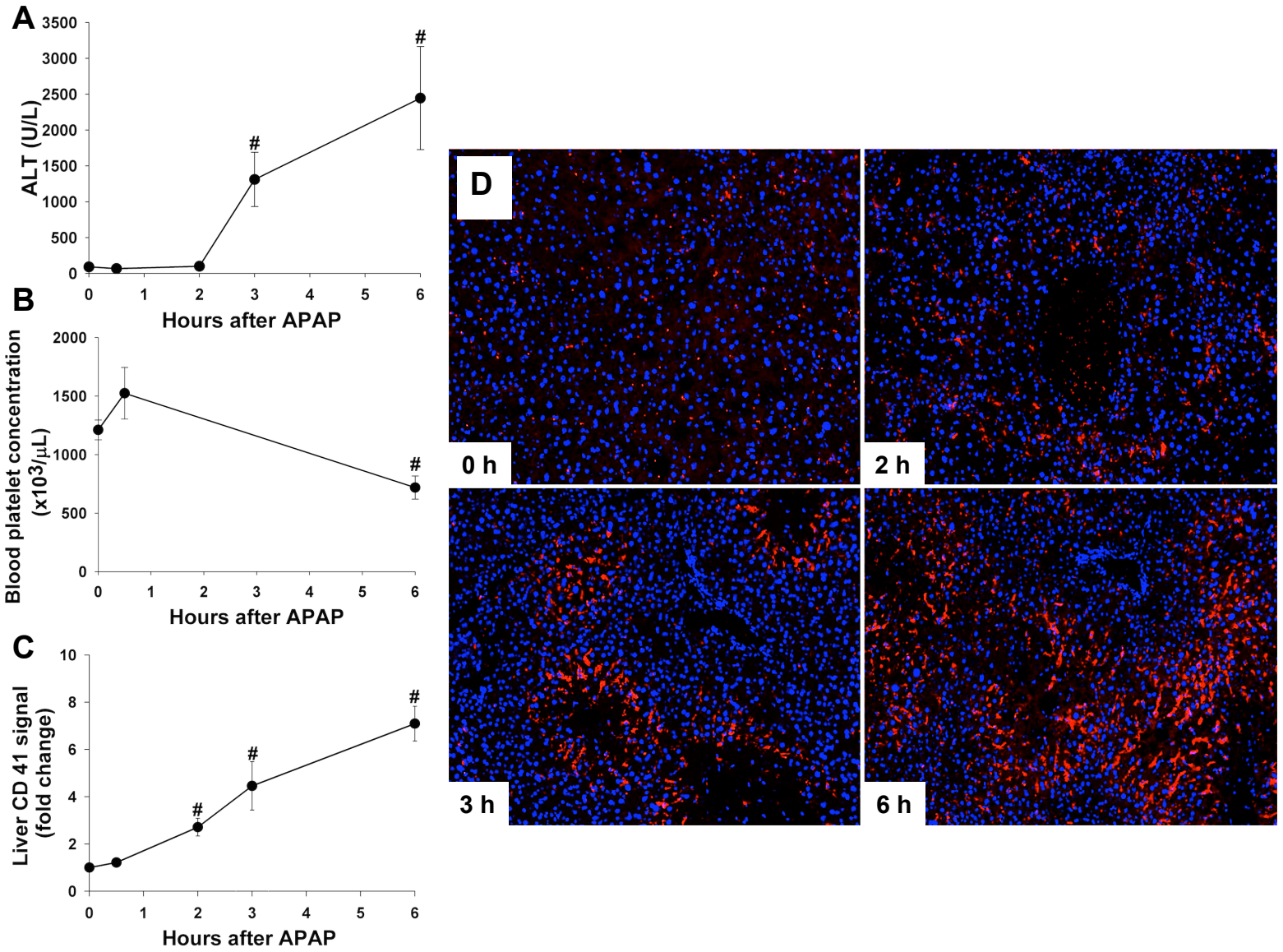

Figure 5: Platelet accumulation during development of APAP-induced liver injury in mice.

Figure 5 (cont'd)

C57BL/6J mice were treated with 300 mg/kg APAP i.p. (A) ALT activity in plasma, (B) Concentration of platelets in blood, (C) Quantification of CD41 signal in the liver at 0.5 h, 2 h, 3 h, and 6 h. Signal intensity of CD41 was normalized to the CD41 signal at time 0. (D) Representative photomicrographs of frozen liver sections labeled with anti-CD41 antibody to identify platelets; DAPI stain was used to identify nuclei. Platelets are pseudocolored red; nuclei are pseudocolored blue. (10 x objective)

[#]Significantly different form the saline-treated group. N=3-7 per group

APAP-induced hepatic platelet accumulation and injury are thrombin dependent

To determine whether thrombin activity contributes to platelet accumulation in APAP-treated mice, we utilized the direct thrombin inhibitor lepirudin (Greinacher et al., 2004). Lepirudin pretreatment significantly reduced hepatic platelet accumulation at both 3 and 6 h after APAP administration (Figure 6A and F). To evaluate liver injury, plasma ALT activity and area of necrosis/degeneration were determined (Figure 6B, C, and G). Hepatic necrosis was characterized as centrilobular coagulative necrosis, and degeneration of hepatocytes was occasionally observed bordering the necrotic area. Lepirudin pretreatment significantly reduced the increase in plasma ALT activity and area of necrosis/degeneration that occurred 6 h after APAP treatment. Also, there was a significant decrease in accumulation of RBCs, mostly adjacent to necrotic areas, in livers of lepirudin-pretreated mice (Figure 6D and G). Histological evaluation suggested that the increase in RBCs was due to congestion within the sinusoids at 3 and 6 h.

Nitrotyrosine-protein adducts arising from NO generation appear early in livers of mice overdosed with APAP (Knight et al., 2001). Thrombin increases expression of inducible NOS in glioma cells (Meli et al., 2001), and it activates endothelial NOS and causes NO release from endothelial cells through activation of PARs (Touyz et al., 2007; Momota et al., 2006). The enhancement of NO production through thrombin might contribute to peroxynitrite formation and consequent nitrotyrosine-protein adducts in liver tissue. Accordingly, IHC labeling for nitrotyrosine-protein adducts was evaluated as a biomarker of peroxynitrite formation in liver tissue. Treatment with lepirudin significantly reduced the nitrotyrosine signal in the livers of APAP-treated mice (Figure 6E).

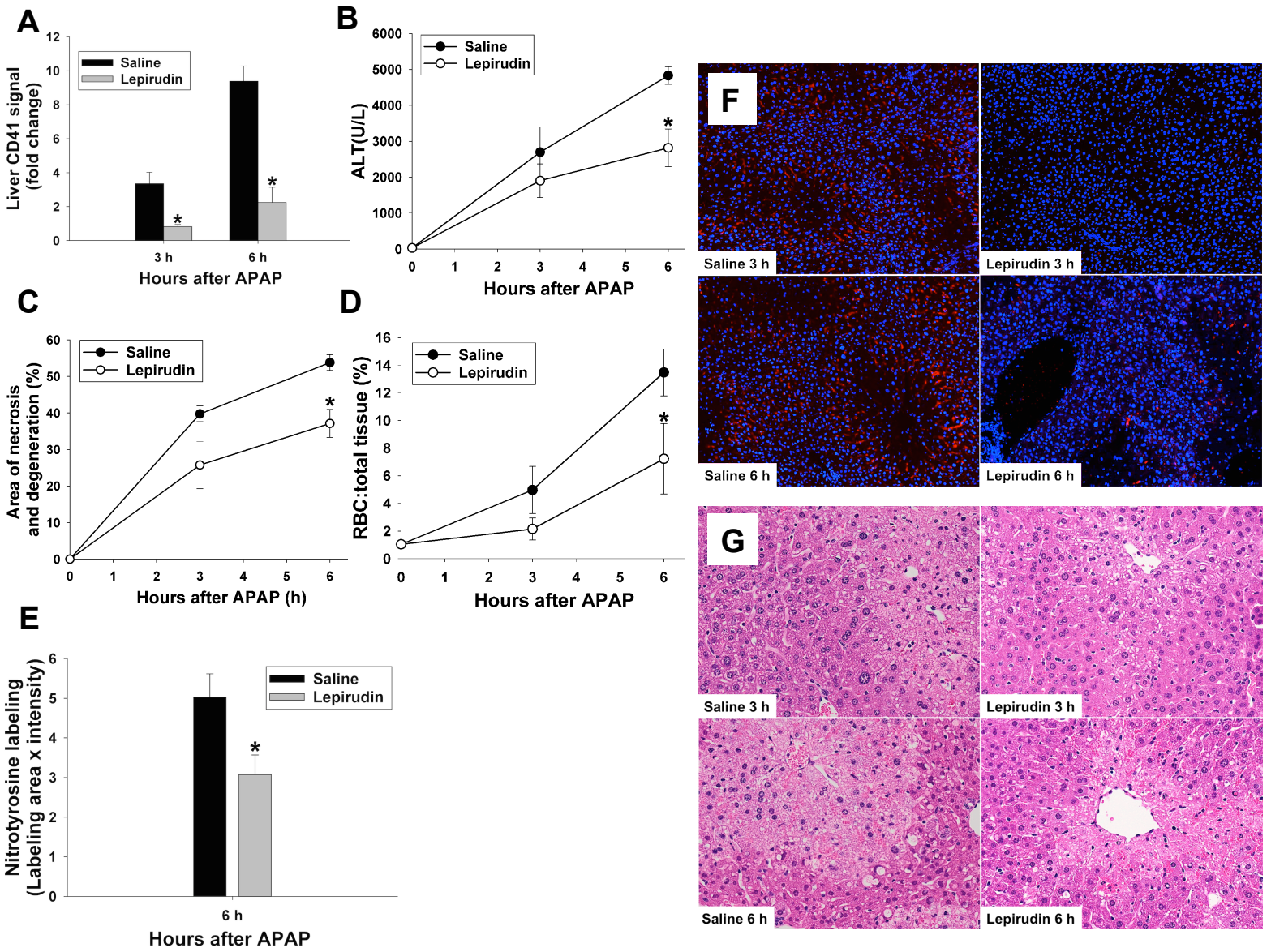

Figure 6: Effect of lepirudin on APAP hepatotoxicity.

Figure 6 (cont'd)

Mice were treated with 300 mg/kg APAP or sterile saline i.p. at time 0 h. 1 mg/kg lepirudin was given i.p. at -0.5 h and +0.5, +2.5, and +4.5 h relative to administration of APAP or saline. (A) Hepatic CD41 signal at 3 h and 6 h, intensity normalized to the CD41 signal of WT mice treated with neither APAP nor lepirudin. (B) ALT activity in plasma. (C) Area of necrosis and degeneration as a percentage of total liver area. (D) RBC to total cell (%) in sections of liver tissue. (E) Nitrotyrosine label quantified as described in Materials and Methods at 6 h. (F) Representative photomicrographs of frozen liver sections labeled with anti-CD41 antibody to identify platelets, using DAPI stain to identify nuclei. Platelets are pseudocolored red; nuclei are pseudocolored blue. (10 x objective) (G) Representative liver sections from each group at 3 h and 6 h after APAP injection. (20 × objective)

*Significantly different from the saline-pretreated group at same time. N=4 per group.

Depletion of GSH has been used extensively as a biomarker for the formation of the reactive metabolite NAPQI (Mitchell et al., 1973B; Jaeschke et al., 1990, 2011; Chiu et al., 2003). APAP administration caused more than 80% reduction in liver GSH, and this was unaffected by lepirudin pretreatment (Figure 7), suggesting that inhibition of APAP-induced liver injury by lepirudin was not a function of altered APAP metabolism.

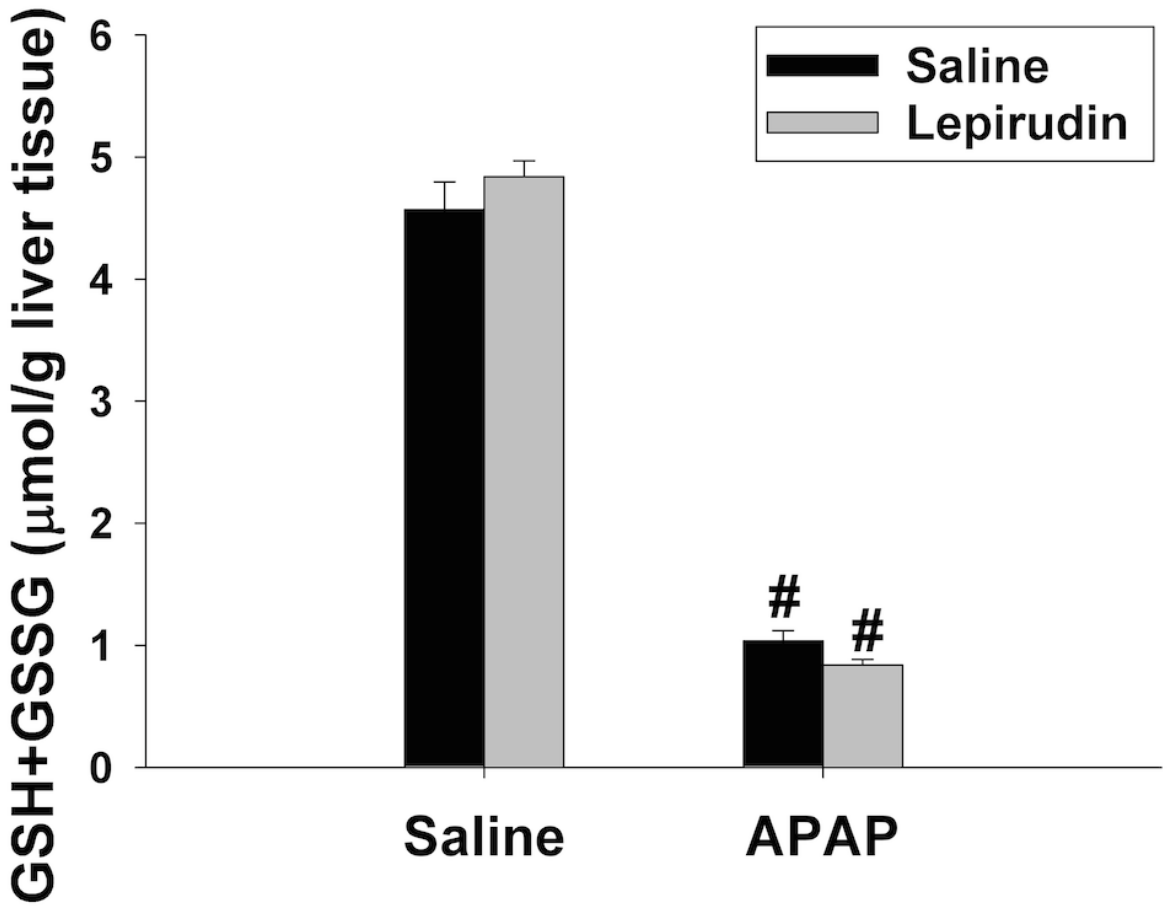

Figure 7: Evaluation of bioactivation of APAP with lepirudin pretreatment.

Glutathione concentration in liver was determined 30 min after administration of APAP (300 mg/kg) or vehicle (saline) from mice treated as described in figure 6. # Significantly different from respective group treated with saline. N = 3 per group.

In a separate study, lepirudin treatment was started 1.5 h after APAP to evaluate the effect of lepirudin after APAP metabolism and NAPQI-protein binding had occurred (Saito et al., 2010). The increase in plasma ALT activity and histopathological changes 6 h after APAP were less severe in mice given lepirudin compared to mice given saline vehicle (Figure 8A and B).

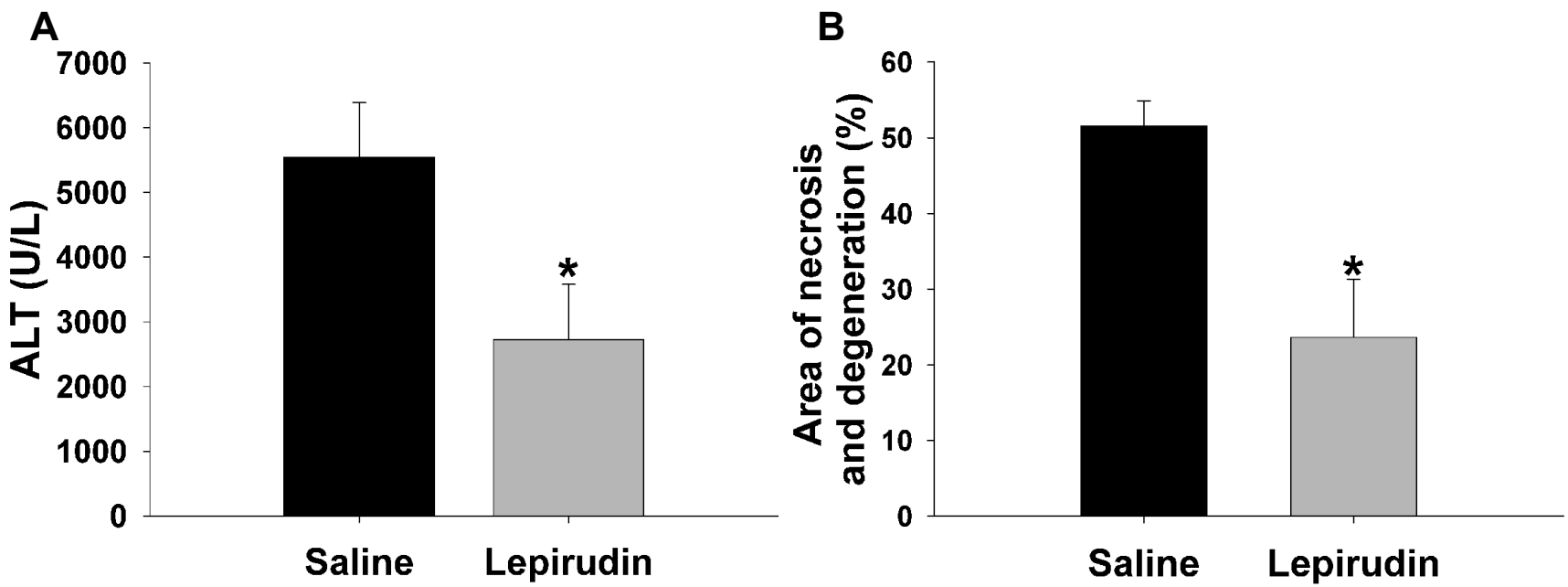

Figure 8: Effect of lepirudin post-APAP on APAP-induced hepatotoxicity.

Mice were treated with 300 mg/kg APAP or sterile saline i.p. at time 0 h. Lepirudin (1.5 mg/kg) was given at +1.5, +2.5 and +4.5 h after APAP or saline. Liver injury was determined 6 h after APAP administration. (A) Plasma ALT activity. (B) Area of necrosis and degeneration. *Significantly different form the saline-pretreated group. N =7 per group.

APAP-induced hepatic platelet accumulation and injury are PAR-4 dependent

Because we found that thrombin activity contributed to hepatic platelet accumulation after APAP overdose, we examined the role of the platelet thrombin receptor, PAR-4 (Coughlin et al., 2005). Early hepatic platelet accumulation (2 h) was significantly reduced in APAP-treated PAR-4$^{-/-}$ mice compared to APAP-treated matching WT mice (Figure 9A), and the increase in plasma TAT concentration was attenuated (Figure 9B). The reduction in hepatic platelet accumulation in PAR-4$^{-/-}$ mice was accompanied by a significant reduction in APAP-induced liver injury, indicated by reduced serum ALT activity and liver necrosis at both 6 and 24 h (Figure 9C, D, and Figure 10). Also, PAR-4 deficiency did not affect hepatic GSH depletion after APAP administration (Figure 11). Accumulation of RBCs in hepatic lesions at 6 h was mainly related to sinusoidal congestion and was more pronounced in PAR-4$^{-/-}$ mice (Figure 9E and Figure 10). In contrast, RBC accumulation at 24 h was predominantly due to hemorrhage and was less in PAR-4$^{-/-}$ mice than in matching WT mice treated with APAP. As with lepirudin pretreatment (Figure 6E), there was a significant decrease in nitrotyrosine labeling in liver tissue collected at 6 h from PAR-4$^{-/-}$ mice (Figure 9F).

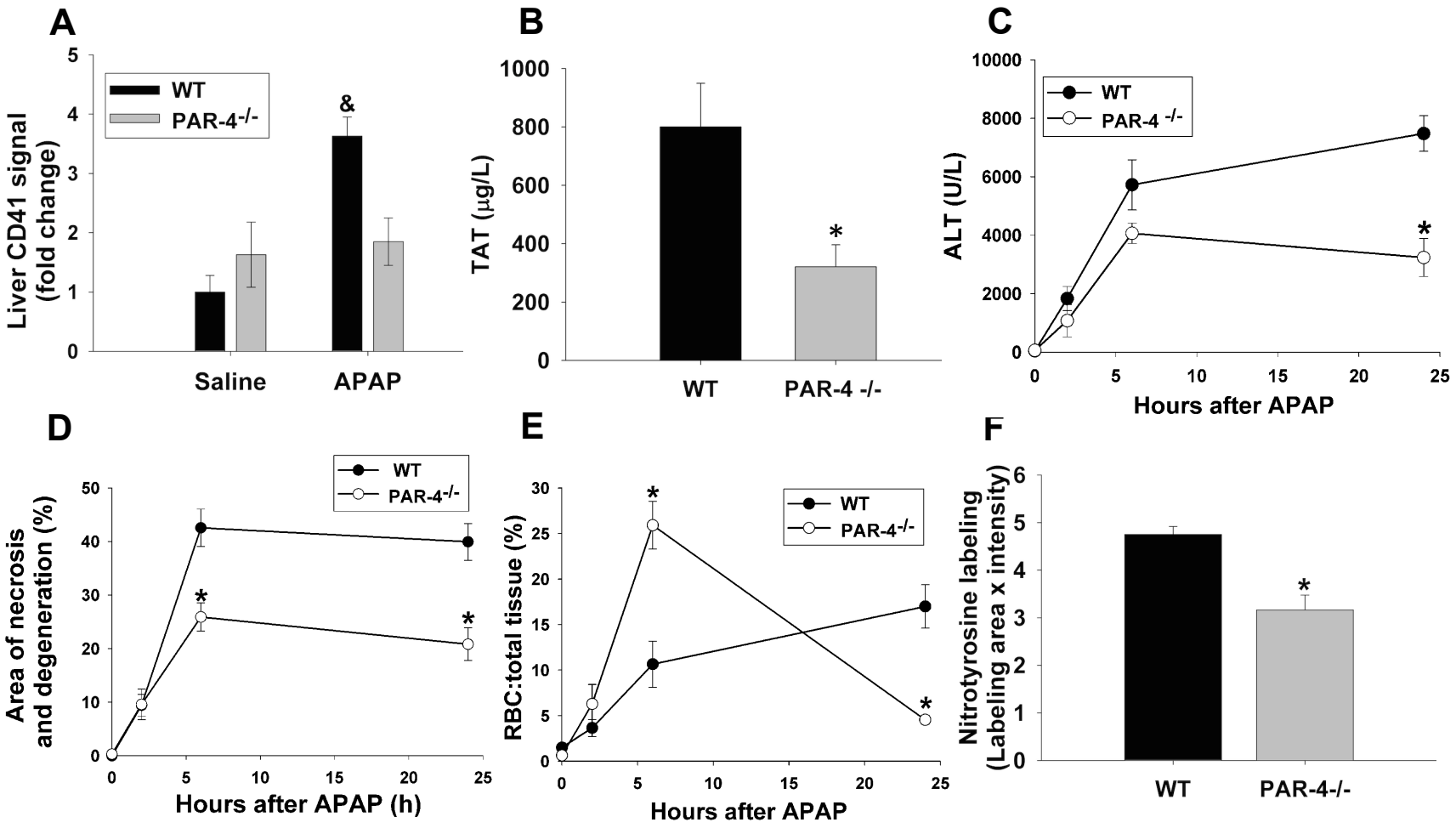

Figure 9: APAP-induced liver injury in WT and PAR-4$^{-/-}$ mice.

Figure 9 (cont'd)

PAR-4 $^{-/-}$ mice and matching WT mice were treated with 300 mg/kg APAP i.p. (A) Quantification of CD41 signal in the liver at 2h. Signal intensity of CD41 was normalized to that of matching WT mice not treated with APAP. (B) TAT concentration in plasma at 2 h after APAP injection. (C) ALT activity in plasma. (D) Area of necrosis and degeneration in the liver. (E) RBC to total liver tissue (%). (F) Nitrotyrosine label quantified in liver sections as described in Materials and Methods at 6 h after APAP injection. $^{\&}$Significantly different from all the other groups. *Significantly different from the matching WT group at same time. N = 3-14 per group.

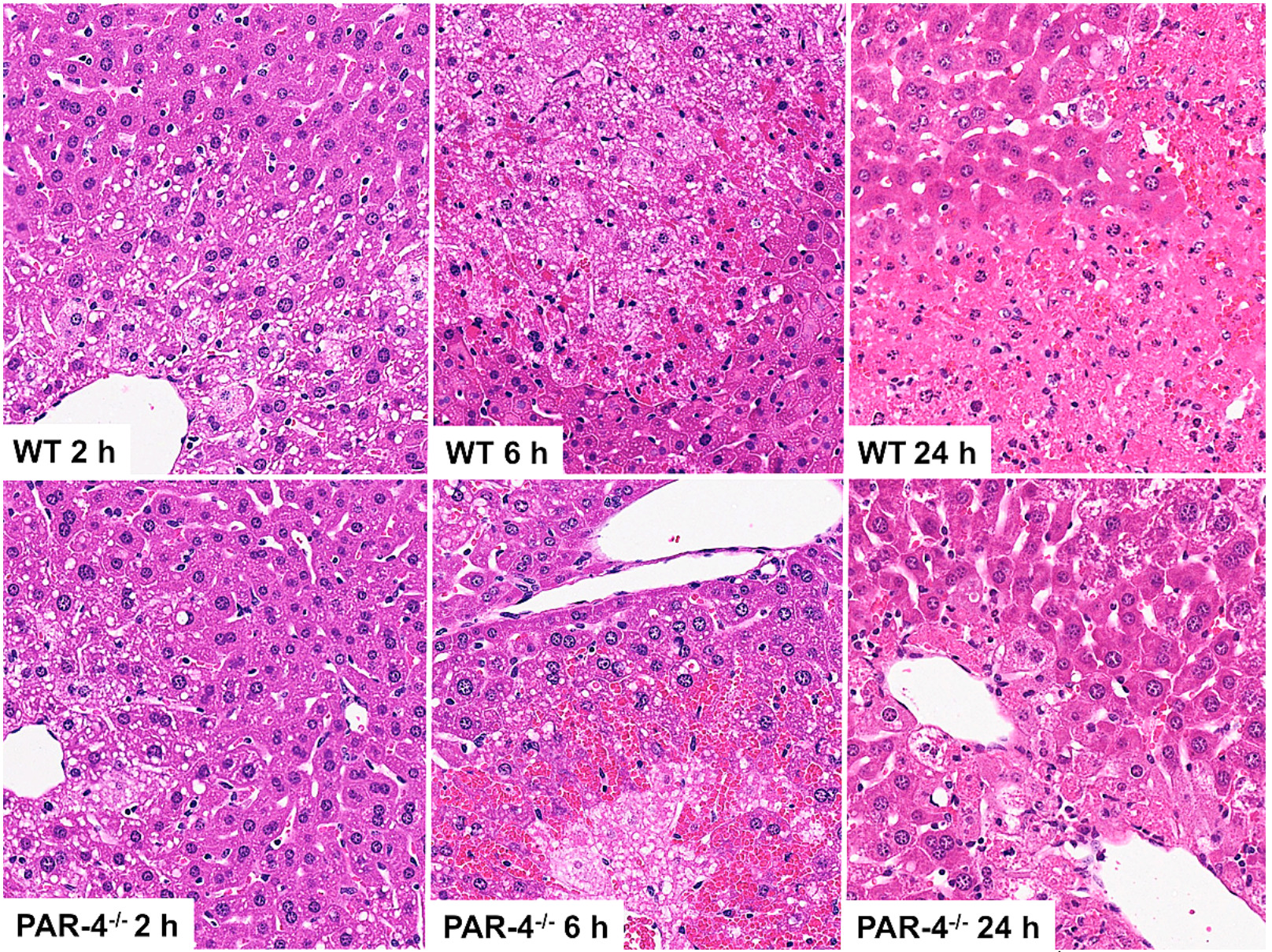

Figure 10: Histopathology of APAP-induced liver injury in WT and PAR-4$^{-/-}$ mice.

Figure 10 (cont'd)

PAR-4 $^{-/-}$ mice and matching WT mice were treated as mentioned in Figure 8. Representative sections taken at 2 h, 6 h, or 24 h from matching WT and PAR-4$^{-/-}$ mice treated with APAP (20 × objective).

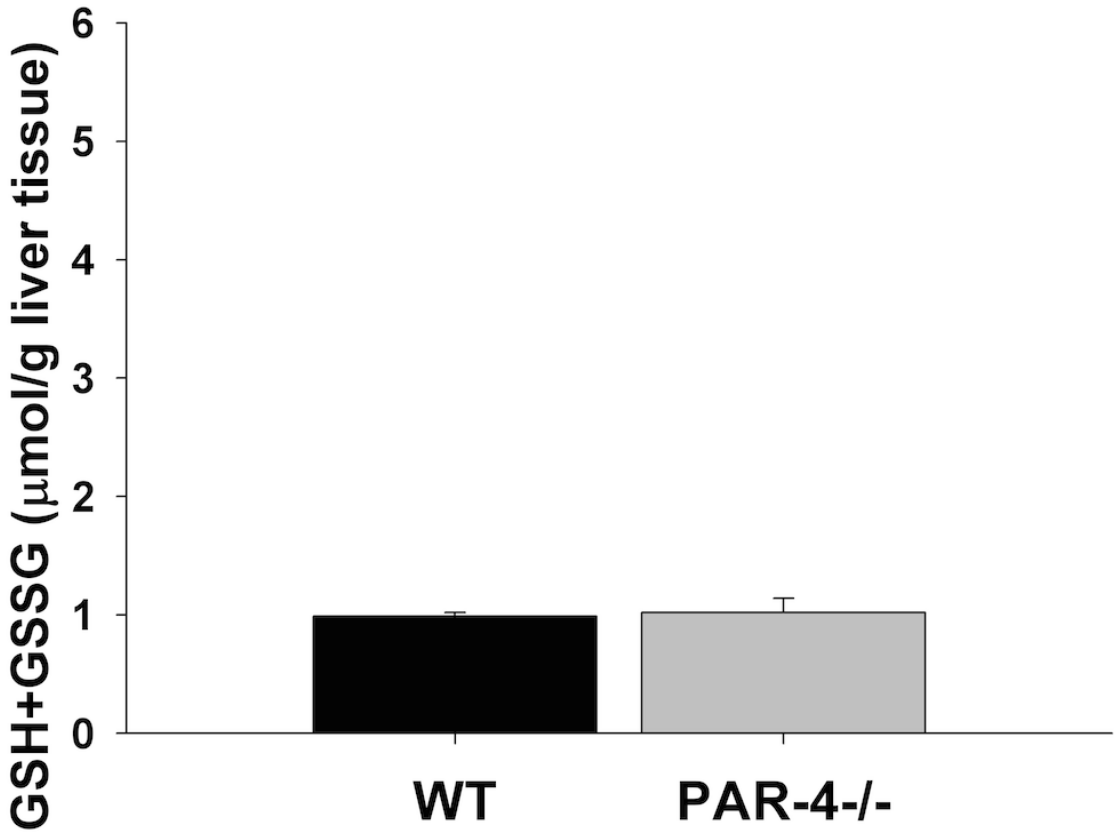

Figure 11: Evaluation of bioactivation of APAP with PAR-4$^{-/-}$ mice. Glutathione concentration in liver was determined 60 min after administration of APAP (300 mg/kg) or vehicle (saline) to PAR-4$^{-/-}$ mice or matching WT mice. N = 4 per group.

Platelets contribute to APAP-induced liver injury and coagulation

Pretreatment with anti-CD41 antibody prior to administration of APAP decreased the platelet concentration in blood at 0 h by greater than 90% compared to control IgG-pretreated mice (Figure 12A). This difference was apparent before and up to 24 h after APAP administration, and corresponded to attenuation of hepatic platelet accumulation and thrombin generation (i.e., plasma TAT concentration) in APAP-treated mice (Figure 12B and C). Platelet depletion significantly reduced APAP-induced liver injury at both 6 and 24 h (Figure 12D, E, and H). Hepatic RBC accumulation apparent in mice given APAP and control IgG was not seen in platelet-depleted mice (Figure 12F and H). Hepatic GSH concentration 30 min after APAP treatment was not affected by platelet depletion (Figure 13A). It has been suggested that antibody pretreatment triggers the induction of protective genes such as HO-1 and metallothioneins (Jaeschke et al., 1990). However, no effect of treatment with either control IgG or anti-CD41 IgG on expression of these genes was observed (Figure 13B and C).

As observed with lepirudin pretreatment and in PAR-4$^{-/-}$ mice, nitrotyrosine signal in liver sections was significantly reduced in platelet-depleted mice compared to control IgG-treated mice at 6 h after APAP administration (Figure 12G).

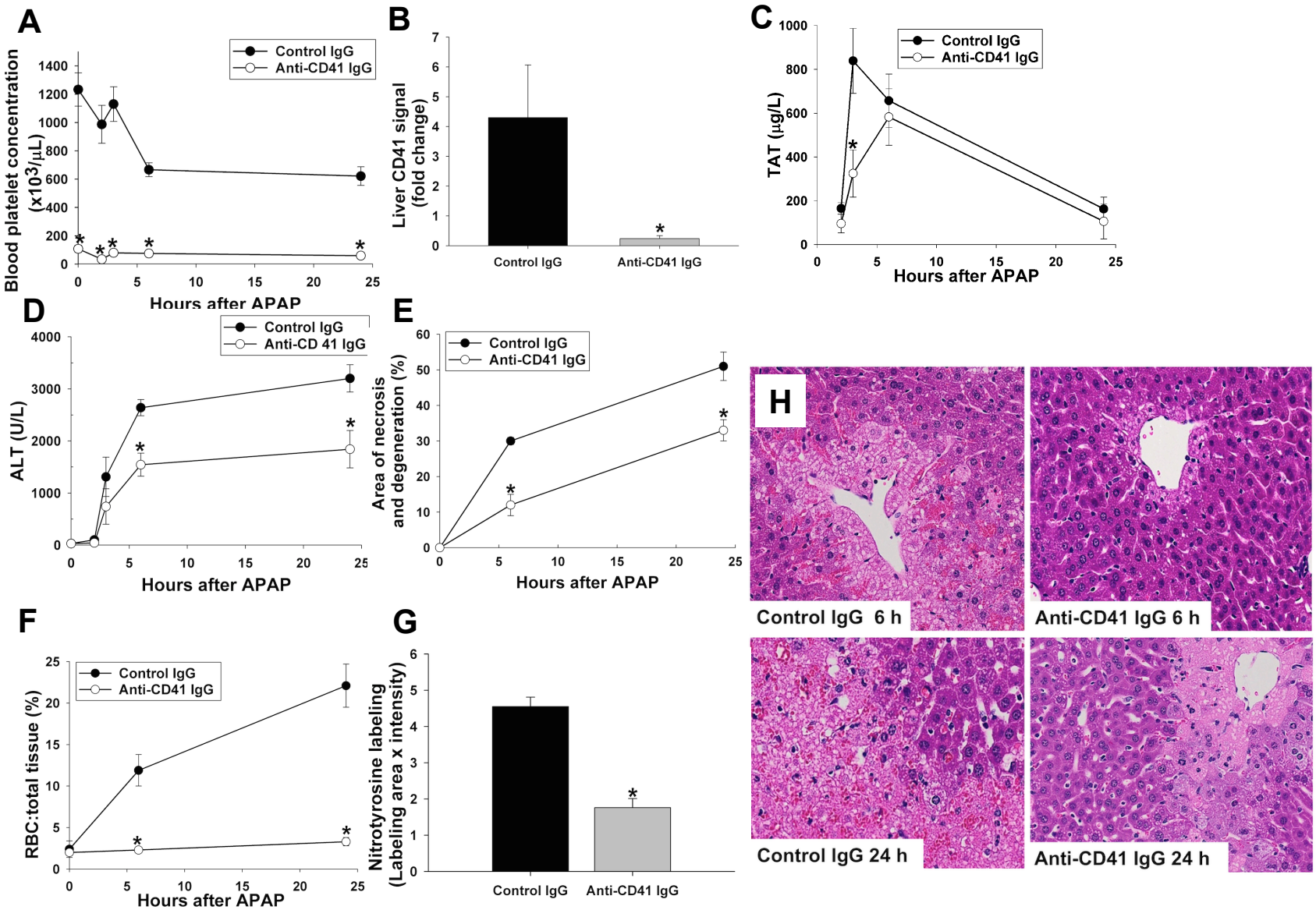

Figure 12: Effect of platelet depletion on APAP-induced hepatotoxicity.

Figure 12 (cont'd)

Mice were treated with 300 mg/kg APAP or sterile saline i.p. at time 0 h. Anti-CD41 antibody (2 mg/kg) was given i.p. 12 h and 2 h before administration of APAP or saline. (A) Blood platelet concentration. (B) Quantification of CD41 signal in the liver at 3 h after APAP injection. Signal intensity of CD41 from different groups was normalized to saline-pretreated group at time 0 h. (C) Concentration of TAT complexes in plasma over time. (D) ALT activity in plasma. (E) Area of liver necrosis and degeneration. (F) RBC to total liver tissue (%). (G) Nitrotyrosine label quantified as described in Materials and Methods at 6 h at after APAP injection. (E) Representative liver sections from each group at 6 h and 24 h after APAP injection (20× objective). * Significantly different from Control IgG group at the same time. N = 3-15 per group.

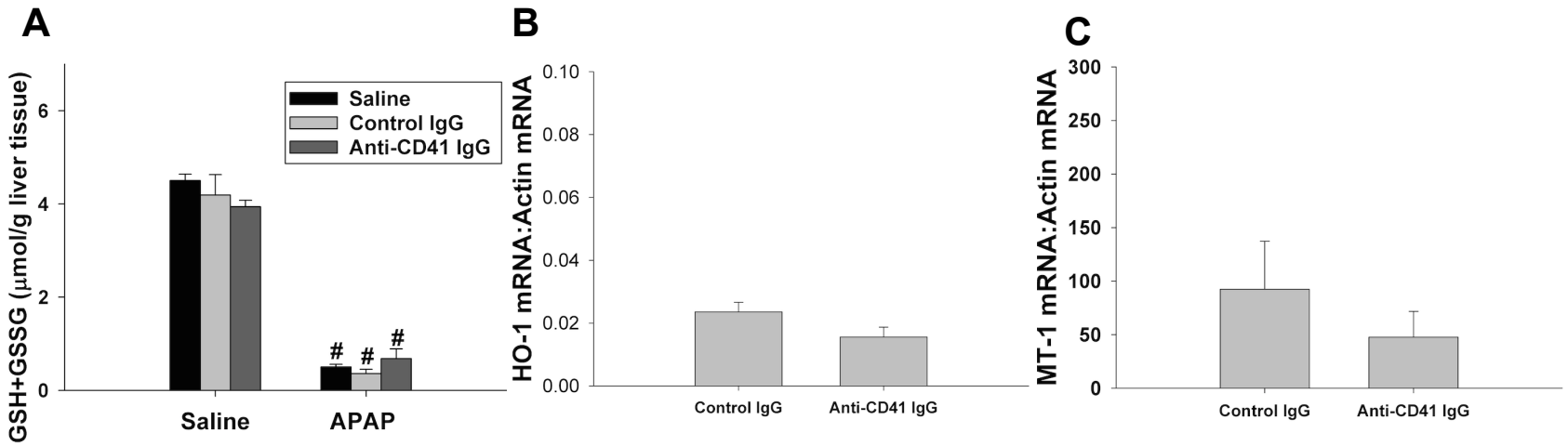

Figure 13: Evaluation of bioactivation of APAP and antibody-related induction of protective genes with anti-CD41 antibody.

GSH level in liver was determined 30 min after administration of APAP (300 mg/kg) or vehicle (saline) to mice. Data are from the (A) platelet depletion studies described in Results. mRNA expression of HO-1 and MT-1 was determined at the time when APAP would have been given in the platelet depletion study. (B) Ratio of HO-1 mRNA to actin mRNA. (C) Ratio of MT-1 mRNA to actin mRNA. # Significantly different from respective group treated with saline. N = 3-5 per group.

2.5 Discussion

APAP overdose is responsible for many cases of liver injury and acute liver failure (Ostapwicz et al., 2002; Kahshab et al., 2007; Fontana et al., 2008; Karvellas et al., 2010). Treatment options are limited, and understanding mechanisms involved in liver damage might provide alternative treatment approaches. Evidence suggests that platelets and the coagulation system contribute to liver damage from APAP overdose (Ganey et al., 2007; Grèen et al., 1989; Guarner et al., 1988; Grypioti et al., 2005, 2006), and we have previously demonstrated that anticoagulant heparin and PAR-1 deficiency reduce liver toxicity from APAP in mice (Ganey et al., 2007). In this study, the role of platelets and the coagulation system were evaluated further.

APAP administration resulted in a decrease in the concentration of platelets in blood and an increase in platelet accumulation in the liver as early as 2 h. This accumulation of platelets within the liver began before an increase in ALT or necrosis was evident, raising the possibility that they contribute to APAP-induced liver damage. At 2 h, before the onset of liver injury, the platelet distribution in liver sections was diffuse, but from 3 h onward, platelets appeared to be associated mainly with the centrilobular regions of the lobules, the areas where necrosis would develop.

As mentioned, we reported previously that the concentration of TAT complexes in blood spiked at the onset of APAP-induced liver injury (Ganey et al., 2007), and injury was ameliorated by pretreatment with anticoagulant heparin, suggesting a role for the coagulation system (Ganey et al., 2007). Heparin inactivates thrombin by increasing its affinity for antithrombin III, but it also affects other coagulation factors (Björk et al., 1982;

Gray et al., 2012) and has numerous nonanticoagulant activities. For example, heparin can inhibit activation of inflammatory cells and interfere with factors released during inflammation (Lever et al., 2012). Accordingly, we tested the effect of lepirudin, a direct, selective inhibitor of thrombin, to determine whether thrombin activity contributes to liver injury and platelet accumulation in APAP-treated mice. Lepirudin cotreatment reduced both the increase in plasma ALT activity and hepatocellular necrosis and decreased platelet accumulation caused by APAP. These results suggest that thrombin plays an important role in the hepatic platelet accumulation accompanying APAP-induced hepatotoxicity in mice. Interestingly, lepirudin also reduced liver injury when it was given 1.5 h after APAP, a time when bioactivation of APAP to NAPQI is nearly complete (Saito et al., 2010). This observation indicates that lepirudin did not reduce toxicity by inhibiting bioactivation of APAP, as confirmed by lack of effect of lepirudin on the loss of glutathione after administration of APAP. Furthermore, it suggests that at least some of the injurious effects of thrombin are not complete by 1.5 h after administration of APAP.

Based on these findings, one might consider use of a thrombin inhibitor to treat APAP hepatotoxicity, but it should be noted that in this study, one of the lepirudin-treated mice given APAP died prior to the sample collection time, with hemoabdomen and pronounced, diffuse hemorrhage in the liver. This mouse might have been more sensitive than the others to thrombin inhibition, with consequent life-threatening hemorrhage. This emphasizes the potential risks of using thrombin inhibitors in the treatment of human APAP poisonings. In contrast, no deaths occurred among the PAR-4$^{-/-}$ mice. Accordingly, selective interference with thrombin receptor(s) rather than global elimination of thrombin's many effects might be a safer option for reducing APAP-

induced liver injury.

One of the critical roles of thrombin is conversion of fibrinogen to fibrin. Although fibrin is deposited in livers of mice after exposure to a hepatotoxic dose of APAP, complete fibrinogen deficiency did not affect early hepatotoxicity, thus suggesting that the harmful role of thrombin is due to another of its actions (Sullivan et al., 2012). In addition to its participation in coagulation, thrombin activates protease-activated receptors. We reported previously that PAR-1-deficient mice experienced less liver injury from APAP than did with matching WT mice (Ganey et al., 2007). In this study, the role of PAR-4, a thrombin receptor that is expressed on mouse platelets (Coughlin 2005), was evaluated. PAR-4$^{-/-}$ mice developed less liver injury 6 h to 24 h after APAP compared to matching WT controls. This protection was observed under conditions in which bioactivation was similar between matching WT and PAR-4$^{-/-}$ mice (Figure 10). These results suggest that PAR-4 plays an important role in the progression of APAP hepatotoxicity in mice. Interestingly, thrombin activation and platelet accumulation in the liver at 2 h were less in PAR-4$^{-/-}$ mice, indicating that PAR-4 activation contributes to these events.

Platelets are stimulated by PAR-4 activation and accumulate in the liver after a hepatotoxic dose of APAP, and deficiency in PAR-4 ameliorates liver damage. To evaluate a potential role for platelets in liver injury, platelets were depleted prior to administration of APAP. Depletion of platelets did not alter bioactivation of APAP (Figure 12A), but liver injury and platelet accumulation were significantly reduced in platelet-depleted mice. Also, the spike in plasma TAT concentration that occurred at 3 h in APAP-treated mice was significantly reduced by platelet depletion. This suggested

that platelets are involved in the early, APAP-induced thrombin generation that occurs in this model. In light of the observation that there was also less thrombin generation in PAR4$^{-/-}$ mice, it seems likely that PAR-4 on platelets is involved in this response.

Evidence exists for the formation of peroxynitrite after exposure to toxic doses of APAP (Hinson et al., 1998; Knight et al., 2001, 2002). Peroxynitrite is thought to arise from NO and O_2^- produced in response to APAP exposure. Resultant nitration of proteins detected by IHC occurs first in sinusoids and later in parenchymal cells (Lever et al., 2012). In addition to sinusoidal endothelial cells and parenchymal cells, platelets contain nitric oxide synthase (NOS) isoforms and can produce NO (Gkaliagkousi et al, 2007). Accordingly, thrombin-activated platelets might contribute to NO release in APAP hepatotoxicity, with consequent formation of peroxynitrite and nitration of proteins. Our evaluation confirmed observations by others that nitrotyrosine-protein adducts accumulate in liver tissue in response to APAP exposure. Early nitrotyrosine labeling (2-3 h) tended to be sinusoidal, whereas later (6 h) signals localized to parenchymal cells. Evidence exists that thrombin can enhance NOS production through thrombin receptors (Meli et al., 2001; Touyz 2007; Momota et al., 2006). Interestingly, lepirudin treatment, deficiency in PAR-4, and platelet depletion each reduced nitrotyrosine adduct labeling at 6 h. These findings suggest that platelets activated by thrombin stimulation of PAR-4 can contribute to peroxynitrite generation in APAP hepatotoxicity.

Accumulation of RBCs due to congestion and hemorrhage was observed in and around the centrilobular lesions of APAP-treated mice. Prior platelet depletion reduced the RBC accumulation, as did treatment with lepirudin, suggesting that thrombin and platelets contribute to the congestion and hemorrhage during the pathogenesis of liver

injury. This result was somewhat surprising, since platelets and fibrin cover defects in endothelium and might therefore be expected to reduce hemorrhage. It could be that formation of platelet plugs in injured sinusoids slowed blood flow and increased microvascular pressure, thereby favoring accumulation of RBCs and their exit from the sinusoidal lumen (i.e., hemorrhage). Congestion and hemorrhage can lead to tissue hypoxia, which occurs after a hepatotoxic dose of APAP (Chandhuri et al., 2011). Interestingly, RBCs can produce and release NO under hypoxic conditions (Nagababu et al., 2003; Allen et al., 2009). Thus, it seems possible that RBCs accumulated in developing lesions could release NO and thereby contribute to peroxynitrite generation and nitration of cellular proteins.

Hepatic RBC accumulation was also decreased at 24 h in PAR-4$^{-/-}$ mice treated with APAP. Unlike the observation at 6 h in platelet-depleted mice, RBC accumulation was enhanced at 6 h in PAR-4$^{-/-}$ mice. The enhanced RBC accumulation at 6 h was related more to congestion than to hemorrhage, whereas hemorrhage predominated at 24 h. It has been reported that PAR-1 and PAR-4 on endothelial cells contribute to vascular relaxation and microvascular patency (Kataoka et al., 2003); accordingly, impaired sinusoidal relaxation in the PAR-4$^{-/-}$ mice might have contributed to the greater congestion seen 6 h after APAP treatment.

In conclusion humans suffering from APAP overdose, evidence exists for activation of the coagulation system and of platelets (Lee 1993; Larson et al., 2005). This is supported by our findings of thrombin activation and platelet sequestration in livers of mice given a hepatotoxic dose of APAP. Hepatocellular necrosis, congestion/hemorrhage, and protein nitration in liver were reduced by a selective

thrombin inhibitor, by PAR-4 deficiency, and by platelet depletion, suggesting that thrombin, the thrombin receptor PAR-4, and platelets contribute to the progression of APAP-induced liver injury. None of these effects appears to be due to impaired bioactivation of APAP by the liver. Our results also suggest that platelets might contribute to the release of NO needed for generation of peroxynitrite and consequent nitration of proteins. Platelet depletion also reduced the early appearance of TAT complexes in plasma, suggesting that platelets contribute to thrombin generation. Previously, we showed that tissue factor is activated very early (30 min) after APAP administration (Ganey et al., 2007). These results coupled with the observation that thrombin inhibition reduced hepatic platelet accumulation suggest an amplification loop in which early activation of tissue factor results in generation of thrombin, which in turn activates platelets that encourage additional thrombin activation. Our previous results also pointed to a role for PAR-1 in the progression of hepatocellular injury. Inasmuch as PAR-1 does not occur on mouse platelets, and since PAR-4 can occur on cells other than platelets, activation of these receptors on NPCs by thrombin is likely important. Although this study points to platelets as important contributors to APAP-induced liver injury, a full understanding of the mechanisms involved in their capacity to promote injury requires additional investigation.

CHAPTER 3:

Influence of vWf and the contribution of NPCs to APAP-induced death of hepatocytes

3.1. Abstract

The previous chapter revealed that thrombin-mediated activation of platelets contributes to amplification of the procoagulant response and to the progression of APAP-induced liver injury in mice. Here we further evaluated the distribution of platelets in APAP hepatotoxicity and association with other cells using transmission electron microscopy (TEM), and the contribution of vWf (one of the key players in platelet adhesion) to APAP hepatotoxicity using vWf- deficient mice. Following that, we assessed the direct and indirect effects of thrombin in vitro by using primary mouse HPCs cocultured with or without liver NPCs.

TEM of liver tissues from APAP-treated mice revealed frequent platelet aggregation in sinusoids around the borders of the necrotic areas, occasional accumulation of RBCs in the sinusoids, and possible association of these platelets with endothelial cells, monocytic cells (Kupffer cells and/or macrophages), and hepatocytes. Previously, thrombin inhibition using lepirudin, PAR-4$^{-/-}$ mice and platelet depletion showed a decrease in liver injury with APAP treatment. Interestingly, there were significant decreases in platelet accumulation in the liver and plasma TAT complexes at early times with these experiments. These results suggested that thrombin-mediated activation of platelets contributes to amplification of the procoagulant response and to the progression of APAP-induced liver injury in mice.

Although the vWf$^{-/-}$ mice had less platelet accumulation in the liver, there was no significant difference in liver injury and plasma TAT complexes compared to the matching WT group. This suggests that vWf contributes to the accumulation of platelets

in the liver with APAP hepatotoxicity, but that vWf-mediated platelet events are not critical to APAP hepatotoxicity. These results also suggested that thrombin might play a more important role in APAP hepatotoxicity compared to platelets. In vitro experiments revealed that thrombin does not have a direct effect to enhance hepatocellular injury in APAP-treated primary HPCs. However, when primary HPCs were cocultured with NPCs, thrombin treatment enhanced the APAP hepatocellular toxicity. This suggests that thrombin likely enhances liver injury indirectly by stimulating liver NPCs.

3.2. Introduction

Previously, we found that APAP overdose in mice caused thrombocytopenia associated with accumulation of platelets in the injured liver. This liver injury was attenuated by administration of the direct thrombin inhibitor, lepirudin, by using mice deficient in PAR-4, or by platelet depletion with an anti-CD41 antibody. These results suggested that thrombin-mediated activation of platelets contributes to amplification of the procoagulant response and to the progression of APAP-induced liver injury.

Others have evaluated the morphological changes in liver cells over time in APAP treated mice using transmission electron microscopy (TEM) and scanning electron microscopy (Dixon et al.1974, Walker et al. 1980, 1983). However, only little has been described about platelets (Walker et al. 1980, 1983). Here we evaluated the distribution of platelets and association with other cells in APAP hepatotoxicity using TEM.

vWf is a large, multimeric glycoprotein synthesized in endothelial cells and megakaryocytes. It circulates in the blood, binding with coagulation factor VIII (in inactive form). It is also present in endothelial cells and in α-granules of platelets. One of the important functions of vWf is to mediate platelet adhesion to sites of vascular injury by binding to subendothelial collagen and to GP1bα on platelets (Figure 14). This is one of the first steps in thrombosis and vWf also contributes to inflammation by acting as a ligand for leukocyte receptors like P-selectin (De Meyer et al. 2012).

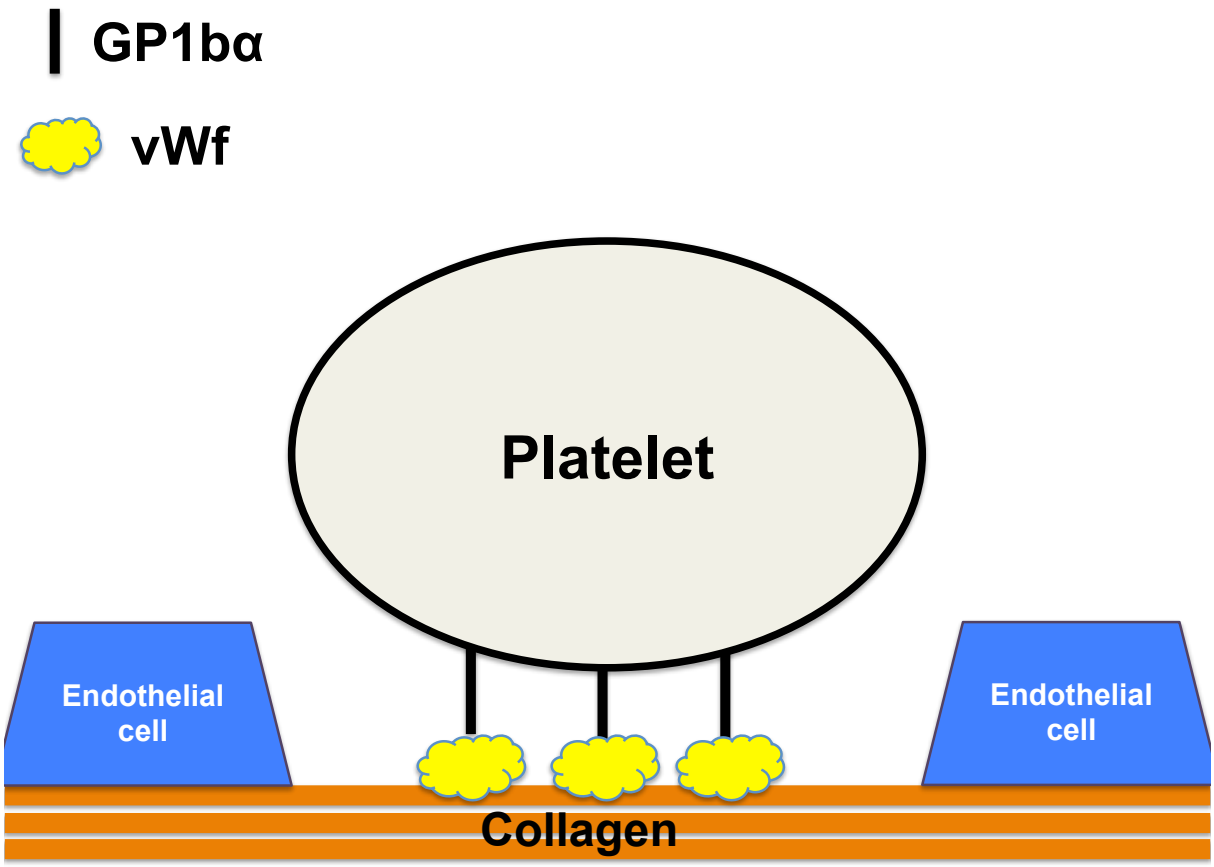

Figure 14: Platelet adhesion to subendothelial collagen

NAC has been used for the treatment of APAP hepatotoxicity. Recently, it has been shown that NAC reduces the size of vWF multimers (the most functional form), the activity of vWF and vWF-dependent platelet aggregation (Chen et al. 2011). This anti-vWF effect might contribute to NAC's protective effect in APAP toxicity. Also, it has been reported that APAP treatment in mice increases vWF (Hsu et al. 2006). Based on our findings in Chapter 2 (PAR-4$^{-/-}$ mice and platelet depleted model) and reports from others, we hypothesized that platelet adhesion through vWf plays a role in APAP hepatotoxicity. To evaluate this hypothesis, we used genetically modified, vWf-depleted mice.

The findings with vWf$^{-/-}$ mice treated with APAP suggest that vWf does not contribute to APAP hepatotoxicity. Interestingly, there were similar significant increases in plasma TAT complexes in both vWf$^{-/-}$ and matching WT groups after APAP treatment, but platelet accumulation in the liver with the vWf$^{-/-}$ group was significantly less than the matching WT group. This was surprising, because in previous experiments with a thrombin inhibitor, PAR-4$^{-/-}$ mice or platelet depletion, there was a decrease in liver injury and an early decrease in plasma TAT complexes and reduced platelet accumulation in the liver. These findings further suggest that thrombin might play a more important role in APAP hepatotoxicity than platelets. Based on these findings we evaluated the direct effect of thrombin on APAP-treated primary mouse HPCs and primary mouse HPCs co-cultured with liver NPCs.

3.3 Materials and Methods

Materials

APAP (A7302) was purchased from Sigma- Aldrich Co. (St. Louis, MO). Anti-CD41 antibody (Rat IgG1, 133902) for platelet immunofluorescence was purchased from Biolegend Inc. (San Diego, CA). Infinity ALT Reagent (TR71121) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). TAT ELISA kit (Enzygnost TAT Micro Kit) was obtained from Siemens Healthcare Diagnostics Inc. (OWMG15 Deerfield, IL). Collagenase from *Clostridium histolyticum* and Prostaglandin E1 (PgE1) were purchased from Sigma-Aldrich (St Louis, MO). Antibiotic-antimycotic (ABAM), Dulbecco's PBS, Alexa fluor 488 Goat Anti-Rat IgG, liver perfusion medium, hepatocyte wash medium, fetal bovine serum (FBS), L-glutamine, and Williams' Medium E (WME) were purchased from Life Technologies (Carlsbad, CA). Type I collagen from rat tail was purchased from BD Bioscience (San Jose, CA). Bovine insulin for cell culture was purchased from Cell Applications (San Diego, CA). Human α thrombin was purchased from Enzyme Research Laboratories (South Bend IN). DNase I was purchased from Roche (Lavel, QC, Canada).

Animals

C57Bl/6J mice (9 weeks of age) used for hepatocyte and NPC isolation and for TEM studies were purchased from Jackson Laboratory (Bar Harbor, ME). $vWf^{-/-}$ (Denis et al., 1998) and matching WT mice of an identical genetic background (C57Bl/6J) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained by homozygous

breeding, and aged-matched males were used for experiments after confirming genotype by PCR analysis. All mice were maintained at 22°C with alternating 12 h light/dark cycle and access to standard chow diet (Teklad 22/5 Rodent Diet 8940: Harlan Laboratories [Indianapolis, IN]) and spring water ad libitum. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee.

Experimental Protocols for TEM study

All mice were fasted for approximately 15 h before being treated with APAP (300 mg/kg) or saline by i.p. injection. At termination of experiments (0, 3, 6 h), mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and liver tissues (approx. 0.5 mm cube) from the left lobe were collected immediately and fixed with 2.5% paraformaldehyde +2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4 °C for 24 hours, post-fixed in 1% osmium tetroxide and dehydrated in a graded acetone series. Samples were infiltrated and embedded in Spurr resin (Polysciences). Thin sections (70 nm thickness) were obtained with a PTXL ultramicrotome (RMC, Boeckeler Instruments, Tucson, AZ) on 200 mesh copper grids stained with uranyl acetate and lead citrate. Sections were imaged using a JEOL 100CX Transmission Electron Microscope (Japan) at a 100 kV accelerating voltage.

Experimental Protocols for vWf study

All mice were fasted for approximately 15 h before being treated with APAP (300 mg/kg) or saline by i.p. injection. At termination of experiments, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and blood and liver tissues were collected immediately. Blood was drawn from the caudal vena cava into a syringe containing 3.2% sodium citrate (final concentration 0.32%) for plasma preparation. The left lateral liver lobe was collected and fixed with 10% neutral-buffered formalin for 24 h, then the formalin was replaced with 70% ethanol for histopathology. The right lobe was collected and embedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) medium and frozen in liquid nitrogen-chilled isopentane for immunofluorescence studies.

Plasma TAT complexes

Citrated plasma samples were thawed at room temperature, and plasma TAT complexes were measured using a TAT ELISA kit.

Liver Injury

Liver injury was estimated from increases in plasma ALT activity, determined spectrophotometrically, and by morphometric evaluation of left lateral liver lobes. Formalin-fixed livers were embedded in paraffin, sectioned 5 μm thick, and stained with hematoxylin-eosin. Slides were scanned and digitized using the Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20× objective. An automated, random sub-sampling of 60 images was further performed on the digitized slides with NewCast

software (Visiopharm, Hoersholm, Denmark) at 200× magnification. Calculation of the percentage of area with hepatocellular necrosis/degeneration (i.e., moderate to severe irregular vacuolar degeneration adjacent to necrosis) was carried out with the STEPanizer software (http://www.stepanizer.com) (Tschanz et al., 2011). Briefly, a point grid was superimposed over the images, and points intersecting normal liver parenchyma and hepatocellular necrosis/degeneration were counted. The percentage of liver tissue with hepatocellular necrosis/degeneration was estimated by dividing the number of points intersecting hepatocellular necrosis/degeneration by the number of total points on liver tissue (i.e., the sum of the points of normal liver parenchyma and hepatocellular necrosis/ degeneration). The accumulation of RBCs in liver tissue was evaluated similarly.

Platelet Accumulation in the Liver

Frozen liver sections cut 8 μm thick were fixed with 4% neutral buffered formalin for 30 min at room temperature, washed 3 times with PBS for 5 min each, and blocked with 10% goat serum in PBS for 1 h. Sections were incubated with anti-CD41 antibody (1:1000 diluted with 10% goat serum in PBS) overnight at 4 °C. Sections were washed 3 times with PBS for 5 min each and incubated with goat anti-rat Alexa-594-conjugated antibody (1:1000 diluted in 10% goat serum and 2% mouse serum) for 3 h at room temperature. Sections were then washed 3 times with PBS for 5 min each and treated overnight with Slow Fade Gold Antifade reagent with DAPI. Slides were photographed using an Olympus IX71 inverted fluorescent microscope, with the following fluorescence

filter settings: CD41-antibody, excitation 535 nm, emission 645 nm; DAPI: excitation 350 nm, emission 460 nm. Quantification of fluorescence was performed using Image J software. The integrated intensity of CD41 fluorescence from at least 3 representative microscopic fields per liver section was measured using a 10× objective, and the average was calculated as one replicate.

Evaluation of platelets with flow cytometry

Platelet-rich plasma was harvested from whole blood within 30 min of collection. It was collected from each blood sample after each of 2–3 repetitive centrifugations at 300 × g for 3 min. PgE1 was added in a final concentration of 1 μM to samples of platelet-rich plasma prior to pelleting by centrifugation. After discarding the plasma, platelets were washed 3 times at 1300 × g for 3.5 min with 37 °C wash buffer (PBS containing 3% bovine serum albumin) and resuspended in 1 mL wash buffer at 37 °C. Platelet concentrations were then measured using a hemocytometer, and adjusted to 100,000 platelets in 100 μL wash buffer. Samples were incubated at room temperature for 45 min with 30 μg anti-CD41 antibody in 100 μL wash buffer. Following this, samples were washed 3 times with wash buffer and incubated for 45 min in a darkroom with 1.5 μg goat anti-rat Alexafluor 488-conjugated antibody in 100 μL wash buffer. Samples were washed 3 times and were reconstituted in 500 μL flow buffer (PBS, 0.1% sodium azide, 2% fetal calf serum).

Data were acquired using a FACSCalibur flow cytometer (Becton, Dickinson and Company) interfaced with a Power Macintosh computer (Apple, Cupertino, CA, USA) and analyzed via instrument-specific computer software (BD CellQuest Pro software, Becton, Dickinson and Company). The electronic settings for erythrocyte and platelet analysis were as follows: forward scatter (FSC) voltage = E00, AmpGain = 2.25; side scatter (SSC) voltage = 304, AmpGain = 1.32, FL1 voltage = 613; FL2 voltage = 700; and FSC threshold = 100. Platelets were gated based on SSC and FSC; the minor population of contaminating erythrocytes and any platelet clumps or large platelets in the erythrocyte region were excluded. For all samples, data were generated on 10,000 events.

Isolation of primary mouse HPC and NPCs

Primary murine HPCs were isolated as previously reported (Klaunig et al., 1981; Bajt et al., 2004) with some modification. Briefly, each mouse was anesthetized with pentobarbital 50 mg/kg i.p., then the abdominal cavity was opened and a 24 ga catheter was placed and secured in the inferior vena cava. The liver was perfused first with 50 mL of liver perfusion medium supplemented with ABAM. The portal vein was severed, and the anterior vena cava was clamped with a hemostat. The liver was then perfused with 40 mL of WME supplemented with ABAM and 5-10 mg type IV collagenase. After the perfusion was completed, the liver was removed, placed in hepatocyte wash medium and gently broken apart with forceps. The cell mixture was filtered through 3 layers of gauze followed by addition of DNase I (20 µg/mL) and centrifugation at 50g for

2 minutes. Supernatant and pellet were separated, the pellet that contained hepatocytes was washed two more times, and cell viability was evaluated with trypan blue exclusion; cell viability of 85% or greater was the criterion for use in experiments. Supernatant that contained NPCs was centrifuged at 350 × g for 10 min once, and supernatant was removed and fresh WME was added. 2.5×10^5 hepatocytes/well with or without 10×10^5 NPCs/well were cocultured in 24 well plates (coated with type I collagen from rat tail (5 μg/cm^2) overnight) with WME supplemented with 1×10^{-7} M insulin and incubated at 37 °C and 5 % CO_2. After an hour, medium was replaced (to remove unattached cells) and incubated for another 4 h. Hepatocytes with or without NPCs were then gently washed twice with PBS followed by reconstitution in WME supplemented with ABAM and 2 mM L-glutamine. APAP (2.5 mM) and thrombin (30 U/mL) were added, and cells were incubated for an additional 18 h.

ALT assay for in vitro hepatocellular injury

Medium was collected, and remaining cells were lysed and collected by adding an equal volume to the medium of 1% Triton X-100 and scraping with a cell scraper. Medium and cell lysate were centrifuged at 660 × g for 5 min. Cytotoxicity was assessed by measuring ALT release into the medium in duplicate using Infinity-ALT reagent. ALT activity in the medium was expressed as a percentage of total ALT activity (i.e., medium plus lysate).

Statistical Analysis

Data are expressed as mean ± SEM. Data that were not normally distributed were subjected to appropriate transformation. Student's t-test was used to compare two means, and analysis of variance was used when more than two means were compared. If a difference was detected with analysis of variance, appropriate post-hoc testing (Holm-Sidak) was performed. If data transformation failed to generate data with a normal distribution, nonparametric tests were performed (Mann-Whitney U test for comparison of two means, and Kruskal-Wallis one-way analysis of variance for multiple comparisons). $P < 0.05$ was set as the criterion for statistical significance.

3.4. Results

Platelets accumulate in the liver after APAP treatment

TEM revealed that liver from mice given vehicle had no platelets observed in the sinusoids (Figure 15A and B). In the livers of mice treated with APAP 3 or 6 h earlier, there were platelets in the sinusoids, mostly in loose aggregates (Figure 15C) and associated with monocytic cells (Kupffer cells or macrophages), endothelial cells and hepatocytes (Figure 15D, E, G and H). Most of the platelets still contained their α and dense granules and platelet accumulation was observed around the border of areas of hepatocellular necrosis (Figure 15F). The numbers of platelets also increased with time and degree of injury.

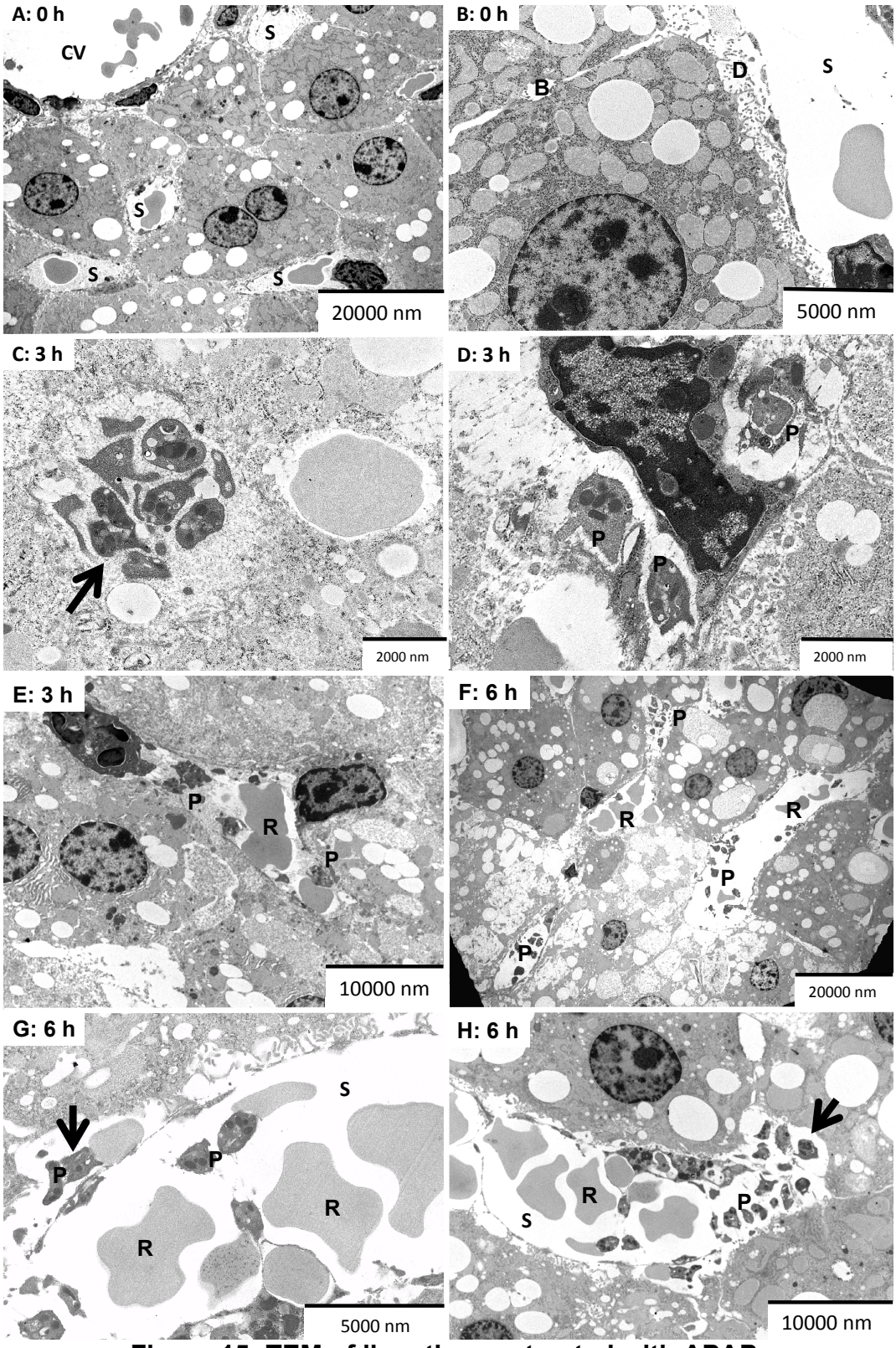

Figure 15: TEM of liver tissues treated with APAP.

Figure 15 (cont'd)

Mice were treated with APAP (300 mg/kg i.p.). Liver tissues were collected at 0, 3, and 6 h. A: Central vein (CV), sinusoid (S). B: Liver before receiving APAP in higher magnification than A. Disse space (D), Bile canaliculus (B). C: Liver 3 h after APAP. Platelets formed an aggregate in the sinus (arrow). D: Liver 3 h after APAP. Platelets (P) associated with monocytic cell (Kupffer cell or macrophage). E: Liver 3 h after APAP. Platelet aggregates (P) and RBCs (R) in the sinusoid. F: Liver 6 h after APAP. Platelet aggregates with RBCs in the sinusoids around the border where hepatocytes are necrotic (hepatocytes in left bottom have loss in density, organelle swelling and nuclear chromatin margination). G: Liver 6 h after APAP. Platelets in loose aggregates and some contacting the endothelial cell lining. Some platelets were observed in the space of Disse (arrow). H: Liver 6 h after APAP. Platelet aggregates likely associated with monocytic cells and hepatocytes. Some platelets are in the space of Disse (arrow). N=2-4 per group.

vWf$^{-/-}$ does not contribute to APAP induced liver injury

Because vWf typically plays a critical role in platelet adhesion to sites of vascular injury, the contribution of vWf was evaluated using vWf$^{-/-}$ mice. Interestingly, liver injury evaluated by ALT and area of necrosis did not show any significant difference between matching WT and vWf$^{-/-}$ groups. This suggests that vWf does not contribute to APAP hepatotoxicity (Figure 16).

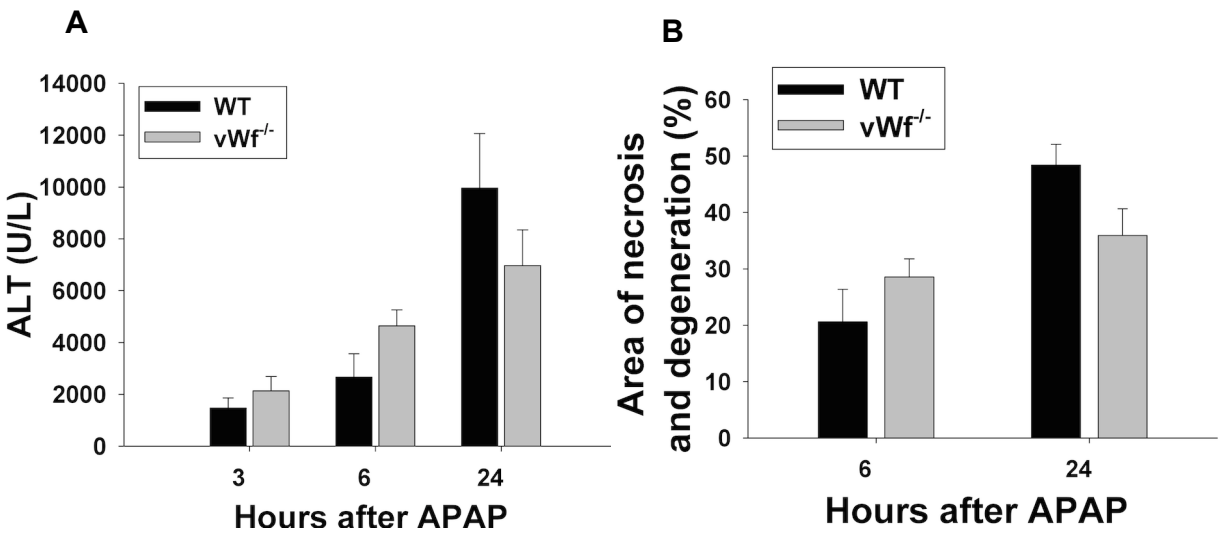

Figure 16: APAP-induced liver injury in WT and vWf$^{-/-}$ mice.

vWf$^{-/-}$ mice and matching WT mice were treated with 300 mg/kg APAP, i.p. A: ALT activity in plasma. B: Area of necrosis and degeneration in the liver. N=4-27 per group.

vWf$^{-/-}$ mice did not have decreased plasma TAT complexes but had significantly decreased platelet accumulation in the liver

There was no significant difference in plasma TAT complexes between the vWf$^{-/-}$ group and matching WT mice at the time the peak in plasma TAT complexes was expected (Figure 17). However, the CD41 signal used to detect platelets in the liver was markedly decreased in vWf$^{-/-}$ mice at the same time that the TAT complex peak was expected (Figure 18A and B). To assess the possibility that platelets in vWf$^{-/-}$ mice might have lower CD41 expression compared to the matching WT mice, flow cytometry was performed on the blood platelets from vWf$^{-/-}$ and matching WT mice using the same anti-CD41 antibody used for platelet labeling in the liver tissue. There were no differences in percentage of positively stained platelets (Figure 18C) or median fluorescence intensity (MdFI) between the groups (Figure 18D).

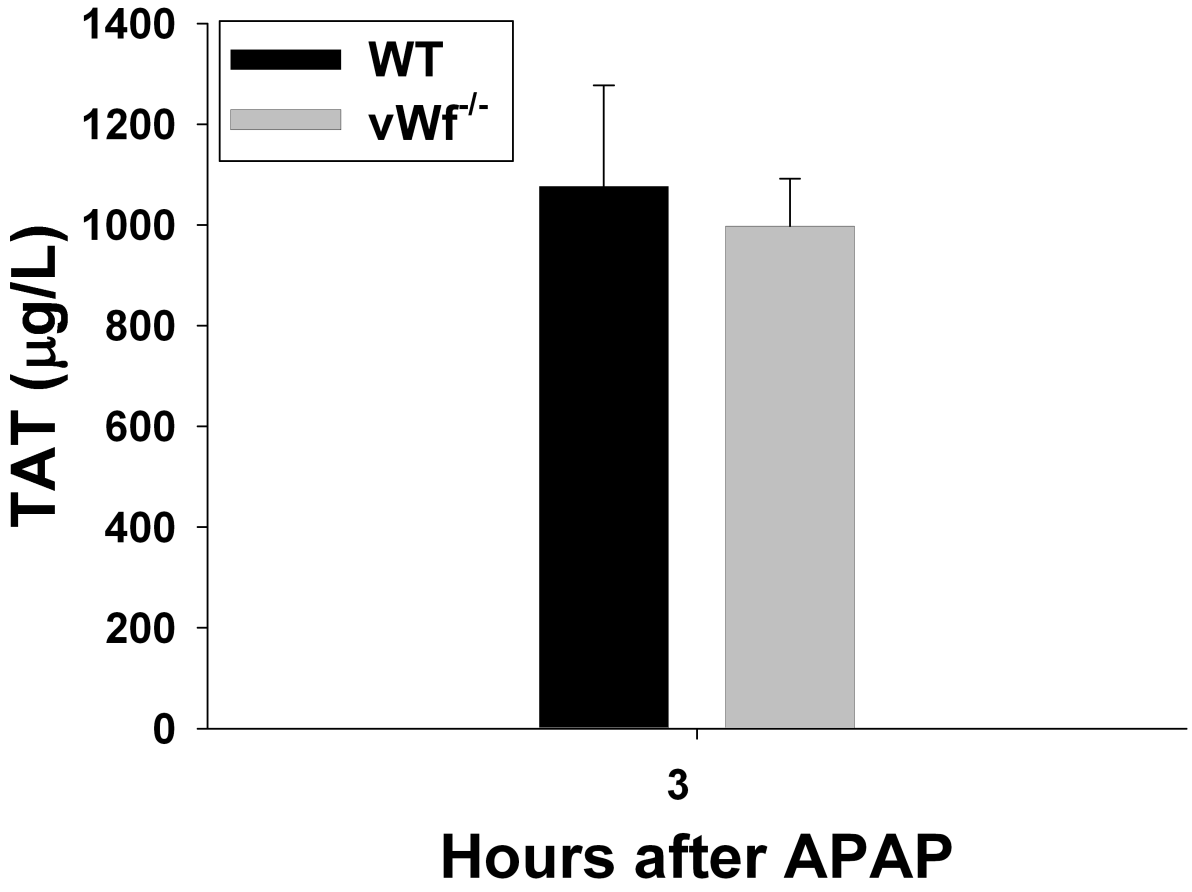

Figure 17: Plasma TAT complex in APAP-induced liver injury in WT and vWf$^{-/-}$ mice.

vWf$^{-/-}$ mice and matching WT mice were treated with 300 mg/kg APAP, i.p.. Plasma TAT concentration 3 h after APAP treatment. N=4-15 per group.

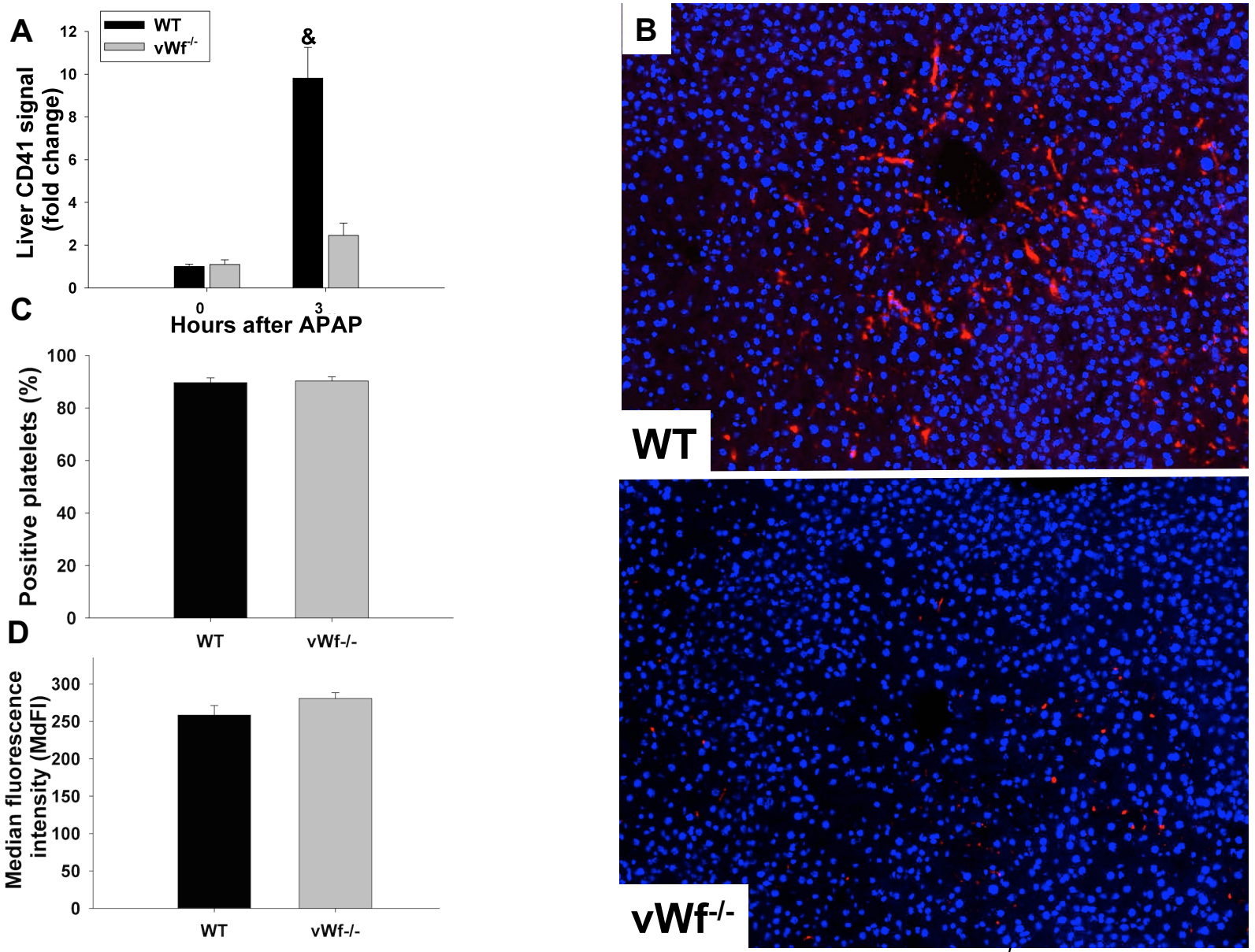

Figure 18: Platelet anti-CD41 signal in APAP-induced liver injury in vWf$^{-/-}$ mice.

Figure 18 (cont'd)

In A and B, $vWf^{-/-}$ mice and matching WT mice were treated with 300 mg/kg APAP, i.p.. A: Quantification of CD41 signal in the liver at 3 h after APAP injection. Signal intensity of CD41 was normalized to matching WT mice at time 0 h. B: Representative photomicrographs of frozen liver sections labeled with anti-CD41 antibody to identify platelets, using DAPI stain to identify nuclei. Platelets are pseudocolored red; nuclei are pseudocolored blue (10× objective). In C and D, platelets were collected from $vWf^{-/-}$ mice and matching WT mice and immunolabeled with anti-CD41 antibody for flow cytometry. C: MdFI of the anti-CD41 signal on platelets was measured. D: Percentage of anti-CD41 positive cells within the platelet population.

A and B: N=4-15, C and D: N=4 per group. $^{\&}$Significantly different from all the other groups.

Thrombin enhances APAP-induced injury to hepatocytes when cocultured with NPCs

The $vWf^{-/-}$ mice had no significant difference in liver injury and plasma TAT complexes; however, they had less platelet accumulation in the liver compared to the matching WT mice. This suggests that vWf contributes to the accumulation of platelets in the liver with APAP hepatotoxicity, but that vWf-mediated platelet events are not critical to APAP hepatotoxicity. These results also suggested that thrombin might play a more important role in APAP hepatotoxicity compared to platelets. The ability of thrombin to injure APAP-exposed hepatocytes directly was evaluated using primary mouse HPCs. Thrombin failed to cause injury to HPCs and did not enhance injury from APAP exposure (Figure 19). However, when the primary mouse HPCs were incubated with NPCs, treatment with thrombin significantly enhanced the ALT release from APAP-exposed HPCs (Figure 19).

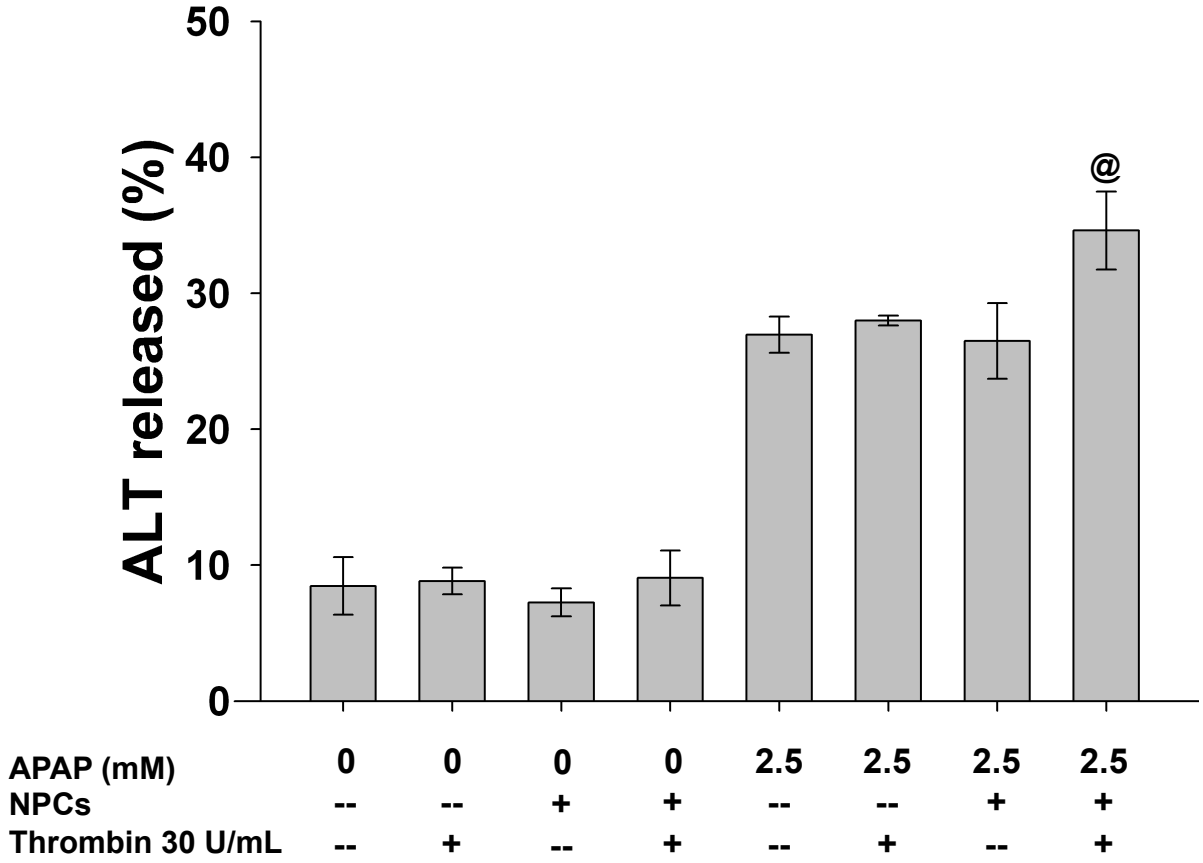

Figure 19: Thrombin effect in primary HPCs cocultured with or without primary NPCs treated with APAP.

Primary mouse HPCs were cultured in the absence and presence of primary NPCs, then exposed to APAP +/- thrombin. Hepatocellular injury was measured 18 h later as % ALT released. @Significantly different from the other APAP 2.5 mM treated groups. N=7 per group.

3.5. Discussion

Platelet accumulation in the liver after a toxic dose of APAP in mice has been confirmed (Chapter 2) by using immunolabeling with a platelet-specific antibody against CD41. However the sinusoidal distribution and association of platelets with other cells in APAP hepatotoxicity were not clear from the immunofluorescence of platelets in the liver or even from published TEM studies (Walker et al., 1980,1983). In our study, TEM evaluation of livers from APAP-treated mice revealed accumulation of platelets in the sinusoids as loose aggregates that appeared to be associated with other cells including endothelial cells, monocytic cells (macrophages and Kupffer cells), and hepatocytes as early as 3 h after APAP treatment. These findings suggested that platelets might contribute to APAP hepatotoxicity by communicating with various liver cells and/or by releasing mediators (i.e. thrombin and others) to the nearby cells to enhance the liver injury. Also, most of the platelet accumulation was observed at the border of the necrotic area.

Interestingly, most of the platelets still contained their granules (α and dense granules) and were only loosely aggregating. There were no aggregates of platelets that were showing fusion of platelets with loss of the granules that is observed commonly with viscous metamorphosis in clot formation (Castaldi et al., 1962). It seemed that the platelets were not fully activated (Castaldi et al., 1962). This might end up with more developed viscous metamorphosis at a later time. However, transient accumulation of RBCs has been reported in other studies (Walker et al., 1983, 1985) and the loose aggregation might relate to this transient RBC accumulation by occluding blood flow to some extent. Further TEM evaluation of platelets at later time points in APAP

hepatotoxicity and with higher concentration of APAP and therefore more severe liver injury might be illuminating.

To further evaluate the importance of platelets, we assessed the contribution of vWf to APAP hepatotoxicity. vWf is known for its important role as one of the first steps in platelet adhesion to injury sites with subsequent platelet activation (Varga-Szabo et al., 2008). Interestingly, $vWf^{-/-}$ mice did not show any difference in liver injury compared to matching WT mice evaluated by ALT release and area of necrosis. Also, plasma TAT complexes did not show any difference at the time that the peak of plasma TAT complexes were observed in matching WT mice. However, there was a significantly less anti-CD41 signal in livers of $vWf^{-/-}$ mice compared to matching WT mice after APAP administration, suggestive of a decrease in hepatic platelet accumulation in the livers of $vWf^{-/-}$ mice. This was surprising, since our previous results indicated that platelets were important to provide their membrane surface as a prominent amplifier for thrombin production. This suggested the possibility that CD41 expression on the platelet surface might be decreased in the $vWf^{-/-}$ mice, so we might not have been detecting all of the accumulated platelets. To confirm that the decreased hepatic anti-CD41 signal from APAP-treated $vWf^{-/-}$ mice was because of decreased platelets, we isolated and immunolabeled these platelets with anti-CD41 for flow cytometric evaluation of the intensity and prevalence of CD41. There was no significant difference in anti-CD41 signal between the platelets of $vWf^{-/-}$ mice and matching WT mice, suggesting that the decreased accumulation of the CD41 signal in the $vWf^{-/-}$ mice was indeed due to decreased platelet accumulation.

Thrombin generation in vWf deficiency seems to be variable and reliant more on the amount of FVIII than vWf (Rugeri et al., 2007). $vWf^{-/-}$ mice have no detectable vWf but still retain around 20% of normal FVIII activity (Denis et al., 1998) compared to <10% FVIII activity in humans, and this remaining 20% of FVIII activity might be sufficient enough to generate thrombin in these $vWf^{-/-}$ mice. However, this does not explain why platelet depletion by anti-CD41 but not by vWf deficiency decreased peak thrombin generation.

Another thing that needs to be considered is the threshold of platelet accumulation in liver needed for thrombin generation. The CD41 signal in liver of $vWf^{-/-}$ mice treated with APAP was around twice that of the $vWf^{-/-}$ mice or matching WT mice without APAP treatment. However, in the earlier platelet depletion study, platelet-depleted mice treated with APAP still had around 20% of the CD41 signal compared to the control mice without APAP. This suggested that there might be more platelets of the $vWf^{-/-}$ mice accumulating in the liver after APAP treatment compared to the platelet-depleted group treated with APAP, and this number of platelets might be enough to generate thrombin in $vWf^{-/-}$ mice even if the platelet accumulation in these mice was significantly decreased compared to the matching WT mice.

These findings suggest that vWf promotes platelet accumulation in the livers of APAP-treated mice, but in $vWf^{-/-}$ mice, platelets are only decreased to an extent that platelet accumulation in the liver does not alter the thrombin generation or injury from APAP exposure. Thrombin might play a more important role in APAP hepatotoxicity than do platelets.

Other things that need to be considered are possible compensatory mechanisms in $vWf^{-/-}$ mice that allow thrombin generation without much platelet accumulation in the liver. Further evaluation of this discrepancy with thrombin generation and platelet accumulation in the liver in $vWf^{-/-}$ is warranted.

Based on these findings, we evaluated the direct and indirect cytotoxic effect of thrombin using primary HPCs and NPCs. In the rat, PAR-1 is present on Kupffer cells and endothelial cells but not on hepatocytes and neutrophils (Copple et al., 2003). However, the presence of thrombin receptors on mouse liver cells is still unclear. A direct effect of thrombin was not observed in primary mouse HPCs treated with APAP. This suggested that primary mouse HPCs might not express PARs as rat hepatocytes do, or that thrombin signaling in mouse HPCs does not enhance APAP induced hepatocyte injury. Primary mouse HPCs cocultured with NPCs with or without thrombin did not show evidence of injury, suggesting that the presence of NPCs by themselves is not injurious and that thrombin does not enhance cytotoxicity in the absence of APAP. Interestingly, there was a modest but significant increase in hepatocellular injury from APAP when primary mouse HPCs cocultured with liver NPCs were exposed to thrombin. These findings suggested that liver NPCs can enhance hepatocellular injury through activation of PARs by thrombin. Some of the likely NPCs candidates would be Kupffer cells and sinusoidal endothelial cells, since thrombin activation of PARs on vascular endothelial cells and macrophages is known to enhance inflammatory cytokine release and NO release (Coughlin 2000; Coughlin 2005; Borissoff et al., 2009; Touyz 2007; Momota et al., 2006; Meli et al., 2001) that might contribute to APAP hepatotoxicity.

Also, we found (Chapter 2) that peroxynitrite formation might be one way that platelets and thrombin contribute to APAP hepatotoxicity.

Based on our findings, it seems that one way platelets contribute to APAP hepatotoxicity is by generating thrombin and that thrombin works indirectly by stimulating liver NPCs to enhance liver injury. Also, the indirect effect of NPCs was significant but only mild (10% increase), so there is still some potential for direct effects of platelets that have not been ruled out. Further evaluation for the main contributing NPC by isolating endothelial cells or Kupffer cells and coculturing with primary HPCs and evaluating thrombin-enhanced hepatotoxicity is warranted. Evaluation of a direct contribution of platelets to APAP-induced hepatocellular toxicity is also warranted, using primary HPCs with or without cocultured NPCs.

CHAPTER 4:

A cytochrome P450-independent mechanism of acetaminophen-induced hepatocellular injury

4.1 Abstract

Liver injury from APAP in mice resembles in most ways that which occurs in humans. For this reason, the mouse model in vivo and primary mouse HPCs in vitro have become the most frequently used models for studies of mechanisms of APAP-induced hepatotoxicity. There is a universal acceptance that APAP hepatocellular injury requires bioactivation to NAPQI by CYPs. Although it seems reasonable to assume that HPC death from APAP in vitro also requires bioactivation by CYPs, this remains unproven, especially over the wide range of APAP concentrations that have been employed.

To test this assumption, we undertook a concentration-response study to evaluate cytotoxicity in the presence and absence of a broad-spectrum CYP inhibitor, 1-aminobenzotriazole (ABT). ABT eliminated formation of APAP-protein adducts, indicating inhibition of bioactivating CYPs. Interestingly, ABT pretreatment eliminated cytotoxicity at small but cytotoxic APAP concentrations ($\leqq$ 5 mM) but not at larger concentrations ($\geqq$ 8 mM), indicating the presence of a CYP-independent mechanism at larger APAP concentrations. This mechanism was delayed in onset relative to CYP-dependent cytotoxicity. We evaluated the possibility that para-aminophenol (PAP), one of the minor metabolites of APAP, contributed to the CYP-independent mechanism. PAP was detected in HPCs exposed to large concentrations of APAP, and a deacetylase inhibiter, bis (4-nitrophenyl) phosphate (BNPP) significantly reduced cytotoxicity.

Conclusion: APAP hepatocellular injury in vitro occurs by at least two mechanisms, a CYP-dependent mechanism at small, cytotoxic APAP concentrations (≦ 5 mM) and a CYP-independent mechanism that predominates at larger concentrations (≧ 8mM) and is slower in onset. PAP likely contributes to the latter mechanism.

4.2 Introduction

Overdose with APAP is the leading cause of drug-induced liver injury and acute liver failure in the U.S. and other developed countries (Larson et al., 2005; Ostapowitz et al., 2002; Khashab et al., 2007; Fontana et al., 2008). As a result, there have been many studies of APAP hepatotoxicity with the ultimate goals of understanding mechanisms of liver injury and developing more effective preventive and therapeutic strategies for APAP overdose. Moreover, results from studies of mechanisms of APAP hepatotoxicity have been used as the basis for approaches to study the toxicity of other drugs and environmental toxicants.

Liver injury from APAP in mice resembles in most ways that which occurs in humans suffering from APAP overdose. For this reason, the mouse model in vivo and primary mouse HPCs in vitro have become most frequently used for studies of mechanisms of APAP-induced hepatocellular injury. The pioneering work of Bernard Brodie and coworkers in mice in the mid 1970s and subsequent studies by others led to universal acceptance that APAP hepatotoxicity requires bioactivation to NAPQI by CYPs (Mitchell et al., 1973a, 1973b; Jollow et al., 1973; McGill et al., 2013). Later, when HPCs were able to be isolated from rodents, cultured, and used for mechanistic studies, it seemed reasonable to assume that hepatocellular death from APAP in vitro also required bioactivation by CYPs, and many studies of mechanisms in vitro have relied upon this assumption. A PubMed search revealed that published studies using isolated primary mouse HPCs have employed a very wide range of APAP concentrations, i.e., from 0.1-50 mM, with 5 mM and 10 mM used most commonly (Figure 20).

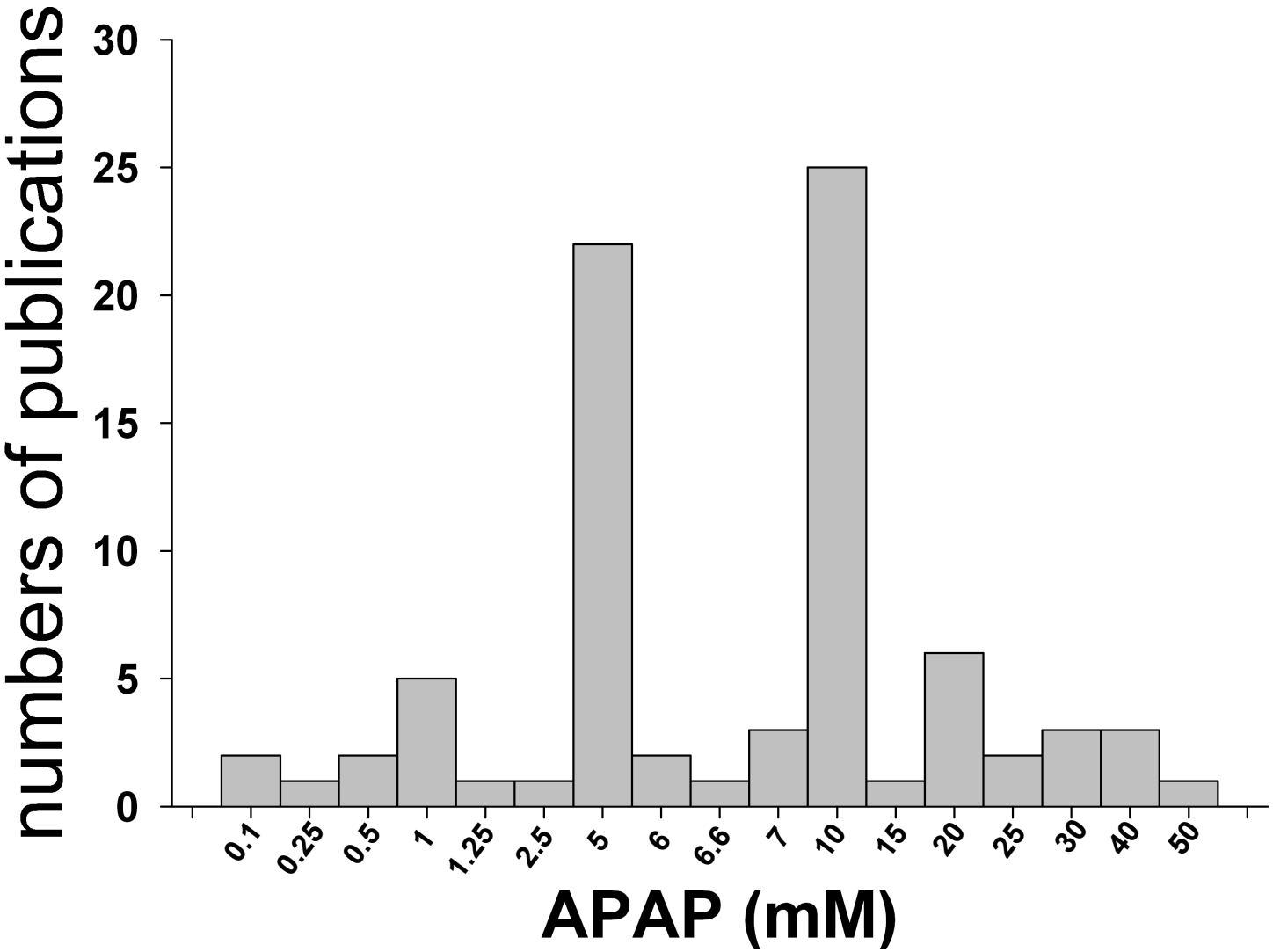

Figure 20: APAP concentrations used for in vitro studies with primary mouse HPCs.

Figure 20 (cont'd)

A PubMed search was conducted using the key words, "acetaminophen" and "mice" and "hepatocyte." Subsequent perusal of the abstracts and publications revealed at least 60 papers that used primary mouse HPCs in the study of APAP cytotoxicity. Some papers employed several concentrations. The number of publications employing each concentration was plotted as a frequency diagram.

This raises a question about what concentrations are the most appropriate ones for use in studies in vitro. Plasma APAP concentrations in human overdose cases vary considerably. They have exceeded 1 mM in patients seen several hours after an acute overdose and who developed liver injury (Dougherty et al., 2012; Prescott et al., 1971). Since APAP is rapidly absorbed and eliminated (t1/2 = 3-8 h in overdosed patients (Prescott et al., 1971)), plasma concentrations soon after ingestion of APAP are likely to be substantially greater, but they would rarely if ever exceed 5 mM (Prescott et al., 1971, 1977). In mouse models of APAP hepatotoxicity, the most typical doses used are 300 or 400 mg/kg; plasma concentration at a dose of 400 mg/kg would not be expected to exceed 3 mM (max. plasma conc. = dose/Vd = [400 mg/kg / 151 mg/mmol] / 0.9 L/kg = 2.9 mM). Based on these values in humans and mouse models, concentrations of 5 mM or less would seem reasonable for use in studies in vitro. In many studies, the rationale for using greater concentrations was not explained. In some studies, DMSO was used as a vehicle to dissolve agents (i.e., MAPK inhibitors) used in the investigation; since DMSO can inhibit APAP-bioactivating CYPs (Arndt et al., 1989), large APAP concentrations were used ostensibly to overcome the CYP-inhibiting ability of DMSO vehicle (Du et al., 2013; Gunawan et al., 2006).

The large APAP concentrations used in some studies in vitro raise the question as to whether initiating mechanisms that operate at small cytotoxic APAP concentrations are the same as at larger APAP concentrations and how the results relate to those obtained in commonly used murine models in vivo or, for that matter, to human toxicity. Accordingly, we undertook a concentration-response study in primary

mouse HPCs to evaluate cytotoxicity in the presence and absence of an inhibitor of CYP-mediated bioactivation of APAP. The result pointed to a CYP-independent mechanism that predominates at larger APAP concentrations. We then considered an alternative metabolic pathway that could lead to APAP cytotoxicity. Since evidence exists for involvement of deacetylation to PAP in the renal toxicity of APAP (Newton et al., 1985A, 1985B), we examined the possibility that APAP deacetylation contributes to the initiation of CYP-independent toxicity to hepatocytes at larger APAP concentrations.

4.3. Material and Methods

Materials

ABT, BNPP, PAP, collagenase from *Clostridium histolyticum*, were purchased from Sigma-Aldrich (St Louis, MO). Antibiotic-antimycotic (ABAM), Dulbecco's PBS, liver perfusion medium, hepatocyte wash medium, FBS, L-glutamine, and WME were purchased from Life Technologies (Carlsbad, CA). ALT reagent was purchased from ThermoScientific (Pittsburgh, PA). Type I collagen from rat tail was purchased from BD Bioscience (San Jose, CA). Bovine insulin for cell culture was purchased from Cell Applications (San Diego, CA).

Animals

Wild-type C57Bl/6J mice (9 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). They were acclimated for at least one week at 22°C with alternating 12 h light/dark cycle and access to standard chow diet (Teklad 22/5 Rodent Diet 8940: Harlan Laboratories (Indianapolis, IN)) and spring water ad libitum. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee.

Isolation and treatment of hepatocytes

Primary murine HPCs were isolated as previously reported (Klaunig et al., 1981; Bajt et al., 2004) with some modification. Briefly, each mouse was anesthetized with 50 mg/kg pentobarbital i.p., after which the abdominal cavity was opened and a 24 ga

catheter was placed and secured in the inferior vena cava. The liver was perfused first with 50 mL of liver perfusion medium supplemented with ABAM. The portal vein was severed, and the anterior vena cava was clamped with a hemostat. Then the liver was perfused with 40 mL of WME supplemented with ABAM and 5-10 mg type IV collagenase. After the perfusion was completed, the liver was removed, placed in hepatocyte wash medium and gently broken apart with forceps. The cell mixture was then filtered through 3 layers of gauze and centrifuged at 50 x g for 2 min. Hepatocytes were washed two more times, and viability was evaluated with trypan blue exclusion; cell viability of 85% was the criterion for use in experiments. 1.25×10^5 cells/well were cultured in 24 well plates (coated with type I collagen from rat tail (5 $\mu g/cm^2$) overnight) with WME supplemented with 10% fetal bovine serum and containing ABAM, 2 mM L-glutamine and 1×10^{-7} M insulin and incubated at 37°C and 5% CO_2. After an hour, medium was replaced (to remove unattached cells) and incubated for another hour. Hepatocytes were then gently washed twice with PBS followed by WME supplemented with ABAM and 2 mM L-glutamine. Inhibitor was added at this point when appropriate. ABT was diluted with PBS (6.7 mg/mL), and the final concentration for ABT was 1 mM. BNPP was diluted with PBS (5 mg/mL) and warmed in a water bath at 40-45°C; the final concentration for BNPP was 1 mM. After another hour of incubation, hepatocytes were washed with PBS once, APAP was added, and cells were incubated for an additional 2.5-18 h. APAP was dissolved in William's medium E supplemented with ABAM and 2 mM L-glutamine. PAP was dissolved in PBS (1 mg/mL) and applied to primary hepatocytes to achieve final concentrations of 100-800 μM.

ALT assay

Medium was collected, and remaining cells were lysed and collected by adding an equal volume of 1% Triton X-100 and scraping with a cell scraper. Medium and cell lysate were centrifuged at 660 x g for 5 min. Cytotoxicity was assessed by measuring ALT release into the medium using Infinity-ALT reagent. ALT activity in the medium was expressed as a percentage of total ALT activity (i.e., medium plus lysate). In the time course study, baseline ALT activity at each time point was subtracted from values obtained at each APAP concentration.

Measurement of APAP-protein adducts

Media and cells attached to the wells of the 24-well plates after 2.5 h and 8 h of APAP treatment were removed using a cell scraper, and contents of 3 replicate wells were combined, sonicated and saved at -80 ^{o}C until analysis. Samples were analyzed for APAP-protein adducts (acetaminophen-cysteine) by high-performance liquid chromatography with electrochemical detection (HPLC-EC) as previously reported (Muldrew KL 2002). Briefly, samples were dialyzed, treated with protease, and precipitated with trichloroacetic acid. The resulting supernatant was injected into the HPLC column using a Model 582 solvent delivery system, and compounds were detected with a Model 5600A CoulArray detector (ESA, Chelmsford, MA). The range of linearity for this method was 0.01 – 20 µmol/L APAP-cysteine in serum. The coefficients

of variation (CVs) for the assay were consistently <10% at concentrations of 0.03, 0.125, 0.5, and 2.0 μmol/L APAP-cysteine adducts. Based on the CVs for the standard curve for the assay, the lower limit of quantitation for the assay was defined as 0.01 μmol/L APAP-cysteine. APAP-cysteine content was reported relative to the protein content of the sample, determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA), and reported as nmol/mg protein. This part of the experiments were performed by Dr. Laura James and Lynda Letzig from department of Pediatrics, University of Arkansas for Medical Sciences, Clinical Pharmacology and Toxicology Section, Arkansas Children's Hospital, Little Rock, AR.

Statistical Analysis

Data are expressed as mean ± SEM. Data that were not normally distributed were subjected to appropriate transformation (i.e., logarithmic, square root, arcsin squared). Student's t-test was used to compare two means, and analysis of variance was used when more than two means were compared. If a difference was detected with analysis of variance, appropriate post-hoc testing (Holm-Sidak) was performed. If data transformation failed to generate data with a normal distribution, nonparametric tests were performed (Mann-Whitney U test for comparison of two means, and Kruskal-Wallis one-way analysis of variance for multiple comparisons). $P < 0.05$ was set as the criterion for statistical significance

4.4. Results

Biphasic concentration dependence of APAP cytotoxicity

A concentration-response study in primary mouse HPCs was performed to evaluate APAP cytotoxicity in the presence and absence of ABT, a broad-spectrum, suicide inhibitor of CYPs. In the absence of ABT, ALT release 18 h after APAP addition to HPCs increased as APAP concentrations increased from 0.1 mM to 1.25 mM, reaching a plateau at about 40% ALT release (Figure 21A). Beyond 5 mM APAP, cytotoxicity increased markedly with APAP concentration. ALT release was essentially complete (90%) at 14 mM APAP (Figure 21B). APAP adducts with cysteinyl residues on proteins were measured as a marker of CYP-dependent bioactivation of APAP to NAPQI. APAP-protein adducts measured 2.5 h after APAP administration increased in parallel with the increase in ALT up to 5 mM APAP, at which concentration they had reached a plateau and showed no further concentration dependence (Figure 21A). In the presence of ABT, the appearance of APAP-protein adducts was eliminated at 2.5 h and 8 h with 2.5 mM APAP (Figure 21B and 22), confirming almost complete inhibition of CYP-mediated APAP bioactivation. In cells treated with 14 mM APAP and treated with ABT, there was a small but statistically significant increase in APAP-protein adducts at only at 8 h (Figure 21B and 22). With ABT pretreatment, concentrations of 5 mM APAP or less produced no statistically significant increase in ALT. However a concentration-dependent increase in ALT release was observed at APAP concentrations of 8 mM and above, despite the elimination of APAP–protein adducts

(Figure 21B). A different CYP inhibitor, metyrapone, also afforded protection from cytotoxicity at small but not large APAP concentrations (Figure 23).

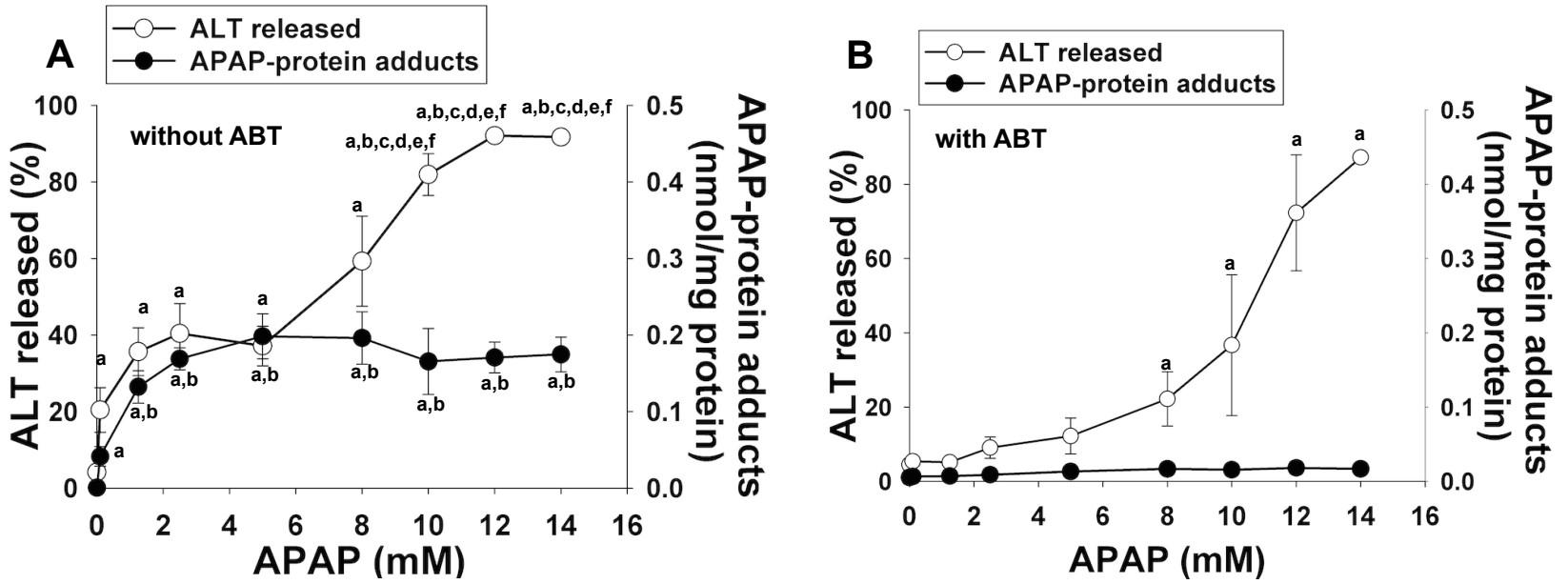

Figure 21: Concentration-dependent ALT release and APAP-protein adduct appearance in primary mouse HPCs exposed to APAP.

Primary mouse HPCs were treated with various concentrations of APAP (0.1 mM to 14 mM) without (A) or with (B) 1 h pretreatment with ABT. ALT release was measured 18 h after the APAP treatment, and APAP-protein adducts were measured at 2.5 h. [a]Significantly different from 0 mM APAP, [b]significantly different from 0.1 mM APAP, [c]significantly different from 1.25 mM APAP, [d]significantly different from 2.5 mM APAP, [e]significantly different from 5 mM APAP, [f]significantly different from 8 mM APAP. N=4

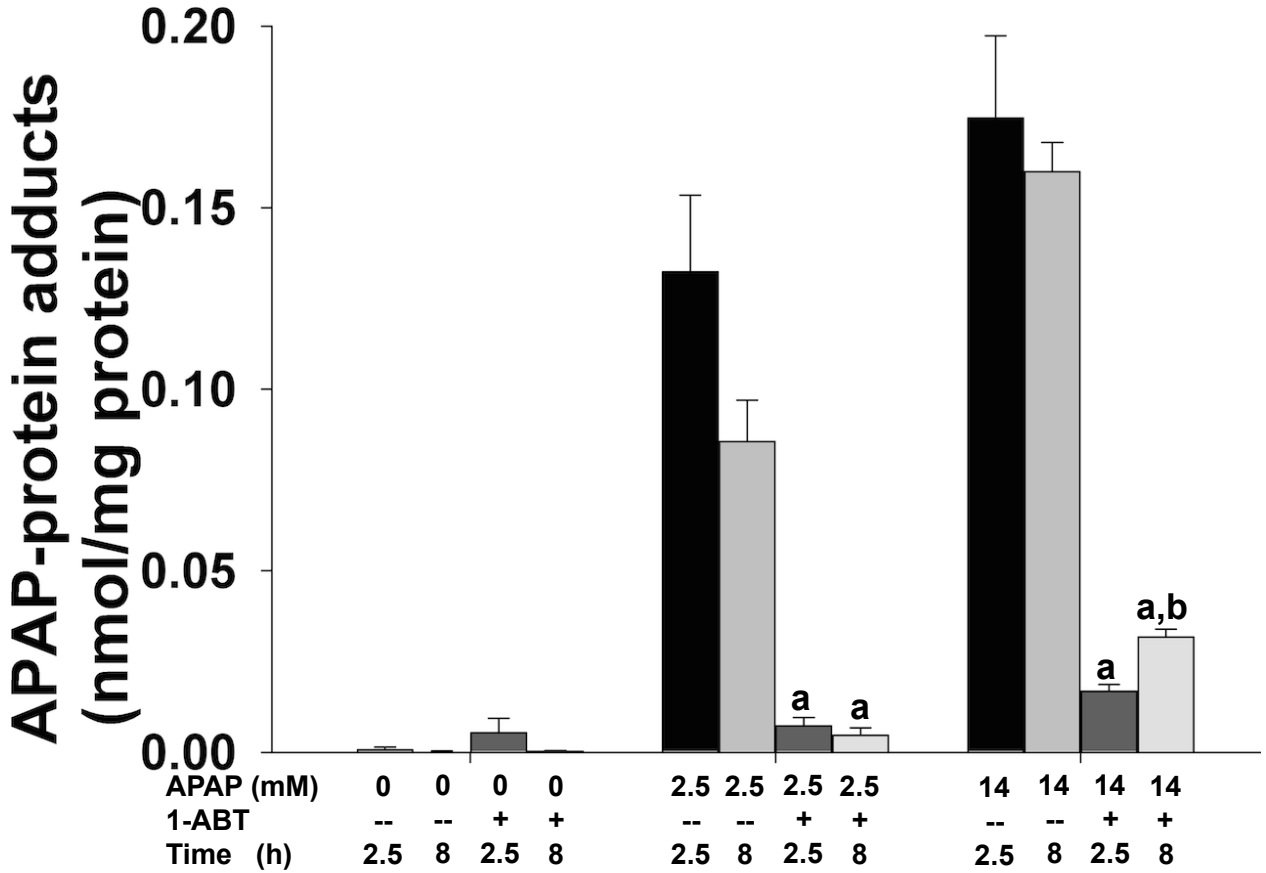

Figure 22: APAP-protein adducts at 2.5 h and 8 h after APAP exposure with or without ABT.

Primary mouse HPCs were treated with 0, 2.5 or 14 mM APAP with or without 1 h pretreatment with ABT. APAP-protein adducts were measured at 2.5 h and 8 h. [a]Significantly different from same time without ABT. [b]Significantly different from same treatment at 2.5 h. N=4

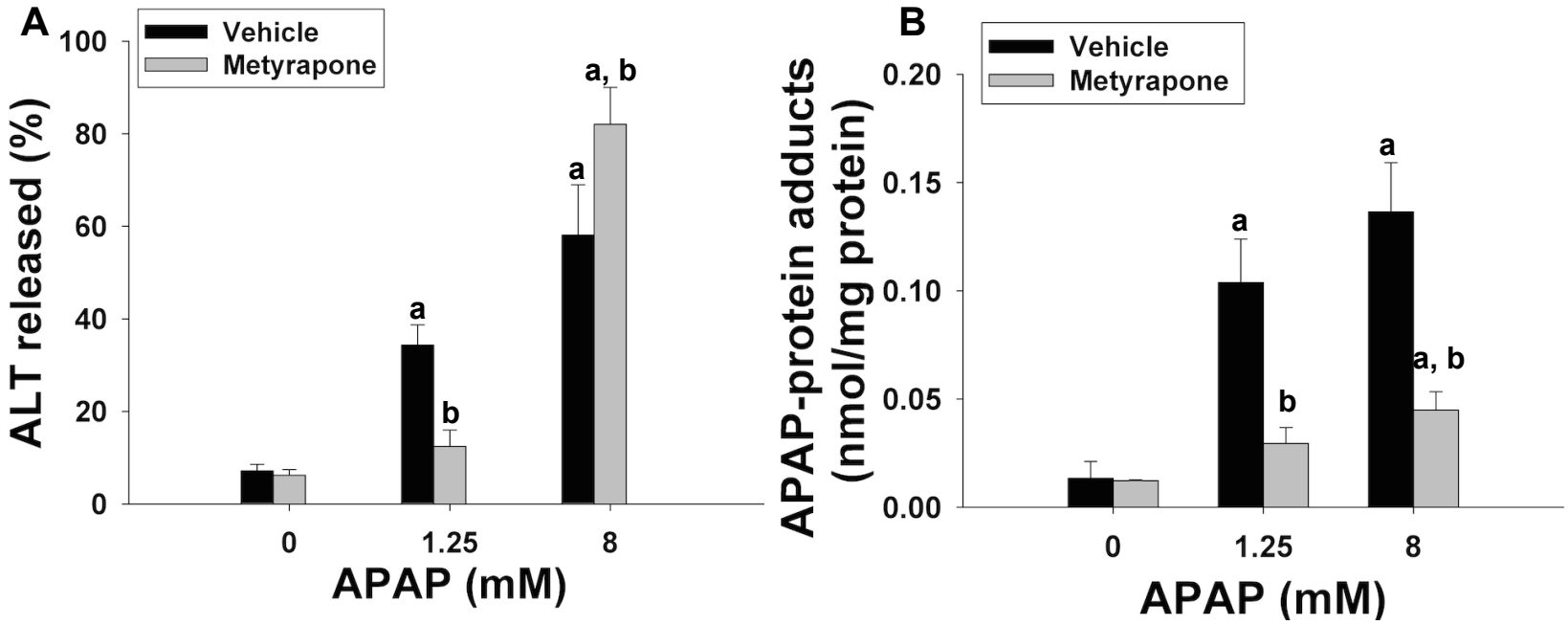

Figure 23: ALT release and APAP-protein adducts after APAP exposure with or without metyrapone.

Primary mouse HPCs were treated with 0, 1.25 or 8 mM APAP with or without 1 h pretreatment with metyrapone. A: ALT release at 18 h, B: APAP-protein adducts at 2.5 h. [a]Significantly different from APAP 0 mM with same pretreatment. [b]Significantly different from same APAP dose without pretreatment. N=4

Development of APAP cytotoxicity over time

Measuring the ALT release over time at various APAP concentrations revealed that at small APAP concentrations (i.e., up to 5 mM), ALT activity in the medium increased within 6 h to about 40% but remained unchanged thereafter (Figure 24A). In contrast, ALT activity continued to increase with time at larger APAP concentrations (i.e., 8 mM and above). In the presence of ABT, there was no significant ALT release at any APAP concentration at 6 h (Figure 24B). Indeed, no significant ALT release occurred at any time at APAP concentrations of 5 mM or less. In contrast, the cytotoxicity at larger concentrations of 8 mM and above did not begin until after 6hr and it progressed with time.

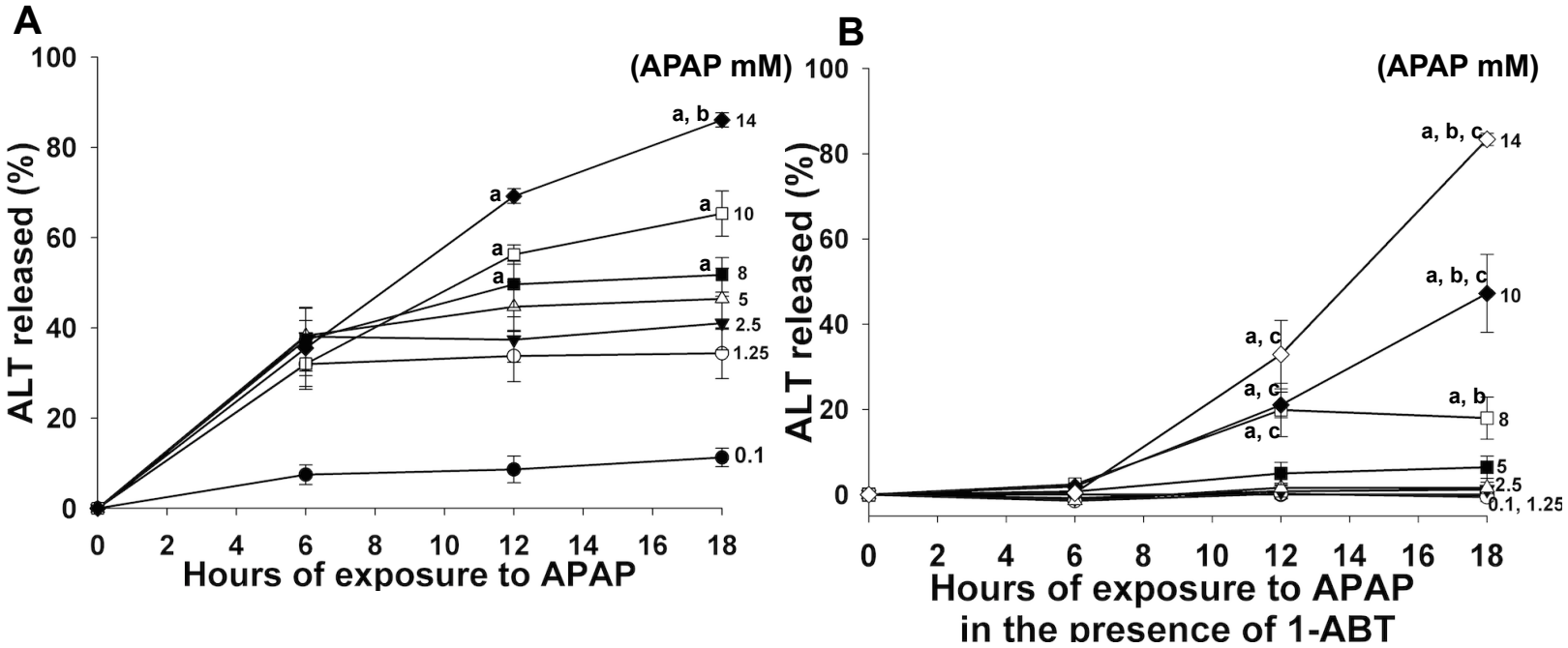

Figure 24: Time course of ALT release from primary mouse HPCs treated with APAP.

Primary mouse HPCs were treated with various concentrations of APAP (0.1-14 mM) without (A) or with (B) 1 h pretreatment with ABT. ALT release was measured at 6 h, 12 h and 18 h. [a]Significantly different from same APAP concentration at 6 h, [b]significantly different from same APAP concentration at 12 h and [c]significantly different from same concentration at time 0 h. N=4

APAP deacetylation contributes to CYP-independent APAP cytotoxicity

The results above pointed to one or more CYP-independent initiating mechanisms of HPC injury at large APAP concentrations in vitro. An intriguing alternative bioactivation pathway was through deacetylation of APAP to form PAP and acetate (McConkey et al., 2009; Nicholls et al., 1997; Zhao and Pickering 2011). Although the contribution of PAP as a deacetylation product has not received attention as a contributor to APAP-induced liver injury, several studies have implicated this metabolite in the nephrotoxicity of APAP, both in animal models and in cultured renal cells. Newton et al. (1985A and 1985B) presented evidence that reactive intermediate(s) of PAP that bind to kidney proteins initiate the renal toxicity of APAP in Fischer 344 rats and that inhibition of APAP deacetylation by BNPP decreased covalent binding of radiolabeled APAP and reduced nephrotoxicity. This suggested that PAP is responsible for the renal toxicity of APAP in F344 rats, and subsequent studies by other investigators (Lock et al., 1993; Klos et al., 1992; Harmon et al., 2006) provided additional evidence for this hypothesis.

With these observations as a backdrop, we examined the possibility that deacetylation to PAP contributes to the initiation of CYP-independent APAP toxicity in HPCs. We evaluated the cytotoxicity in primary mouse HPCs treated with BNPP. BNPP by itself caused no cytotoxicity (Figure 25A), and it did not influence cytotoxicity caused by 2.5 mM APAP (Figure 25B). However, it reduced significantly the cytotoxicity caused by either 10 or 14 mM APAP (Figure 25C and 25D). In contrast, ABT treatment eliminated the cytotoxicity caused by 2.5 mM APAP. It also reduced that caused by 10

mM APAP but was alone without effect at 14 mM APAP (Figures 25C and 25D). Interestingly, primary mouse HPCs treated with both BNPP and ABT experienced less cytotoxicity upon exposure to 10 or 14 mM APAP than occurred with either inhibitor alone.

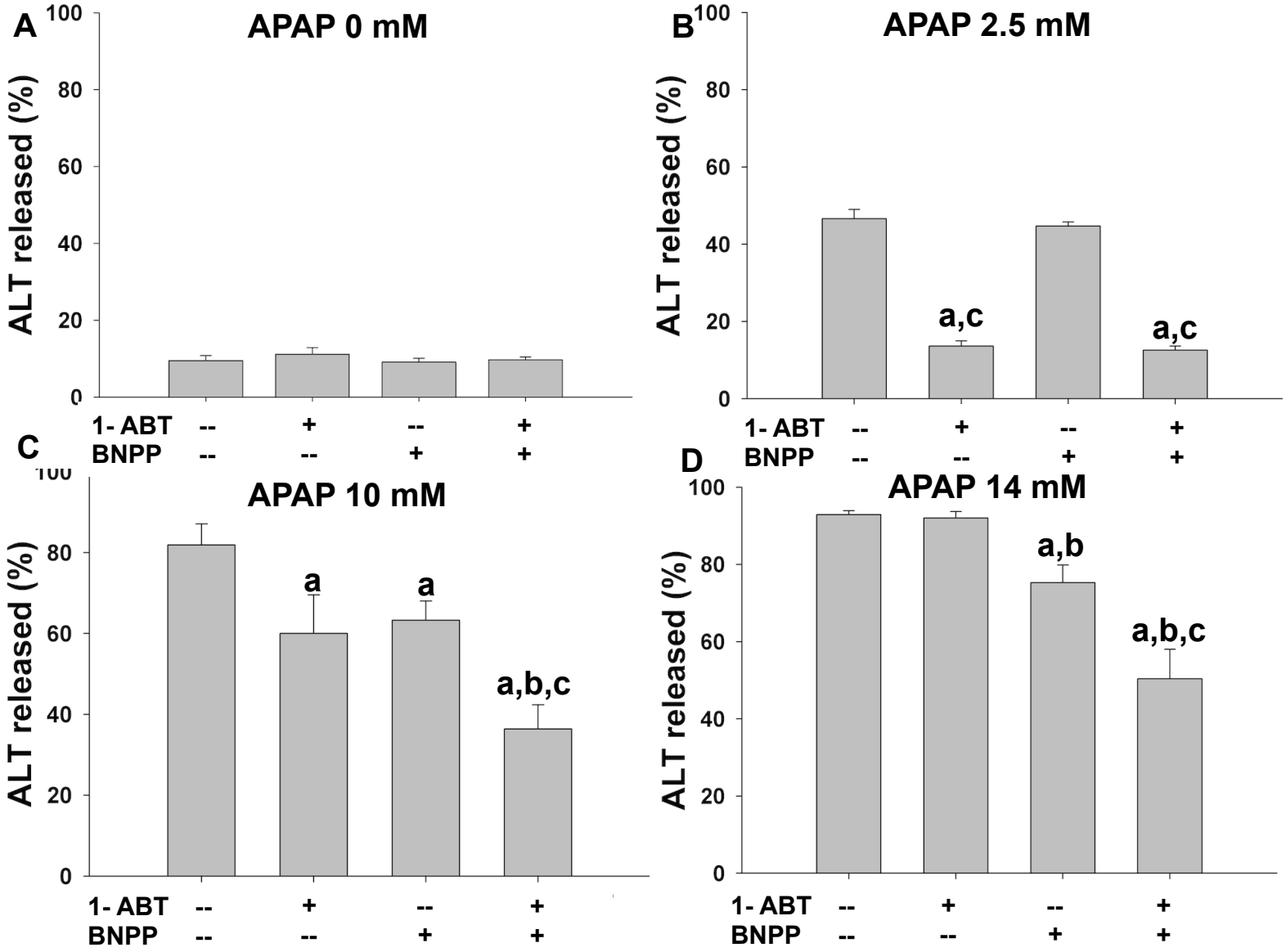

Figure 25: Effect of inhibition of CYP and/or deacetylation on ALT release from HPCs treated with APAP.

Figure 25 (cont'd)

Primary mouse HPCs were treated with 0 (A), 2.5 (B), 10 (C) or 14 mM (D) APAP after 1 h pretreatment with or without ABT (CYP inhibitor) and/or BNPP (deacetlyase inhibitor). [a]Different from -ABT/- BNPP, [b]different from +ABT/- BNPP, [c]different from -ABT/+BNPP. N=5-6

PAP is cytotoxic to primary mouse HPCs

Finally, we determined whether exposure to PAP directly was cytotoxic to HPCs. PAP caused a dose-dependent increase in ALT release from HPCs at concentrations above 200 μM and ALT was significantly increased from the base line at 300 μM PAP (Figure 26). The group that was pretreated with ABT had results (Figure 26) that paralleled those of the group treated only with PAP.

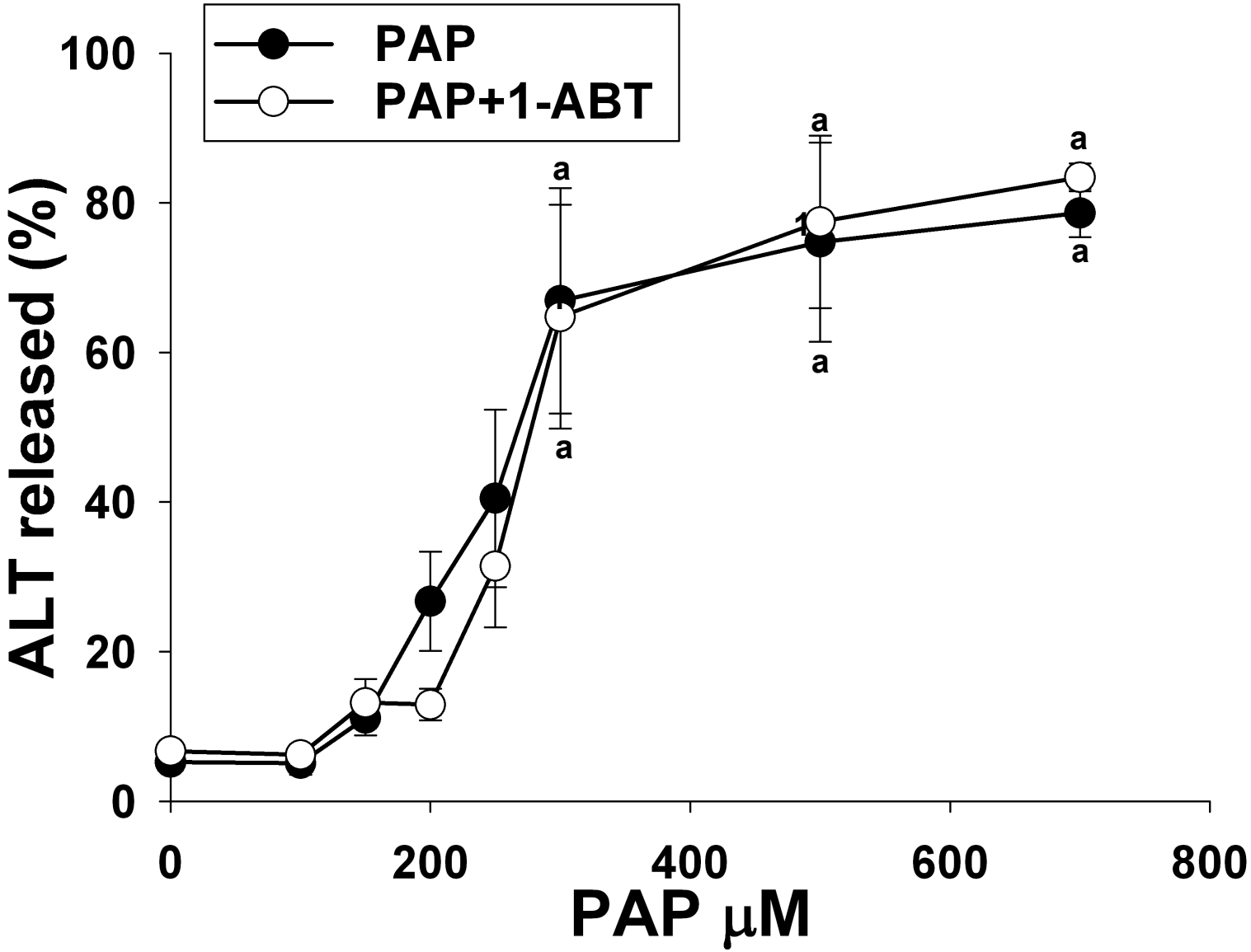

Figure 26: Concentration-dependent ALT release in primary mouse HPCs exposed to PAP.

Figure 26 (cont'd)

Primary mouse HPCs were treated with various concentrations of PAP (100 μM to 700 μM) with or without1 h pretreatment with ABT. ALT release was measured 18 h after the APAP treatment. [a]Significantly different from same group at 0 μM PAP.

Cells exposed to high concentration APAP (i.e. at and above 8 mM) and PAP (i.e., at and above 250 μM) turn brown in color

Another interesting observation was that the hepatocytes that were treated with high APAP concentrations (i.e., >8 mM) were brown in color when pellets from lysed APAP-treated hepatocytes were observed (Figure 27). Also, pellets of lysed PAP-treated hepatocytes had an increase in brown color starting from the concentration (i.e., 250 μM) at which hepatocellular injury was observed (Figure 27).

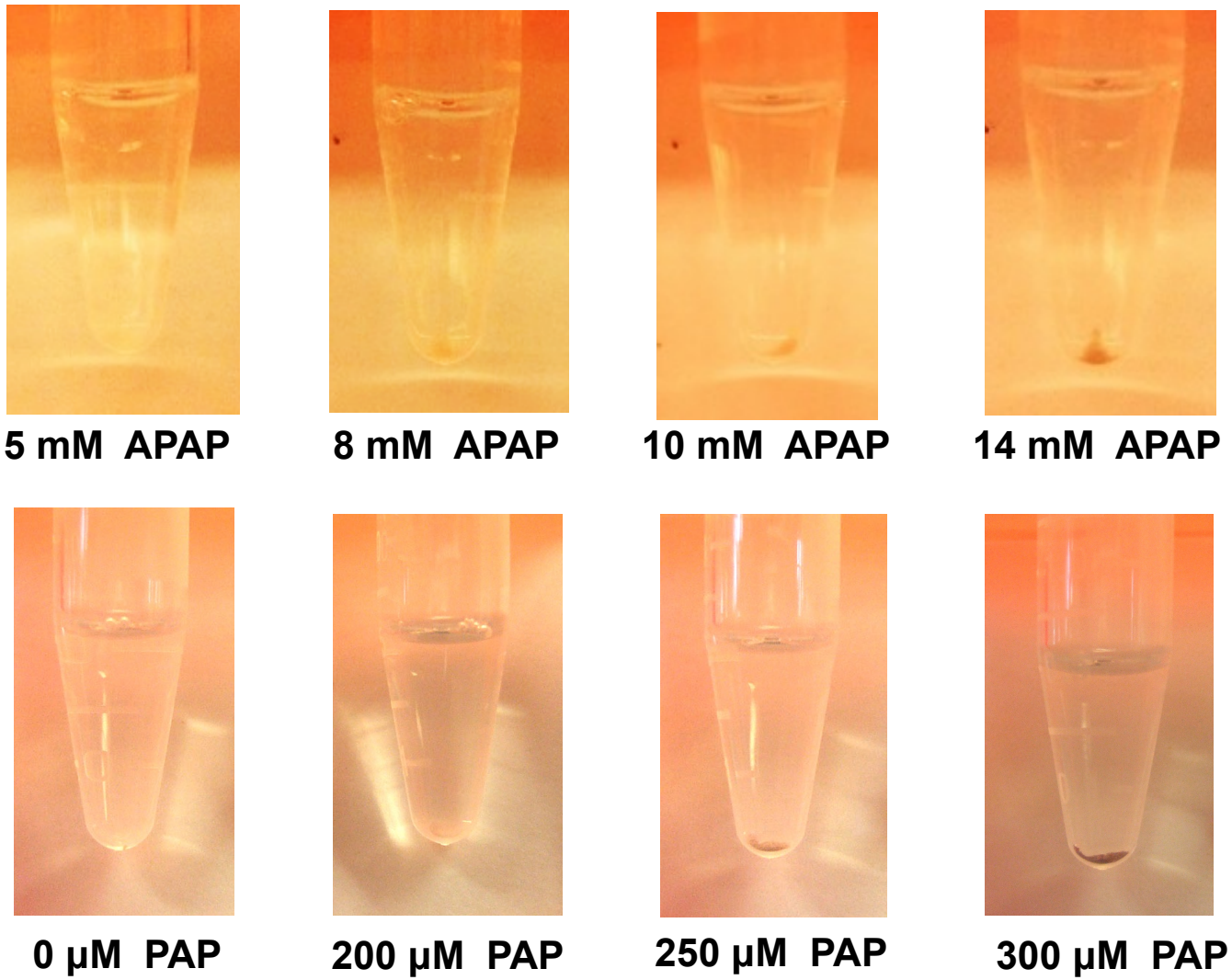

Figure 27: Centrifuged tubes of lysed hepatocytes, 18 h after treatment.

Figure 27 (cont'd)

Top row, pellets of lysed APAP-treated hepatocytes are increasingly brown at APAP concentrations of 8 mM and above. Bottom row, pellets of lysed PAP-treated hepatocytes are increasingly brown at PAP concentrations of 200 μM and above.

4.5. Discussion

Throughout the several decades of investigating APAP toxicity in vitro, it has been assumed that initiating mechanisms that pertain at small but toxic APAP concentrations (i.e., 5 mM or less) are the same as those operative at larger concentrations. Finding no definitive evidence for this assumption in published reports, we undertook a concentration-response study in primary mouse HPCs to evaluate cytotoxicity in the presence and absence of a broad-spectrum, suicide inhibitor of CYP-mediated APAP bioactivation (ABT). In the absence of ABT, the concentration-response relationship for HPC injury as marked by ALT release at 18 h was biphasic, raising the possibility that more that one mechanism was at play. Interestingly, the concentration of APAP-protein adducts increased in a manner that matched the cytotoxicity up to 5 mM APAP and then increased no further with increasing APAP concentration. This result further suggested the existence of two initiating mechanisms, one operating at small APAP concentrations (i.e., 5 mM or less) and another that predominated at larger APAP concentrations (i.e., at or above 8 mM). CYP inhibition with ABT prevented the production of APAP-protein adducts and also eliminated cytotoxicity at APAP concentrations of 5 mM or smaller; however, the marked increase in cytotoxicity at larger APAP concentrations remained despite inhibition of CYP-initiated APAP bioactivation. These results clearly point to at least two mechanisms that contribute to APAP cytotoxicity: a CYP-dependent mechanism at small, cytotoxic APAP concentrations (i.e., 5 mM or less) and a CYP-independent mechanism that predominates at larger concentrations (i.e., at or above 8 mM).

A time-course study revealed that the cytotoxic response at small APAP concentrations began early but was essentially complete by 6 h; however, injury continued to progress with time at larger concentrations. In contrast, when the CYP-dependent component was eliminated by ABT, the cytotoxicity at larger concentrations did not begin until after 6 h, and it progressed with time thereafter. Together, these results point to the occurrence of a rapidly developing, CYP-dependent mechanism of cell injury that predominates at smaller, cytotoxic APAP concentrations and that is limited both in degree and duration, as well as a later-developing, CYP-independent mechanism that predominates at larger APAP concentrations and can result in complete cell killing. Since NAPQI is formed by CYP-mediated metabolism, the CYP-independent mechanism cannot be due to NAPQI. That the CYP-dependent cytotoxicity is limited in magnitude makes sense in light of reports that NAPQI can inactivate the CYPs involved in its formation from APAP (Snawder et al., 1994). Hepatocellular heterogeneity in CYP concentrations or in NADPH cofactor might also contribute to this limitation of cytotoxicity by having differences in NAPQI production. These observations strongly suggest that larger APAP exposures invoke a CYP-independent mechanism of toxicity that differs from the initiating mechanism at smaller APAP exposures.

Since evidence exists for involvement of deacetylation of APAP to PAP in the renal toxicity of APAP, we examined the possibility that deacetylation contributes to the initiation of CYP-independent APAP toxicity in HPCs. Deacetylation of APAP in liver is known to occur, but rapid reacetylation of PAP back to APAP by N-acetyltransferases

(NATs) in a “futile cycle” occurs at smaller APAP concentrations, so that little PAP accumulates (Nicholls et al., 1997; Zhao and Pickering 2011). Accordingly, the deacetylation pathway has been viewed as a minor one with little importance in APAP-induced HPC injury. However, its contribution may have been underestimated. First, the metabolism of APAP by CYPs is limited by inactivation of CYPs by reactive NAPQI (Snawder et al., 1994), thereby potentially slowing APAP elimination and prolonging its availability for deacetylation to PAP. Second, in HPCs compromised by the early acting CYP-dependent mechanism, ATP production is slowed (Harmon et al., 2006; Burcham et al., 1991; Andersson et al., 1990). The resultant decrease in ATP availability would be expected to slow NAT-dependent reacetylation of PAP, since formation of acetylCoA as a donor molecule for this reaction requires ATP. The slowing of NAT-mediated reacetylation coupled with the prolonged availability of APAP would favor accumulation of PAP. At large APAP concentrations, inhibition of both CYPs and deacetylases reduced cytotoxicity more than either alone. Accordingly, it may be that PAP produced by deacetylation of APAP at large concentrations acts on cells stressed by NAPQI to result in pronounced cell death. If PAP indeed contributes to APAP cytotoxicity, it should injure HPCs at concentrations less than those caused by APAP, indeed, direct addition of PAP to HPCs was injurious at concentrations far less (approximately 1/40th) than concentrations of APAP needed to cause CYP-independent cytotoxicity. Also, because ABT pretreatment did not affect PAP-induced ALT release, injury from PAP is not related to metabolism of PAP to APAP in this model.

Interestingly, cells exposed to large, but not small cytotoxic APAP concentrations (i.e. at and above 8 mM) turned brown in color, as did HPCs exposed to cytotoxic concentrations of PAP (i.e., at and above 250 μM). It has been suggested that PAP in solution undergos oxidation in neutral and alkaline conditions and can polymerize to yield a brown color over time, and others have suspected that this is the likely cause of brown urine and of pigmentation of stored serum from APAP overdosed patients (Clark et al., 1986; Brown et al., 1983). These observations suggest that the brown coloration of the cells from exposure to large concentrations of APAP could be caused by PAP. Together, these results strongly suggest that PAP contributes to the CYP-independent cytotoxicity observed at larger APAP concentrations. Currently, detection of PAP in the primary mouse HPCs treated with APAP in various concentrations is ongoing by gas chromatography mass spectrometry to further evaluate the involvement of PAP in this model.

In conclusion, our data point to at least two mechanisms that contribute to APAP cytotoxicity: (1) a CYP-dependent mechanism that operates at small, cytotoxic APAP concentrations (i.e., 5 mM or less) and that occurs rapidly and is limited both in degree and duration, and (2) a CYP-independent mechanism that predominates at larger concentrations (i.e., at and above 8 mM) and that is slower to develop and highly lethal. Deacetylation of APAP to PAP appears to be a contributor to the CYP-independent mechanism. These results clearly indicate that a CYP-independent initiating mechanism predominates upon prolonged exposure of HPCs to APAP concentrations above 5 mM, and that the assumption that formation of NAPQI is solely responsible for hepatocellular

death at such concentrations is incorrect. The proposed pathway for these 2 mechanisms is presented in the attached figure 27.

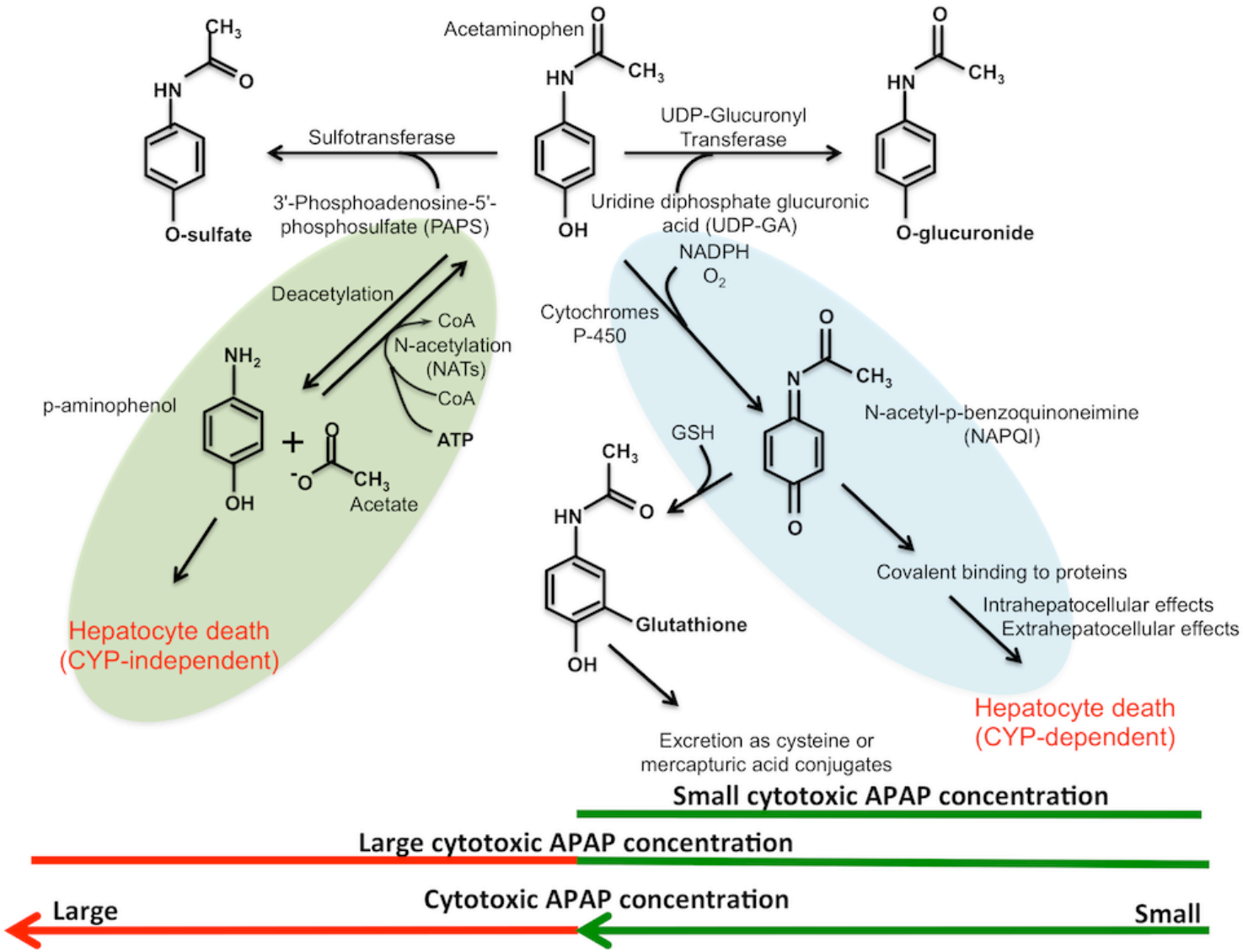

Figure 28. Proposed pathways for APAP-induced HPC injury.

Figure 28 (cont'd)

At low toxic APAP concentrations, conjugation pathways are overwhelmed and the rate of toxic NAPQI formation by CYPs (blue shading) increases, leading to HPC stress and early death of some HPCs. The deacetylation pathway (green shading) initially produces minimal PAP because PAP is rapidly reacetylated by NATs back to APAP. However at high concentrations of APAP, a CYP-independent mechanism predominates in killing and PAP contributes to this by its toxic mechanisms.

This CYP-independent mechanism might explain some confusing results in published reports. For example, some previous in vivo and in vitro findings suggest that protection from certain approaches (Receptor interacting Protein Kinase 3 (RIP3) KO (Ramachandran et al., 2013) and use of cyclosporine A (Kon et al., 2004 and 2007)) were seen early with high concentrations of APAP but diminished later, early protection may have been CYP-dependent but later loss of protection might have been due to the late CYP–independent mechanisms. Whether the CYP-dependent and CYP-independent initiating mechanisms activate similar or different cell death signaling pathways remains to be determined.

CHAPTER 5:

Summary and Conclusions

5.1 **Role of thrombin and platelets in APAP hepatotoxicity**

Most of my dissertation research focused on evaluating the contributions of platelets and thrombin to APAP hepatotoxicity. In order to assess the hypothesis that "Platelets and thrombin contribute to APAP hepatotoxicity", platelet involvement in APAP overdose was evaluated first. Hepatic platelet distribution was assessed by using TEM and immunolabeling of platelets with anti-CD41 IgG. Other studies employed a combination of pharmacologic (lepirudin), genetic (PAR-4$^{-/-}$, vWf$^{-/-}$), cell-specific depletion (anti-CD41 IgG), and in vitro coculture strategies.

5.1.1 **Platelets accumulate in the liver before significant liver injury in APAP hepatotoxicity**

APAP administration resulted in a decrease in the concentration of platelets in blood and an increase in platelet accumulation in the liver as early as 2 h (Figure 5B, C, D), and this accumulation started before an increase in ALT or necrosis was evident. Platelets appeared to be mainly associated with the centrilobular regions of the lobules, the areas where necrosis would ultimately occur (Figure 5D).

Based on TEM, livers from mice not treated with APAP had no platelets in the sinusoids. On the other hand, TEM of the APAP-overdosed mice revealed increased numbers of platelets, mostly in sinusoid as loose aggregates (Figure 15C-H). Platelets tended to be distributed along the border between intact and necrotic areas (Figure 15 F), and they appeared to contact endothelial cells and macrophages and/or Kupffer

cells (Figure 15D, E ,G, H). These findings also raised interest in the possibility that platelets contribute to APAP-induced liver damage.

5.1.2 **Thrombin plays a role in APAP hepatotoxicity**

In previous studies in this laboratory, heparin was used to evaluate the contribution of coagulation to APAP hepatotoxicity, as heparin promotes the removal of thrombin from circulation via antithrombin (Ganey et al., 2007). However, heparin is also known to affect other coagulation factors and has other nonanticoagulation activities (Lever et al., 2012). Therefore, in the studies described here, we used lepirudin, a direct, selective inhibitor of thrombin (Greinacher 2004), to determine more specifically if thrombin activity contributes to liver injury. Lepirudin pretreatment reduced liver injury (Figure 6B,C, F) and also decreased platelet accumulation caused by APAP (Figure 6A and G). These results suggest that thrombin plays an important role in the hepatic platelet accumulation accompanying APAP-induced hepatotoxicity in mice.

Protection was also observed when lepirudin was given 1.5 h after APAP, a time when bioactivation of APAP to NAPQI is nearly complete. This observation suggests that lepirudin did not reduce toxicity by inhibiting bioactivation of APAP and that at least some of the injurious effects of thrombin are not complete by 1.5 h after administration of APAP. This finding suggests the potential for thrombin inhibition in the treatment of APAP hepatotoxicity. However, one of the lepirudin-treated mice given APAP died prior to the sample collection time, with hemoabdomen and pronounced diffuse hemorrhage

in the liver. This mouse might have been more sensitive than the others to thrombin inhibition, with consequent life-threatening hemorrhage. It is well known that one of the main clinical symptoms of APAP hepatotoxicity in human patients is coagulopathy (Lee 1993; Larson et al., 2005,) and this emphasizes the potential risks of using thrombin inhibitors for the treatment of human APAP hepatotoxicity.

5.1.3 **Thrombin receptor PAR-4 plays a role in APAP hepatotoxicity**

The role of PAR-4, a thrombin receptor that is expressed on mouse platelets, was evaluated using PAR-4$^{-/-}$ mice (Sambrano et al., 2001). PAR-4$^{-/-}$ mice developed less liver injury after APAP compared to matching WT controls (Figure 9C,D and Figure 10). Interestingly, there was significantly less thrombin generation (decreased TAT) and platelet accumulation in the liver in PAR-4$^{-/-}$ mice (Figure 9A and B), indicating that PAR-4 activation contributes to these events.

5.1.4 **Platelet depletion decreases APAP hepatotoxicity**

Platelets accumulate in the liver after a hepatotoxic dose of APAP, and both a direct thrombin inhibitor and deficiency in PAR-4 ameliorates liver damage and decreases platelet accumulation in the liver after APAP. To further evaluate a potential role for platelets in liver injury, platelets were depleted prior to administration of APAP using anti-CD41 IgG. Liver injury and platelet accumulation were significantly reduced in

platelet-depleted mice (Figure 12B, D, E, H). Also, the increased plasma TAT complex concentration was significantly reduced by platelet depletion (Figure 12C). These findings suggest that one role of platelets in APAP-hepatotoxicity is as an amplifier of thrombin generation.

5.1.5 **vWf does not contribute to APAP hepatotoxicity**

vWf is one of the critical components of primary hemostasis, tethering platelets to sites of vascular injury, particularly under conditions of high shear stress (Sazbo etal. 2008). Based on our findings suggesting contributions of thrombin and platelets to APAP hepatotoxicity, we used $vWf^{-/-}$ mice to evaluate the contribution of vWf in platelet adherence and accumulation in APAP hepatotoxicity. Interestingly, there was no significant difference in liver injury or plasma TAT concentrations between $vWf^{-/-}$ mice and matching WT mice. However, there was a significant decrease in hepatic platelet accumulation. We also confirmed by flow cytometry that the platelets of $vWf^{-/-}$ mice and matching WT mice expressed similar amounts of CD41, so the decreased accumulation was not related to less detectable CD41 signal. Therefore, vWf appears to promote platelet accumulation in the livers of APAP-treated mice, but its absence does not appear to alter thrombin generation or injury. It could be that the amount of platelet accumulation in the liver affects thrombin generation. The CD41 signal in $vWf^{-/-}$ mice treated with APAP was around twice that of the $vWf^{-/-}$ mice and matching WT mice without APAP. On the other hand, with the platelet depletion study, platelet-depleted

mice treated with APAP still had only around 20% of the CD41 signal compared to the control mice without APAP. This suggests that there might be more platelets in the vWf$^{-/-}$ mice accumulating in the liver after APAP treatment compared to the platelet-depleted group treated with APAP and this number of platelets might be enough to generate thrombin in these vWf$^{-/-}$ mice even if the platelet accumulation in the vWf$^{-/-}$ mice was significantly decreased compared to the matching WT mice.

These findings suggest that vWf promotes platelet accumulation in the liver of APAP-treated mice, and that the decrease in platelet accumulation in the liver of vWf$^{-/-}$ mice was not to the level to influence thrombin generation or injury from APAP exposure. These results suggest further that thrombin plays an important role in APAP hepatotoxicity, and that one of the roles of platelets in APAP hepatotoxicity might be thrombin generation.

5.1.6 **Thrombin enhances APAP-induced injury of hepatocytes co-cultured with non-parenchymal cells**

To further evaluate the contribution of thrombin, we isolated mouse HPCs and treated them with APAP and thrombin. There was no significant difference in hepatocyte injury with or without thrombin. On the other hand, when we co-cultured the NPCs with primary HPCs, there was a significant increase in hepatocyte injury with thrombin exposure. This suggests that thrombin does not directly enhance APAP hepatocellular injury, but more likely works indirectly through NPCs.

5.1.7 **Platelets and thrombin promote peroxynitrite generation in APAP hepatotoxicity**

Peroxynitrite formation after exposure to toxic doses of APAP is suggested as one of the main contributors to APAP hepatotoxicity (Hinson et al., 1998; Cover et al., 2005). In addition to sinusoidal endothelial cells and parenchymal cells, platelets contain NOS isoforms and can produce NO (Gkaliagkousi et al, 2007), and thrombin can enhance NOS production through thrombin receptors (Meli et al., 2001; Touyz 2007; Momota et al., 2006). Based on these findings, we evaluated the hepatic accumulation of nitrotyrosine-protein adducts during APAP exposure in lepirudin, PAR-4 deficiency, and platelet-depletion models in vivo. Interestingly, lepirudin treatment, PAR-4 deficiency, and platelet depletion all reduced nitrotyrosine adducts. These findings suggest that platelets and thrombin can contribute to peroxynitrite generation in APAP hepatotoxicity.

Transient accumulation of RBCs is known to occur with APAP hepatotoxicity due to congestion and hemorrhage (Rose 1969; Walker et al., 1980; Walker et al., 1981; Walker et al. 1983; Walker et al., 1985; Francavilla et al., 1989; Ito et al., 2003). Platelet depletion and treatment with lepirudin reduced the RBC accumulation, suggesting that thrombin and platelets contribute to the congestion and/or hemorrhage during the pathogenesis of liver injury. RBC accumulation was also decreased at 24 h in PAR-4$^{-/-}$ mice treated with APAP. However with PAR-4$^{-/-}$ mice, RBC accumulation (congestion) was enhanced at 6 h. It has been reported that PAR-1 and PAR-4 on endothelial cells contribute to vascular relaxation and microvascular patency (Kataoka et al., 2003);

accordingly, impaired sinusoidal relaxation in the PAR-4$^{-/-}$ mice might have contributed to the greater congestion seen 6 h after APAP treatment.

The roles of congestion and hemorrhage in APAP hepatotoxicity are still not clear (Walker et al., 1981; Walker et al. 1983; Walker et al., 1985). However, RBCs can produce and release NO under hypoxic conditions (Nagababu et al., 2003; Allen et al., 2009). Thus, it seems possible that RBCs accumulating in developing lesions could release NO and thereby contribute to peroxynitrite generation and nitration of cellular proteins.

5.1.8 **Conclusion**

All of our results suggest that thrombin, the thrombin receptor PAR-4, and platelets contribute to the progression of APAP-induced liver injury. Also, our results suggest that platelets and thrombin might contribute to the release of NO needed for generation of peroxynitrite. Thrombin does not appear to enhance hepatocellular injury directly but more likely plays a role by stimulating NPCs in the liver.

Previously, we showed that tissue factor is activated very early (30 min) after APAP administration (Ganey et al., 2007). This and our present results suggest an amplification loop in which early activation of tissue factor results in generation of thrombin, which in turn activates platelets that encourage additional thrombin activation that activates NPCs, leading to enhanced NO production. Our previous results also pointed to a role for PAR-1 in the progression of hepatocellular injury. PAR-1 does not occur on mouse platelets, and since PAR-4 can occur on cells other than platelets,

activation of these receptors on NPCs by thrombin is likely important. Also, there are possible additional roles for platelets in addition to thrombin generation, such as adhering to sites of injury and forming platelet aggregates to cause RBC congestion that might lead to NO release and hypoxia. The proposed roles of platelets and thrombin are summarized in figure 27.

APAP hepatotoxicity is the most common cause of ALF in developed countries. However, treatment options for this hepatotoxicity are still limited. Although treatment with lepirudin after APAP significantly decreased hepatic injury, as discussed in Chapter 2 and in this summary, potential hemorrhagic complications may limit use of thrombin inhibitors and other direct coagulation inhibitors in human patients. Further evaluation of the mechanisms of platelet and thrombin enhancement of APAP-induced liver injury is therefore warranted.

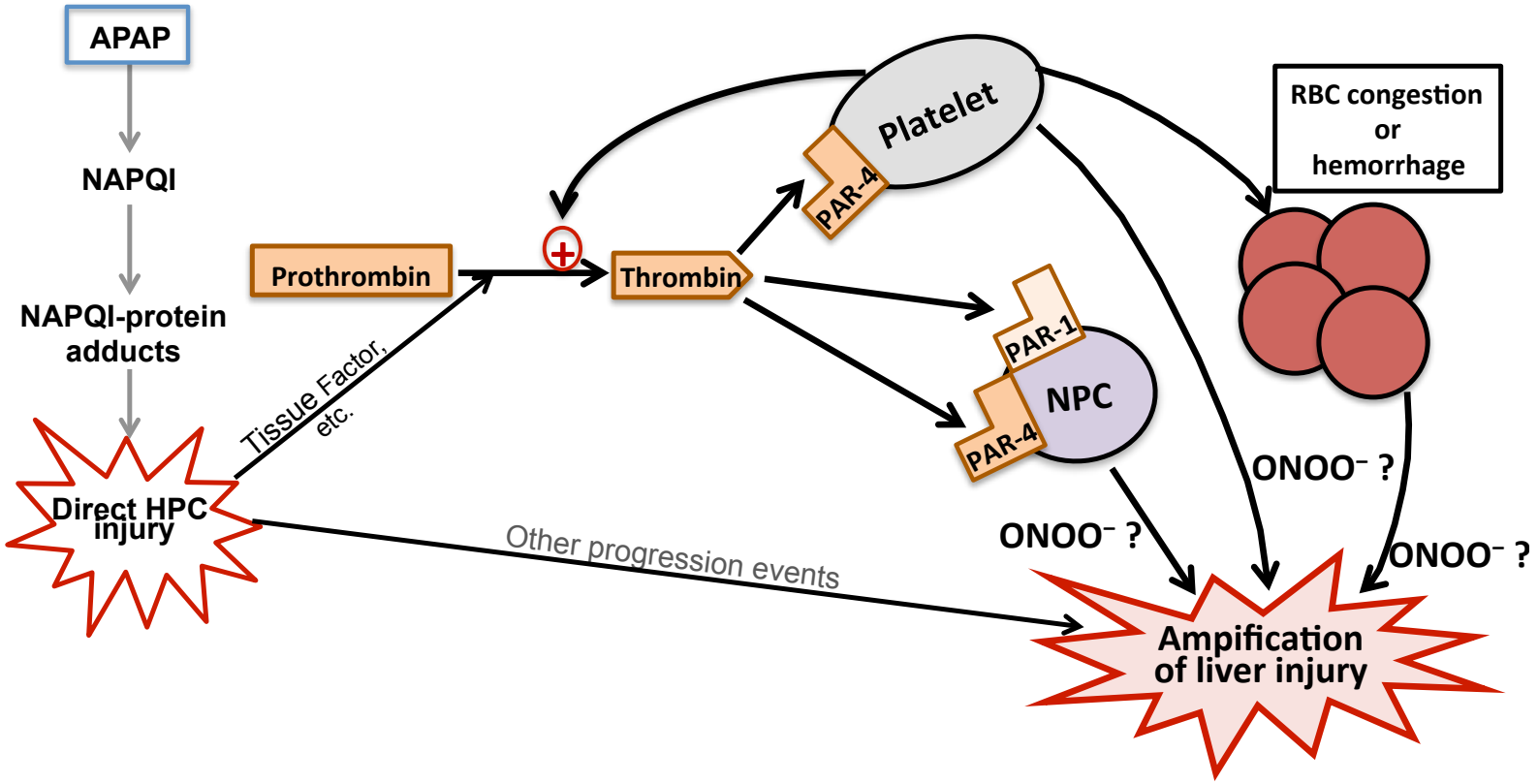

Figure 29: Proposed pathways leading to amplification of liver injury by platelets and thrombin.

Figure 29 (cont'd)

Our results suggest that platelets and PAR-4 contribute to amplification of thrombin generation and to the progression of liver injury caused by acetaminophen, and that the contribution of thrombin to liver injury is mediated impart indirectly through PARs associated with NPCs. Direct effects of platelets on hepatocytes and the contribution of congestion and hemorrhage secondary to platelet aggregation also need to be considered. Also, our results suggest that platelets and thrombin can contribute to the generation of peroxynitrite ($ONOO^-$).

5.2 **A cytochrome P450-independent mechanism of acetaminophen-induced hepatocellular injury**

My other main line of research started with trying to determine the appropriate concentration of APAP to use in primary mouse HPCs, since I was interested in exploring direct effects of thrombin in vitro. I was surprised when my PubMed search revealed how many different concentrations had been used for primary mouse HPCs (from 0.1-50 mM, with 5 mM and 10 mM used most commonly (Figure 20)). Because of uncertainty about which would be most appropriate, we decided to perform a concentration-response study in primary mouse HPCs, and that led to this line of investigation.

5.2.1 **Biphasic concentration dependence of APAP cytotoxicity**

The concentration-response relationship for primary mouse HPC injury measured by ALT release was biphasic (Figure 21A). APAP-protein adducts were also measured and increased in a manner that matched the cytotoxicity up to 5 mM. However, APAP adduct concentration remained the same above 5 mM APAP, even as the cytotoxicity increased (Figure 21A). These results raised the possibility that more than one mechanism was at play (i.e., one operating at low APAP concentrations and another that predominated at higher APAP concentrations).

5.2.2 **CYP-independent APAP hepatotoxicity in high concentration (i.e., 8 mM and above)**

We next used a pan-CYP inhibitor, ABT, and showed that it minimized the APAP-protein adducts, confirming effective inhibition of CYP-mediated APAP bioactivation (Figure 21B and 22). Up to the concentration of 5 mM, APAP produced no increase in ALT after ABT exposure, but a concentration-dependent increase in ALT release was observed at APAP concentrations of 8 mM and above (Figure 21B). These findings suggested that there is more than one initiating mechanism that can contribute to cell death from APAP. One of them is a CYP-dependent mechanism operative at small APAP concentrations and the other is a CYP-independent mechanism that predominates at larger concentrations.

5.2.3 **Early, low concentration CYP-dependent mechanism and late, high concentration CYP-independent mechanism**

A time-course study with APAP revealed that the cytotoxic response at low APAP concentrations began early and was complete by 6 h (Figure 24A). However, injury continued to progress with time at higher concentrations (Figure 24A). When pretreated with ABT, there was no cytotoxicity at low APAP concentrations but, at higher concentrations, injury did not begin until after 6 h and progressed with time (Figure 24B). This suggests that the CYP-dependent mechanism(s) of cell injury that predominate at lower concentrations contribute to injury rapidly and to a limited degree. On the other

hand, CYP-independent mechanisms at higher APAP concentrations develop more slowly and can result in complete cell death.

5.2.4 **APAP deacetylation contributes to its CYP-independent cytotoxicity**

We were next interested in evaluating the main contributor to this CYP-independent APAP cytotoxicity. Based on pathways of APAP metabolism, we hypothesized that one of the minor metabolites of APAP, PAP, played a role. PAP has been reported to be involved in renal injury with APAP in rats (Newton et al., 1985A, 1985B) and cell toxicity in cultured renal cells (Lock et al., 1993; Klos et al., 1992; Harmon et al., 2006). PAP is formed by deacetylation of APAP, so we used a deacetylation inhibitor, BNPP, to see if BNPP would decrease cell injury at high concentrations of APAP, with or without ABT. ABT reduced cytotoxicity caused by 10 mM APAP (Figure 25C) but was without effect at 14 mM APAP (Figure 25D). BNPP did not influence cytotoxicity caused by 2.5 mM APAP (Figure 25B). However, it significantly reduced the cytotoxicity caused by either 10 or 14 mM APAP (Figure 25C and 25D). Interestingly, HPCs treated with both BNPP and ABT experienced less cytotoxicity upon exposure to 10 or 14 mM APAP than occurred with either inhibitor alone. Why this decrease in cytotoxicity with both inhibitors appeared to be additive at 10 mM APAP and synergistic at14 mM APAP is not clear, but the findings suggest that deacetylation contributes to HPC toxicity at high APAP concentrations.

5.2.5 **PAP is cytotoxic to primary HPCs**

As our next step, we determined if PAP is directly cytotoxic to HPCs. PAP caused a dose-dependent increase in ALT release starting at concentrations above 200 μM and was significantly increased from baseline at 300 μM (Figure 25). Also, ABT pretreatment did not alter the PAP hepatotoxicity suggesting that PAP-induced hepatotoxicity is not related to the metabolism of PAP to APAP in this model. If PAP indeed contributes to APAP cytotoxicity, it should injure HPCs at concentrations less than those caused by APAP, since PAP is one of the metabolites of APAP; indeed, direct addition of PAP to HPCs was injurious at concentrations far less (approximately1/40th) than concentrations of APAP needed to cause CYP-independent cytotoxicity. This finding further supports that PAP might contribute to APAP hepatotoxicity.

5.2.6 Cells exposed to high concentration APAP and cytotoxic concentration of PAP turn brown in color

Interestingly, cells exposed to large, but not small, cytotoxic APAP concentrations (i.e. at and above 8 mM) turned brown in color, as did HPCs exposed to cytotoxic concentrations of PAP (i.e., at and above 250 μM) (Figure 26). PAP in solution undergoes oxidation in neutral and alkaline conditions and may polymerize to yield a brown color over time, and others have suspected that this is the likely cause of brown urine and pigmentation of stored serum with APAP overdosed patients (Clark et al., 1986; Brown et al., 1983). These observations suggest that brown coloration of the

cells that exposed to high concentrations of APAP (i.e., at and above 8 mM) could be related to PAP.

5.2.7. **Conclusion**

Our data show that at least two mechanisms contribute to APAP cytotoxicity. One is a CYP-dependent mechanism that operates at small, cytotoxic APAP concentrations and that occurs rapidly and is limited both in degree and duration. This mechanism is related to CYP-dependent NAPQI production. The reason for the limitation in degree and duration could be related to NAPQI inhibition of CYPs (Snawder et al., 1994) and to hepatocellular heterogeneity of CYPs that could lead to differences in NAPQI production on hepatocytes. The second is a CYP-independent mechanism that predominates at higher APAP concentrations and that is slower to develop and highly lethal. Deacetylation of APAP to PAP appears to be a contributor to the CYP-independent mechanism and might enhance cell death by its own mechanism or by working on cells already stressed by NAPQI. The proposed pathway for these 2 mechanisms is presented in Figure 28 (Chapter 4). Whether the CYP-dependent and CYP-independent initiating mechanisms activate similar or different cell death signaling pathways remains to be determined.

5.3. **Knowledge gaps and future studies**

Both lines of study described above, one regarding the contribution of platelets and thrombin to APAP hepatotoxicity (Chapters 2 and 3) and one regarding a CYP-independent mechanism of APAP hepatotoxicity (Chapter 4), yielded novel findings about APAP hepatotoxicity. However, neither provides detailed mechanistic insight into how liver injury and hepatocellular death occur.

The findings presented in the platelet and thrombin studies suggest that thrombin generation by the platelet is important and thrombin can contribute to hepatocellular death by working on NPCs. Many of the findings suggest thrombin as a key player in our studies; however, direct effects of platelets on hepatocytes and NPCs have not yet been evaluated. These could be evaluated by using a co-culture system with platelet-rich plasma and different platelet fractions (i.e., membrane versus cytosol) to further evaluate if either the cell membrane binding proteins or components from the cytosol component contributes to APAP induced hepatocyte injury. It would also be interesting to use conditional knockout mice that we can completely deplete of platelets and treat with APAP to see if the protection occurs.

Even if thrombin works with NPCs to enhance hepatocellular injury, we have not evaluated which NPCs are the main contributors. This could be evaluated by co-culturing different cell types of the NPCs with hepatocytes, and treating them with thrombin. Also, the contribution of each thrombin receptor on NPCs has not been evaluated. Thrombin receptors that are activated by thrombin are only PAR-1, 3, and 4. To further evaluate the role of each receptor, thrombin-specific agonists could be used

on isolated NPCs and primary HPCs treated with APAP and thrombin to characterize the cell types and thrombin receptors important in APAP hepatotoxicity.

This series of studies suggested that NO production by thrombin and/or platelets might contribute to liver injury. It has been reported that platelets and thrombin can contribute to the increase in NO production. It would be interesting to quantify NO in a coculture model treated with APAP and thrombin.

As discussed in the 4.1.7, it seems possible that RBCs accumulated in developing lesions could release NO and thereby contribute to peroxynitrite generation and nitration of cellular proteins. Further evaluation of RBCs and their NO release would be interesting if an appropriate model (in vivo and in vitro) could be found.

It is also important to evaluate other contributors to APAP hepatotoxicity downstream from thrombin-receptor signaling and platelets. As described in Chapter 2, post-APAP lepirudin was protective, but a mouse bled significantly and died during the experiment. This may be a critical problem for APAP-hepatotoxicity patients that often suffer coagulopathy. If future experiments can identify downstream contributors that do not negatively influence the coagulation system, these contributors might lead to alternative treatments for APAP hepatotoxicity.

The second main study (Chapter 3 or 4) showed evidence that there are at least two initiating mechanisms for APAP hepatotoxicity in primary mouse HPCs. One occurs at low APAP concentrations and is mainly CYP-dependent, while the other occurs at high concentrations and involves also a CYP-independent mechanism. One of the CYP-independent mechanisms is suggested to occur through PAP and deacetylation, but a

detailed mechanism of how these contribute to APAP hepatotoxicity has not yet been evaluated. It has been suggested that a PAP-SG metabolite formed in kidney cells treated with PAP is toxic to the cells (Klos et al.,1992; Fowler et al., 1991). Further evaluation of the mechanism of hepatocellular death and of hepatic accumulation of PAP and PAP-SG would be interesting.

Further evaluation of the mechanism of cell death by PAP and comparison to what has been suggested in APAP hepatotoxicity would be interesting. Since some previous findings suggest that protection from certain approaches (RIP3 KO (Ramachandran et al., 2013) and use of cyclosporine A (Kon et al., 2004, 2007)) was seen early with high concentrations of APAP but diminished later, early protection may have been CYP-dependent but later loss of protection might have been due to the late CYP–independent mechanisms.

Also, further evaluation of a possible contribution of PAP to APAP hepatotoxicity in an in vivo mouse model is suggested. This could include experiments using BNPP to assess for inhibition of liver injury at high doses of APAP or PAP after pretreatment with ABT; such experiments would help determine if the in vitro finding is relevant to in vivo APAP hepatotoxicity.

The protection that was seen with primary hepatocytes treated both with ABT and BNPP was only partial. Searching for a possible 3rd mechanism of cell injury at high concentrations of APAP is warranted if there is significant ALT release with ABT and BNPP pretreatment and PAP accumulation is similar to controls.

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