MECHANISMS REGULATING TISSUE FACTOR:FACTOR VIIA-DEPENDENT COAGULATION IN LIVER DISEASE

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ABSTRACT

MECHANISMS REGULATING TISSUE FACTOR:FACTOR VIIA-DEPENDENT COAGULATION IN LIVER DISEASE

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Over 50 million people in the world have a chronic liver disease. Interestingly, as a result of liver disease, patients often acquire complex alterations in their coagulation cascade. The coagulation cascade plays a critical role in preventing hemorrhage after vascular injury through the formation of a blood clot. There is also evidence suggest that the activation of the coagulation cascade worsens the progression of liver disease. However, the mechanisms underlying the activation of coagulation during liver disease are poorly understood. If the mechanisms whereby coagulation is activated during liver disease were known, one could develop targeted strategies to inhibit pathologic coagulation in patients with liver disease.

The coagulation cascade is controlled by a complex composed of tissue factor (TF) and its plasma ligand, factor FVIIa (FVIIa). Within the liver, the TF:FVIIa complex exists in a non-procoagulant, 'encrypted' state on hepatocytes. Prior studies focusing on encrypted TF:FVIIa have identified several potential mechanisms underlying the activation of TF:FVIIa. However, the *in vivo* relevance of these mechanisms has remained unclear, because examples of TF:FVIIa activation triggered by (patho)physiologically-relevant mediators are lacking.

A major focus of the work described in this dissertation was to identify activators of coagulation during liver injury. Interestingly, bile acids, cholesterol metabolites produced in the liver, accumulate within the liver parenchyma and plasma during liver disease. Exploring the possibility for bile acids to activate coagulation, I found that pathologically relevant concentrations of the bile acid, glycochenodeoxycholic acid, increased hepatocyte TF:FVIIa activity. Expanding

on this finding, I tested the hypothesis that bile acids directly increase TF:FVIIa activity by using TF:FVIIa relipidated in unilamellar vesicles and soluble TF:FVIIa. The bile acids, glycochenodeoxycholic and taurochenodeoxycholic acid, directly increased the activity of relipidated and soluble TF:FVIIa. Interestingly, it was found that not all bile acids act through the same mechanism. Taurocholic acid did not directly activate TF:FVIIa but increased hepatocyte TF:FVIIa activity through the non-apoptotic externalization of phosphatidylserine *in vitro*. These results indicate that bile acids increase TF:FVIIa procoagulant activity through both direct and indirect mechanisms.

Although prior studies have focused on hepatocyte TF, it is unknown if hepatocyte TF is universally required to activate coagulation after challenge with hepatotoxicants. Another focus of this work was to determine the role of hepatocyte TF in acute and chronic challenge with carbon tetrachloride (CCl_4). Contrary to my hypothesis that hepatocyte TF drives coagulation after CCl_4 challenge, there was no change in the activation of coagulation in mice deficient of hepatocyte TF versus control mice after acute and chronic challenge with CCl_4. This suggests another cellular source of TF is driving coagulation after CCl_4 challenge. Notably, deficiency in hepatocyte TF did not affect the development of fibrosis. In summary, contrasting other models of liver injury, this suggests hepatocyte TF does not drive coagulation after CCl_4 challenge.

In summary, these results indicate that bile acids increase TF:FVIIa procoagulant activity, which suggests the possibility for bile acids to activate coagulation during liver disease. Additionally, it was found that hepatocyte TF does not contribute to the activation of coagulation in all models of liver injury, which suggests the potential for another cellular source of TF that is driving coagulation. Overall, this work expands our knowledge of how coagulation is regulated within the liver and identifies potential activators of coagulation that increase during liver disease.

To my friends and family

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KEY TO ABBREVIATIONS

ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANIT	alpha-napthylisothiocynate
BACS	bile acid:CoA synthase
BAT	bile acid:amino acid transferase
BDL	bile duct ligation
BSEP	bile salt exporting protein
CCl_4	carbon tetrachloride
COL1A1	collagen type 1 alpha 1
CYP27A1	cytochrome P450 27A1
CYP7A1	cytochrome P450 7A1
CYP7B1	cytochrome P450 7B1
CYP8B1	cytochrome P450 8B1
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FIX	coagulation factor IX
FVIIa	coagulation factor VIIa
FXa	coagulation factor Xa
FXR	farnesoid X receptor
GCDCA	glycochenodeoxycholic acid
HBS	HEPES buffered saline

HBSA	HEPES buffered saline with albumin
HPC	hepatocyte
i.p.	intraperitoneal
NTCP	sodium taurocholate cotransporting polypeptide
PAR	protease activated receptor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDI	protein disulfide isomerase
PDVF	polyvinylidene fluoride
PS	phosphatidylserine
SPR	surface plasmon resonance
TAT	thrombin-antithrombin
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TF	tissue factor

CHAPTER 1

Introduction

Introduction to liver disease

There are several prominent causes of liver disease including but not limited to: obesity, environmental factors (e.g., alcohol consumption), and viral hepatitis infection.[1-3] A large epidemiological study documented that viral infections represented the lowest cause of liver disease whereas non-alcoholic fatty liver disease (NAFLD) represented the largest portion of cases.[4] NAFLD is defined as the excessive accumulation of fat within the liver (5-10% by weight) and as the name implies, develops in people that do not consume alcohol excessively.[5] NAFLD includes fatty liver, non-alcoholic steatohepatitis (fatty liver with inflammation), and fibrosis/cirrhosis. Liver fibrosis, the excessive deposition of collagen within the liver, commonly occurs as the result of persistent liver injury. If the cause of fibrosis is not addressed, fibrosis eventually progresses into cirrhosis. As previously mentioned, environmental factors such as chronic alcoholism have been shown to cause liver disease and subsequent cirrhosis. Indeed, chronic alcoholism was estimated to be responsible for 50% of the reported deaths by cirrhosis in 2014.[6] In addition to chronic liver diseases (i.e., NAFLD), acute liver injury can cause a loss in liver function that occurs rapidly. One example is acetaminophen toxicity, which is one of the leading causes of acute liver injury in humans.[7] Overall, regardless of the underlying cause, both acute and chronic liver disease can lead to liver failure and ultimately death. In 2018, the global mortality of liver disease was approximately 2 million individuals.[8] Complications that accompany liver disease are liver cancer, infection, jaundice, and organ failure.[9-11] Unfortunately, liver transplantation is the only cure for end stage liver disease, and in the United States it has been reported that more than 10,000 people are waiting for liver transplants each year. Interestingly, secondary to their disease, patients often have accompanied changes in their blood coagulation cascade leading to the activation of coagulation. This activation is important to consider as studies

have documented the effect of blood coagulation on the progression of liver disease. [12, 13] Through the exploration of the underlying mechanisms whereby the blood coagulation cascade impacts the development of liver disease, new therapeutics may be identified. In this dissertation, I will be focusing on how the primary activator of the blood coagulation cascade is controlled within the liver and the potential mechanisms whereby coagulation is activated during liver disease.

Introduction to blood coagulation

The hemostatic system plays an important role in preventing blood loss upon vascular damage. Primary hemostasis occurs when blood vessels are damaged and subendothelial proteins (e.g., collagen) are exposed, causing circulating platelets in the blood to adhere and aggregate at the site of injury.[14] This platelet plug prevents hemorrhage into the tissue through the formation of a physical barrier. Simultaneously, secondary hemostasis begins with the activation of the blood coagulation cascade. The blood coagulation cascade is a series of enzymatic activation events involving serine proteases that culminates in formation of a fibrin polymer, which among other functions serves to stabilize the platelet plug.[15] The coagulation cascade is activated by the complex of the transmembrane receptor tissue factor (TF) and its ligand factor VIIa (FVIIa), which circulates in plasma (Figure 1).[16] Tissue factor (TF) is the primary activator of coagulation; therefore, TF activity must be tightly controlled. TF is constitutively expressed on subendothelial cells (e.g., fibroblasts),[17, 18] which restricts FVIIa from engaging with TF. This physical separation of TF from plasma FVIIa by the endothelium is critical for controlling TF activity but also allows for the rapid binding of FVIIa to TF upon endothelial injury (Figure 2). Thus, this pattern of TF expression forms a 'hemostatic envelope' around blood vessels, limiting activation of TF:FVIIa to sites of vessel injury.[18, 19] Upon binding of plasma FVIIa to TF, the TF:FVIIa complex converts

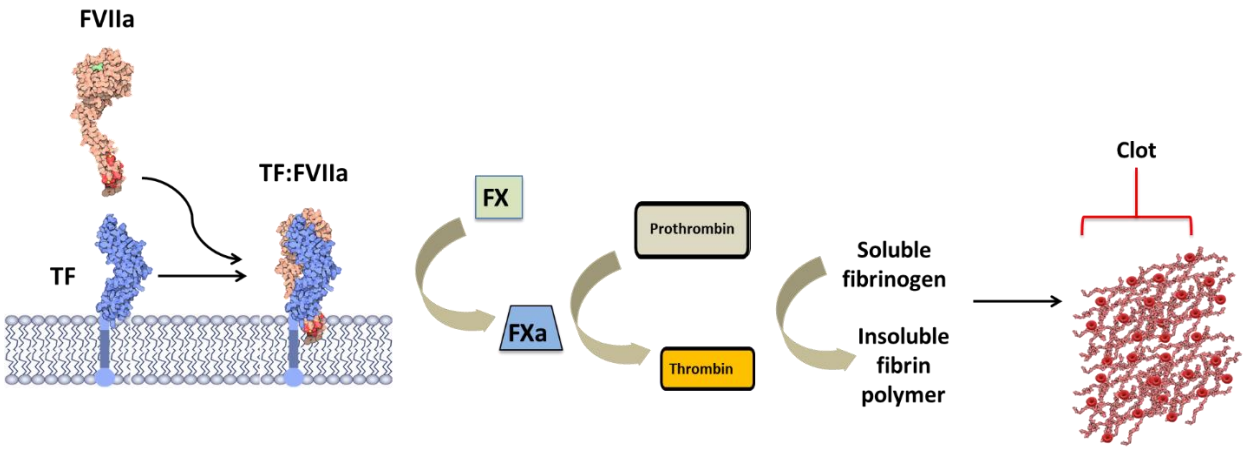

Figure 1. The binding of FVIIa to TF begins the coagulation cascade. TF is a transmembrane protein that binds to plasma FVIIa. The TF:FVIIa complex then converts FX to FXa and activates FIX to FIXa (not pictured). FXa converts prothrombin to thrombin, and thrombin cleaves soluble fibrinogen to insoluble fibrin polymer, a critical component of blood clots. PDB ID: 1dan from Banner, D.W., D'Arcy, A., Chene, C., Winkler, F.K., Guha, A., Konigsberg, W.H., Nemerson, Y., Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* 380: 41-46

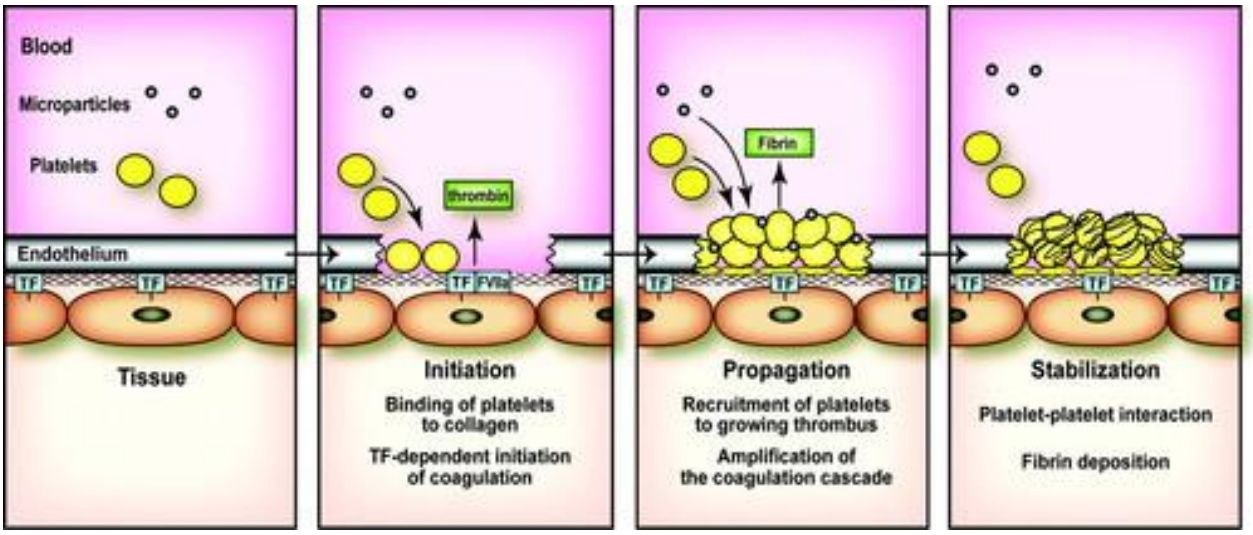

Figure 2. The formation of a blood clot at the site of injury. Normally, TF is separated from blood containing FVIIa by an endothelial barrier. Upon injury to the endothelium, primary hemostasis begins with the binding of platelets to exposed extracellular matrix proteins, forming a platelet plug. Disruption to the endothelial barrier allows, FVIIa to bind TF initiating the coagulation cascade, which leads to the production of thrombin. Thrombin produces fibrin and fibrin stabilizes the platelet plug. This image was reproduced with permission. Nigel Mackman et al. "Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis." *Arteriosclerosis, thrombosis, and vascular biology* 27.8 (2007): 1687-1693.

plasma coagulation factor X (FX) and factor FIX (FIX) into their activated forms (FXa and FIXa respectively).[20] This enzymatic step generates thrombin by direct FXa activation of prothrombin, and by the activation of additional serine proteases (i.e., coagulation factor V and VIII) that ultimately amplifies thrombin generation.[21] Thrombin is a critical component of the coagulation cascade, as it cleaves soluble plasma fibrinogen into insoluble fibrin, which in turn stabilizes the platelet plug. In order to further control the activation of coagulation, there are anticoagulant proteins in the blood (e.g., antithrombin) that bind to serine proteases and inactivate them.[22] Additionally, upon formation of the blood clot, another process begins to prevent excessive clot formation. (i.e., fibrinolysis). Overall, coagulation is a tightly controlled process that serves to localize clot formation to the injured vessel where TF is exposed without hindering blood flow.

Coagulation plays a critical role in preventing bleeding after vascular injury. However, coagulation also plays a role in the development of thrombosis, which occurs when a clot occludes a blood vessel. Arterial and venous thrombosis are a common complication of numerous diseases including cardiovascular diseases, obesity, and cancer.[23-26] Arterial thrombosis is the major cause of stroke and myocardial infarction, whereas venous thrombosis leads to pulmonary embolism, which occurs when parts of the occluding blood clot break off and travel to the lung resulting in disrupted blood flow.[27, 28] Taken together, this makes thrombosis a leading cause of death in the developed world.[29, 30] Standard oral anticoagulation to reduce the risk of thrombosis was once limited to use of warfarin, which reduces the synthesis of the active forms of several coagulation factors (e.g., FX and FVII) by the liver.[31] Another example of an anticoagulant is heparin, which increases the affinity of antithrombin to the serine proteases, thrombin and FXa, ultimately inactivating them. Low-molecular weight forms of heparin have been developed that increase the affinity of antithrombin to specifically inhibit FXa (e.g., enoxaparin).[32] More recently, orally

bioavailable small molecule inhibitors have been developed that inhibit specific coagulation proteases (i.e., thrombin and FXa).[33, 34] In summary, controlling coagulation is critical to prevent pathologic coagulation while still maintaining the ability to stop hemorrhage after vascular injury.

Coagulation in liver disease

The liver is the primary site of synthesis for many of the pro and anticoagulant proteins constituting the coagulation cascade.[35] As such, acute and chronic liver diseases are often associated with changes in coagulation.[36, 37] Routine diagnostic coagulation tests (e.g., prothrombin time), frequently used to diagnose bleeding disorders in patients, suggest that patients with liver disease display a bleeding tendency.[38] However, because patients with liver disease have altered liver function (e.g., synthesis of proteins), they have a reduction in plasma levels of both pro and anticoagulant proteins produced by the liver.[39] Indeed, patients with liver disease have a delicately rebalanced hemostatic system, characterized by equivalent reductions in procoagulant and anticoagulant factors.[40-42] This delicately rebalanced hemostatic system is disturbed by complications during liver disease and tipped towards bleeding or clotting. Because routine tests (e.g., prothrombin time) do not necessarily account for a reduction in both pro and anticoagulant proteins, they do not adequately predict the bleeding risk for patients with liver disease.[38, 43] Indeed, other assays (e.g., thrombin generation assay) that can account for the reduction in pro and anticoagulant proteins have been used to improve our understanding of coagulation changes in liver disease patients. Surprisingly, despite past dogma, patients with liver disease do not appear to have a bleeding risk, and instead display a normal to hypercoagulable (i.e., clotting risk) state.[38, 44]

Although patients with liver disease are in a normal to hypercoagulable state, this does not mean that coagulation is actively occurring. To determine whether coagulation is occurring *in vivo*,

products of coagulation can be measured. For example, a small fragment removed during conversion of prothrombin to thrombin (i.e., prothrombin fragment 1.2) can be measured in blood of patients as an indicator of activated coagulation.[45] Thrombin itself has such a short half-life in plasma, because it rapidly associates with antithrombin.[46] This complex, termed thrombin-antithrombin (TAT), can be measured in plasma as a marker of activated coagulation, because thrombin-antithrombin has a longer plasma half-life than thrombin.[47] Because measuring formation of blood clots is not always practical, the byproducts of fibrin clot degradation (e.g., d-dimer) are more routinely measured as a reflection of clot formation.[48] Measuring specific products of coagulation in blood provides a method to determine whether coagulation has occurred.

There is evidence of increased coagulation activity in patients with acute liver toxicity and chronic liver disease.[49-53] However, the consequences of increased coagulation activity in patients with liver disease are not fully understood. Some studies suggest that coagulation can play in role in the progression of liver fibrosis, which is the excessive deposition of extracellular matrix proteins (e.g., collagen) within the liver. For example, patients with a genetic polymorphism (e.g., factor V Leiden) that increases the risk for thrombosis have increased progression of hepatitis C-mediated liver fibrosis.[12] Similarly, a prothrombin mutation (i.e., G20210A), which causes the excessive production of prothrombin, increased the progression rate of fibrosis in patients with viral hepatitis.[13] Additionally, hepatitis infected patients with hemophilia, which is a hypocoagulable condition, displayed a slower disease progression compared to patients with hepatitis without hemophilia.[54] Overall, these studies suggest that genetic predisposition to either hyper- or hypocoagulability is linked to the progression of pathologies (e.g., liver fibrosis). Furthermore, patients with cirrhosis (a condition in which the liver cannot repair itself) are at increased risk of developing portal vein thrombosis, which is a blockage of the major source of

blood into the liver (i.e., the hepatic portal vein).[44, 55] The potential for portal vein thrombosis to worsen the progression of liver disease has prompted some studies examining the use of anticoagulants in patients with cirrhosis. Therapeutic anticoagulation with enoxaparin (low-molecular-weight heparin) prevented portal vein thrombosis in cirrhotic patients.[56] Remarkably, anticoagulation also delayed hepatic decompensation, which occurs when the remaining functional liver mass can no longer meet demand. While limited, this is consistent with the idea that coagulation contributes to the progression of liver disease.

Activation of coagulation has been observed in rodent models of both acute and chronic liver injury.[57-67] For example, in studies modeling acute acetaminophen overdose in rodents, there was an increase in plasma TAT complexes, suggesting an increase in the activation of coagulation. Additionally, acute exposure to the hepatotoxicant, alpha-naphthyl isothiocyanate (ANIT) significantly increased plasma TAT levels and fibrin(ogen) deposition within the livers of mice.[61] Together, this suggests that acute hepatotoxicant exposure can increase coagulation. Furthermore, studies involving chronic exposure to hepatotoxicants have documented activation of coagulation. For example, chronic exposure to ANIT significantly increased plasma TAT levels, and another study documented an elevation in plasma TAT and hepatic deposition of fibrin(ogen) after bile duct ligation.[67, 68] Overall, various rodent models of liver injury have documented concurrent activation of coagulation, which suggests that liver injury is connected to coagulation.

Prior studies suggest that TF contributes to various features of liver pathology in mouse models of liver injury and disease. In mice that express low levels of TF (~1% of normal levels; low TF mice), there was evidence of a decrease in liver injury compared to mice heterozygous for TF after an acute hepatotoxic dose of acetaminophen.[57] Low TF mice also had a significant reduction in liver injury compared to heterozygous mice after administration with ANIT.[60] This

suggests that TF plays a role in the development of liver injury after exposure to a hepatotoxicant. Moreover, in a non-alcoholic induced steatohepatitis model, low TF mice had a reduction in liver injury and inflammation compared to mice heterozygous for TF.[66] Additionally, TF also contributed to liver fibrosis in a model of ANIT-induced liver injury.[68] Moreover, a neutralizing antibody against TF (1H1) reduced liver injury in a model of severe hepatitis.[69] Overall, this suggests that TF plays a role in the progression of liver injury and fibrosis.

Studies have documented potential mechanisms whereby coagulation contributes to the development of liver fibrosis. One potential mechanism involves the blockage in hepatic blood flow by a blood clot. This results in tissue ischemia (i.e., limited blow flood), which causes parenchymal extinction and the eventual replacement of hepatocytes with collagen (i.e., fibrosis).[70, 71] Studies have documented that pharmacological inhibition of thrombin and FXa significantly attenuates liver pathology in a variety of experimental settings.[64, 72-75] Another potential mechanism whereby coagulation could affect the progression of liver disease involves the activation of hepatic stellate cells, which are cells largely responsible for the deposition of collagen during fibrosis. The mechanisms linking coagulation to stellate cell activation includes the serine proteases, FXa and thrombin. FXa and thrombin cleave and activate protease activated receptors (PARs), which are tethered-ligand G-protein coupled receptors present on various cells including stellate cells. Cleavage of PARs by serine proteases is thought to lead to stellate cell activation and subsequent collagen deposition.[76-78] Taken together, models of acute and chronic liver injury have revealed potential pathways whereby coagulation contributes to the development of liver disease.

Although several studies link activation of the coagulation cascade to the pathogenesis of liver disease, the precise triggers for coagulation activation in the liver microenvironment remain unknown. If we understood the precise mechanisms underlying the activation of coagulation in

patients with liver disease, we could potentially reduce the pathologic consequences of coagulation in patients with liver disease with new therapies that do not carry the same risks as existing anticoagulants. An important gap in our understanding of mechanisms controlling coagulation in the liver is how TF expression and activity is regulated in the liver.

Regulation of tissue factor in the liver

In most tissues, a continuous endothelial barrier separates subendothelial cells expressing TF from plasma FVIIa to control the activation of coagulation. This underlies the concept of the 'hemostatic envelope', in which disruption to the endothelial barrier activates coagulation at a site of vascular injury (i.e., where TF is exposed). Interestingly, TF is expressed in a tissue-dependent manner, with high levels of TF present in several tissues wherein bleeding would be quickly fatal (i.e., the brain, heart, lung). [18] TF is also expressed within the liver, albeit at low levels.[18] This presents a unique challenge, because in addition to traditional vascular endothelium, the liver contains specialized sinusoidal endothelial cells. These specialized endothelial cells have fenestrations (i.e., pores), which allows for the exchange of fluid and proteins between blood and hepatocytes.[79] This fenestrated endothelium may present a challenge to the notion of a 'hemostatic envelope' if TF is expressed on cells underlying the fenestrated endothelium. Furthermore, the liver produces coagulation factors, including FVII/VIIa, which would allow FVIIa will be in direct contact with cells expressing TF. Overall, the liver expresses low levels of TF in a unique environment wherein there is a fenestrated endothelium in the organ responsible for producing coagulation factors, which suggests a unique mechanism whereby TF activity is controlled within the liver.

TF expression within the liver is very precarious as the sinusoidal structure could potentially allow for a direct interface of components of plasma with cells expressing TF. Defining

which cells in the liver express TF might explain how TF activity is controlled. Notably, there are several types of cells present within the liver, but the most prominent cells are hepatocytes, which constitute 80% of the liver mass.[80] Hepatocytes carry out many necessary functions, including the synthesis of coagulation factors.[81] Interestingly, prior studies have shown that hepatocytes can convert FX to FXa, and later it was found that hepatocytes express TF.[82-84] The liver is a seemingly dangerous place to express TF as the fenestrated endothelium would allow plasma FVIIa to bind hepatocyte TF. Furthermore, several studies have shown that hepatocyte TF contributes to coagulation during liver injury.[67, 84] Notably, under normal conditions, pathologic coagulation does not occur within the liver, which suggests that the physical separation of TF from FVIIa is not the only mechanism controlling TF activity. Taken together, this suggests that hepatocyte TF activity must be regulated by a mechanism other than physical separation from FVIIa. Surprisingly, it has been documented that hepatocyte TF is bound to FVIIa but remains in an inactivate state.[84] This suggests that, in contrast to the 'hemostatic envelope', the binding of FVIIa to TF is not sufficient to activate coagulation. Therefore, there must be another mechanism whereby TF:FVIIa activity is controlled within the liver.

There is evidence that the TF:FVIIa complex can exist in a state with limited to no procoagulant activity, which is commonly referred as "encrypted".[85] Encryption is defined as the posttranslational suppression of TF:FVIIa procoagulant activity, which can be activated rapidly without a requirement for *de novo* synthesis.[86-89] A mechanism such as encryption would be necessary to control TF:FVIIa activity within the liver microenvironment where hepatocyte TF is in constant contact with plasma FVIIa. Previous studies found that the activity of cellular TF:FVIIa can be increased by mechanical stress (e.g., freeze-thawing) or treatment with various compounds

(e.g., calcium ionophores).[87-92] From these studies, potential mechanisms whereby TF:FVIIa becomes activated have been proposed.

One potential mechanism shown to control TF:FVIIa activity is the externalization of the membrane phospholipid, phosphatidylserine.[86, 93, 94] Normally, phosphatidylserine is asymmetrically distributed, wherein most phosphatidylserine is held in the inner leaflet of the outer cell membrane. The levels of phosphatidylserine are kept low in the outer leaflet by an active process involving coordinated regulation of flippase and scramblase enzymes.[95] Externalization of phosphatidylserine to the outer leaflet of the cell membrane is perhaps most commonly associated with induction of apoptosis but is also induced by a number of stimuli that elicit cell activation. For example, treatment of cells with calcium ionophores increases the levels of intracellular calcium, prompting a series of intracellular events coupled to phosphatidylserine externalization.[96, 97] Although its role as a flag for phagocytic cells is perhaps the most well appreciated, externalized phosphatidylserine on the cell surface contributes to coagulation reactions.[98, 99] As a transmembrane protein complex, the TF:FVIIa complex contains specific domains that enable interactions with phospholipids,[100] and phosphatidylserine interactions increase TF:FVIIa procoagulant activity.[87, 101, 102] Together this highlights how the regulation of phosphatidylserine localization at the cell membrane ultimately has a major impact on the regulation of coagulation reactions initiated by TF:FVIIa

Another mechanism thought to play a role in the activation of TF:FVIIa involves chemical modifications to the TF protein. It is thought that formation of a disulfide bond (i.e., Cys^{186}-Cys^{209}) on the TF protein controls the procoagulant activity of TF:FVIIa complex.[103] It hypothesized TF:FVIIa activity is mediated by protein disulfide isomerase (PDI), which is an oxidoreductase that acts like a molecular switch to turn on TF:FVIIa activity. Evidence suggests that PDI forms a

disulfide bond on the TF protein,[104] and neutralizing antibodies against PDI inhibited TF:FVIIa activity *in vitro*.[105] However, in the crystal structure of TF, the area at which PDI acts (i.e., Cys^{186}-Cys^{209}) is between TF and FVIIa; therefore, it is unclear how PDI would activate a preexisting TF:FVIIa complex.[106] Additionally, there is a debate as to whether PDI is responsible for turning TF:FVIIa procoagulant activity on or off.[104, 105] Interestingly, there appears to be an interplay between PDI- and phosphatidylserine-mediated mechanisms, as many of the stimuli used to activate TF:FVIIa have been shown to involve phosphatidylserine.[89, 92, 107]

The ideal location to study the mechanism whereby TF:FVIIa is activated would be in the liver, because it is necessary for TF:FVIIa to be in a non-procoagulant state because of the liver microenvironment. Although it is known that hepatocyte TF is activated during models of liver injury, the precise mechanisms controlling TF:FVIIa activity in the liver are unknown. Because hepatocytes are the primary source of TF within the liver, it is entirely possible that injury to hepatocytes ultimately leads to the activation of coagulation. Moreover, apoptosis has been associated with phosphatidylserine externalization and an increase in hepatocyte TF:FVIIa activity.[108, 109] However, one cannot exclude the potential for mechanisms independent of hepatocellular injury. Defining the mechanisms controlling hepatocyte TF:FVIIa procoagulant activity would reveal how activation of coagulation is regulated within the liver and could potentially identify the basis for coagulation that accompanies liver disease.

A challenge with determining the mechanisms controlling TF:FVIIa procoagulant activity in liver is that there is little known about physiologically relevant mechanisms controlling encryption of TF:FVIIa procoagulant activity. Indeed, many of the foundational studies evaluating these mechanisms have focused on non-physiologic stimuli (e.g., calcium ionophores, freeze-thawing, $HgCl_2$). In the context of liver injury, it is possible that TF:FVIIa activity is increased by

as yet to be identified mediators that increase during injury. Indeed, the identification of a physiologically relevant activator of coagulation would allow the identification of mechanisms whereby TF:FVIIa is activated during liver disease., and perhaps reveal novel strategies to prevent pathologic coagulation in the injured liver.

Liver anatomy, physiology, and bile acid synthesis

One of the primary roles of the liver is metabolism, and there are numerous cells types that aid in this process. Hepatocytes are the primary metabolic cells present within the liver as they express several cytochrome P450s, which allows them to breakdown various molecules (e.g., xenobiotics, steroids, fats). The liver receives a dual blood supply from the hepatic artery and the portal vein, the latter brings intestinal contents into the liver.[110] As previously mentioned, the liver contains fenestrated endothelial cells, which form the wall of blood vessels throughout the liver. Macromolecules in the blood pass through the fenestrated endothelial cells to be processed by the liver. The liver also produces proteins (e.g., albumin, coagulation factors), which can pass through the sinusoidal endothelium into system circulation. In addition to the liver's role in metabolism, the liver is largely responsible for the synthesis of bile, which helps facilitate the excretion of metabolites from the liver.[111] Bile is produced in hepatocytes and is excreted into the bile canaliculi, which forms between adjacent hepatocytes. The bile canaliculi eventually become the bile ducts, which is composed of ciliated epithelial cells called cholangiocytes. Cholangiocytes play an essential role in the formation of bile and separating constituents of the bile from the rest of the liver. [112, 113] From the bile ducts, bile can be stored in the gallbladder, a specialized organ that controls bile release into the intestines.

A major component of bile is bile acids. Bile acids are cholesterol metabolites produced by hepatocytes and are ultimately delivered to the intestines to aid in breakdown of dietary fats.[114,

[115] Cytochrome P450 7A1 (CYP7A1) and 27A1 (CYP27A1) are key enzymes involved in synthesis of the two primary bile acids, cholic acid and chenodeoxycholic acid, by hepatocytes (Figure 3).[116] These primary bile acids provide a basic template onto which additional modifications are made, such as conjugation with taurine or glycine, ultimately synthesizing a diverse bile acid pool (Figure 4).[117, 118] Two major enzymes are involved in the amino acid conjugation process, bile acid:CoA synthase (BACS) and bile acid:amino acid transferase (BAT). [119] Bacteria in the gastrointestinal tract can further modify primary bile acids into secondary bile acids (e.g., deoxycholic and lithocholic acid) through dehydroxylation.[120] These modifications change the chemical characteristics of the bile acid; for example, more hydrophobic bile acids (i.e., chenodeoxycholic acid derivatives) are more effective at solubilizing fats than more hydrophilic bile acids (i.e., cholic acid derivatives).[121]

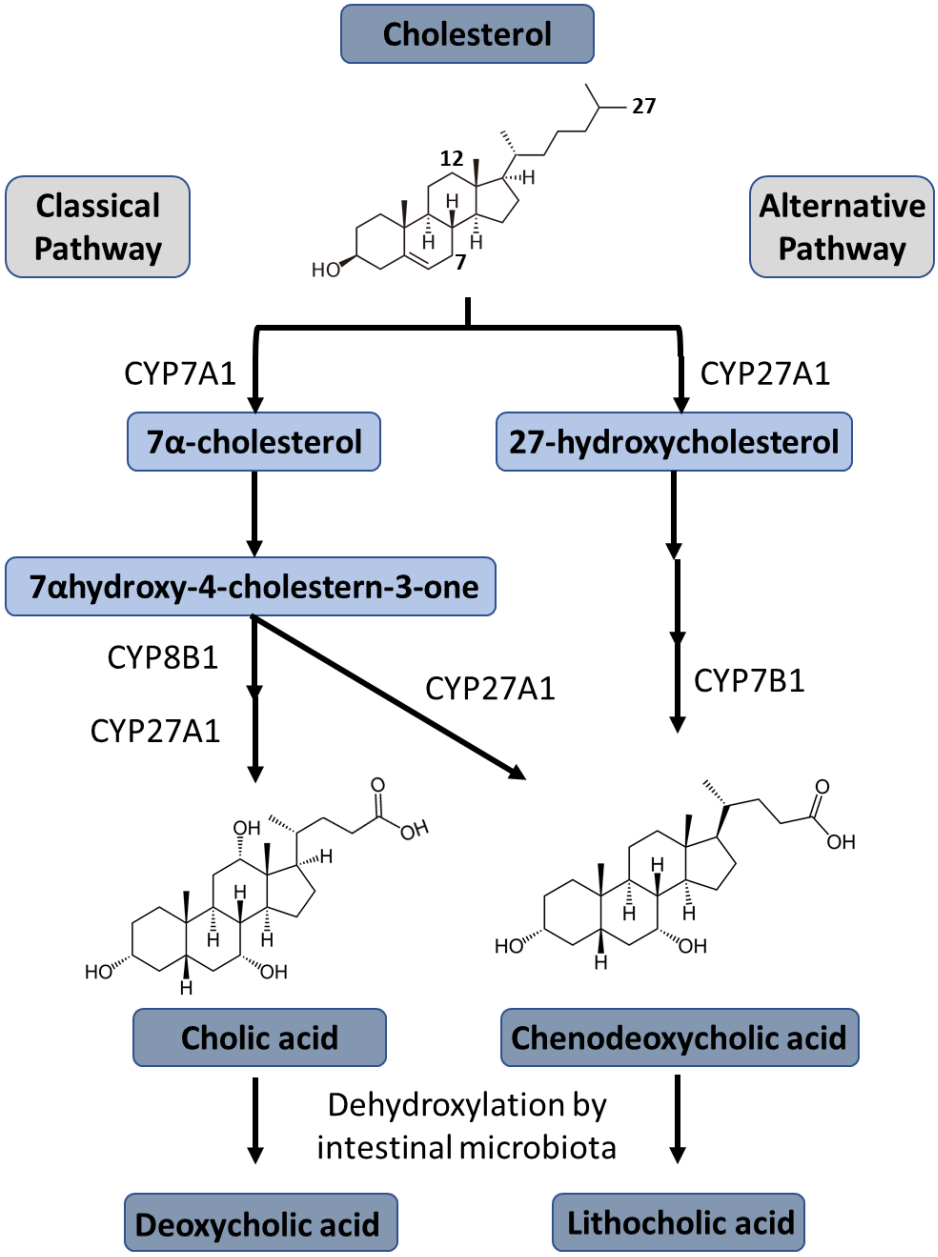

Figure 3. Bile acid synthesis. Bile acids are synthesized within hepatocytes through the breakdown of cholesterol. The primary bile acids, which differ by the presence of a hydroxyl group on cholic acid, are synthesized through are two major pathways. The classic pathway synthesizes cholic acid with the key enzyme CYP7A1, which adds a hydroxyl group to the 7th carbon. The alternative pathway synthesizes chenodeoxycholic acid with the key enzyme CYP27A1, which adds a hydroxyl group to the 27th carbon. Cytochrome 8B1 (CYP8B1) is responsible for adding the hydroxyl group to the 12th carbon to make cholic acid. Cytochrome 7B1 (CYP7B1) adds a hydroxyl group to the 7th carbon to make chenodeoxycholic acid. In the intestines, the 7th carbon of cholic acid and chenodeoxycholic acid is dehydroxylated by bacteria into their secondary bile acids, deoxycholic acid and lithocholic acid, respectively.

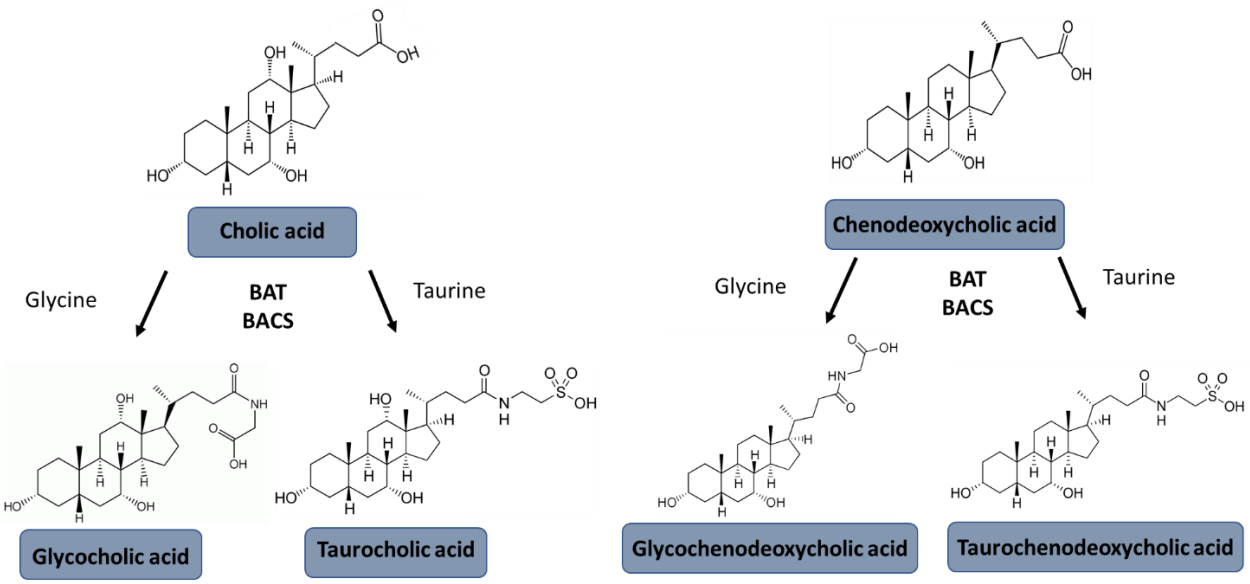

Figure 4. Taurine and glycine conjugation of the primary bile acids. Two enzymes involved in bile acid conjugation are bile acid:CoA synthase (BACS) and bile acid:amino acid transferase (BAT), which catalyze the formation of the amino acid conjugated bile acids. This results in the conjugated forms of cholic acid (taurocholic and glycocholic acid) and chenodeoxycholic acid (taurochenodeoxycholic and glycochenodeoxycholic acid). In addition to the primary bile acids, the secondary bile acids can also be conjugated (not pictured).

Because of their detergent-like effects, bile acid synthesis and transport are well controlled to prevent high concentrations of bile acids within the systemic circulation. Bile acid synthesis is controlled by the nuclear receptor, farnesoid X receptor (FXR), for which some bile acids (e.g., chenodeoxycholic and deoxycholic acid) are natural ligands.[122, 123] FXR controls the expression of enzymes involved in bile acid synthesis (i.e., *Cyp7a1* and *Cyp27a1*). In this way, bile acid levels are self-regulating, as bile acid activation of FXR reduces bile acid synthesis. Once bile acids are synthesized, hepatic transporters (e.g., bile salt exporting protein, BSEP) transport bile acids into the bile canaliculi.[124] As previously mentioned, bile canaliculi eventually coalesce into bile ducts, which ultimately lead to the gallbladder. Bile acids are concentrated in the gallbladder—some achieving millimolar concentrations— before being secreted into the intestine to break down fats. More than 90% of bile acids are recovered from the intestine by a process called enterohepatic circulation, which allows bile acids to be reabsorbed into hepatocytes.[125, 126] In summary, this process allows for the tight control of bile acid synthesis and transport between the liver and the intestine to limit the concentrations of bile acid present in the systemic circulation.

Bile acids in liver disease

An increase in plasma bile acid concentration is observed in numerous liver diseases.[127-130] One such condition is obstructive cholestasis, which occurs when the bile duct is blocked by physical impediments such as a stricture, gallstone, or tumor. This blockage causes pressure to build up in the bile duct, leading to rupture of bile duct epithelial and eventual release of bile acids into the liver parenchyma and blood.[129, 130] In addition to the release of bile acids at the level of bile ducts, there is also a release of bile acids at the level of hepatocytes. This is caused by the rupture of the apical membrane of hepatocytes, which allows for the leakage of bile acids into the parenchyma.[131] There is also an increase in plasma bile acids in other diseases associated with

biliary injury (i.e., primary biliary cholangitis).[132] However, plasma bile acids also increase during conditions in which bile duct epithelial damage is not a primary feature, including non-alcoholic steatohepatitis and acute liver failure.[133-135] It is thought that bile acids increase in these conditions because liver function (e.g., bile acid transport) is disrupted, ultimately causing bile acids to leak into the parenchyma.[136, 137] Overall, a breakdown of normal bile acids transport leads to an increase in plasma levels of bile acids.

As a consequence of their detergent effects, bile acids are thought to contribute to liver injury through cytotoxic effects. Studies have documented the cytotoxic effect of bile acids (e.g., chenodeoxycholic acid).[138-140] However, it is important to note that these studies used high concentrations of bile acids that are not found in the blood of patients with liver disease. [129] Indeed, pathologically relevant concentrations of bile acids did not induce injury *in vitro*.[129, 141] Bile acids are thought to generate a proinflammatory response, as prior studies have documented that treating hepatocytes with bile acids increases the expression of proinflammatory mediators.[129, 142] This suggests that bile acids have a more complex effect on the development of liver injury than just damaging hepatocytes. During numerous liver diseases there is both an increase in plasma bile acids and changes in coagulation. Moreover, the presence of TF:FVIIa on hepatocytes suggests that TF:FVIIa would be exposed to high concentrations of bile acids during liver disease. Yet, the potential for bile acids to activate coagulation is largely unknown.

Although limited, a few studies suggest a possible connection between bile acids and the activation of coagulation. It was reported that preoperative biliary drainage in cholestatic patients decreased plasma thrombin-antithrombin complexes.[143] This suggests that a component of the bile, perhaps bile acids, may be increasing the activation of coagulation. Notably, bile acids are the largest solute component within bile.[144] Additionally, there is evidence that fibrin(ogen) deposits

near bile ducts in clinical and experimental setting of cholestasis, which suggests coagulation activation localized to areas in which the bile acid concentration may be highest.[145] Furthermore, a study found that increased levels of bile acids were associated with the pathological progression of viral hepatitis-induced cirrhosis.[146] This may be explained by bile acids activating coagulation, which ultimately leads to disease progression. Overall, this suggests that bile acids could be potential activators of coagulation and further studies may elucidate the mechanisms whereby this bile acid-mediated coagulation occurs.

The effect of bile acids on coagulation

Although concentrations of bile acids increase in the liver parenchyma and blood in several liver disease conditions, their potential to activate coagulation has not been well documented. Previous studies focused on the ability of high concentrations of bile acids to substitute for phosphatidylserine in coagulation reactions.[147, 148] The idea that bile acids could substitute for phosphatidylserine was founded on the observation that bile acids at high concentrations form micelles, which were then thought to be able to support coagulation reactions. One study investigated the potential for taurocholic acid to increase the generation of thrombin through the formation of the prothrombinase complex, which is composed of activated factor V, FXa, and a charged membrane (e.g., phosphatidylserine-containing).[147] Interestingly, taurocholic acid (~2 mM) could substitute for a charged membrane in the prothrombinase complex and generate thrombin.[147] Although these concentrations are well above the serum levels in patients and mice (1uM and 1mM respectively), it is possible that these levels occur near bile ducts during liver injury.[130, 149] Another study found that glycocholic acid (2 mM) could support the generation of thrombin.[148] Together, these studies suggest that high concentrations of bile acids can increase thrombin generation. In addition to studies evaluating thrombin generation, studies have also

determined whether bile acids could increase the activity of coagulation proteases, specifically thrombin. One study documented that cholic acid (2 mM) and glycocholic acid (2 mM) increased thrombin's catalytic activity as determined by its ability to cleave substrates.[150] Another study documented that glycochenodeoxycholic acid (>5 mM) and taurocholic acid (>5 mM) enhanced thrombin's ability to cleave a substrate.[151] Overall, these studies suggest that bile acids could affect coagulation proteins (e.g., thrombin). These studies suggest the possibility that bile acids may affect other coagulation proteins (i.e., TF:FVIIa).

A common feature of liver diseases is an increase in bile acid concentration in the blood, owing to various mechanisms disrupting normal bile acid synthesis or distribution.[130, 152, 153] Additionally, bile acids have well recognized functions (e.g. lipid metabolism, regulation of bile acid synthesis) , and can elicit a variety of effects through activation of specific cell surface and nuclear receptors (e.g., FXR and Takeda G-protein coupled receptor 5).[154, 155] However, the potential for bile acids to increase TF:FVIIa procoagulant activity has been largely overlooked. There are several potential ways bile acids could impact TF:FVIIa activity. After bile duct ligation, bile acids achieve high concentrations near hepatocytes close to bile ducts, an event associated with marked hepatocellular necrosis.[156] Hepatocytes are the primary source of TF in the liver; therefore, hepatocellular injury may ultimately lead to coagulation. Furthermore, there is the potential for bile acids to modify the cellular membrane independent of cellular death. As a product of their detergent-like structure, bile acids may alter membrane phospholipid concentrations, resulting in a phosphatidylserine-mediated increase in TF:FVIIa activity. Moreover, certain bile acids have been shown to increase the activity of enzymes responsible for regulating the levels of cellular membrane components (e.g., phospholipids).[157, 158] Taken together, this suggests that bile acids could potentially increase TF:FVIIa procoagulant activity through phosphatidylserine

externalization. Moreover, there is the potential for bile acids to substitute for phosphatidylserine in coagulation reactions, albeit at lower levels than previously tested.[147] If anything, this warrants a systematic investigation starting at the beginning of the coagulation pathway (i.e., TF:FVIIa activity). Identifying the potential for bile acids to increase hepatocyte TF activity would offer the opportunity to develop targeted strategies to inhibit pathologic coagulation in patients with liver disease.

Overview of AIMs

In this dissertation, I will be focusing on how TF procoagulant activity is controlled within the liver and the potential mechanisms whereby coagulation is activated during liver disease. The work in this dissertation was driven by the need to identify physiologically relevant activators of coagulation during liver disease. The outcomes of these studies have the potential to identify bile acids as activators of coagulation, and the potential for other cellular sources of TF to activate coagulation in liver disease. Defining the mechanisms whereby TF is activated will increase our knowledge on the regulation of coagulation within the liver.

Aim 1: Determine the mechanism whereby bile acids increase TF:FVIIa procoagulant activity. The second chapter of this dissertation will focus on identifying physiologically relevant activators of encrypted TF:FVIIa procoagulant activity. My hypothesis is that bile acids increase TF:FVIIa activity. Therefore, these studies will examine the possibility for bile acids to activate TF:FVIIa procoagulant activity through the use of *in vivo* and *in vitro* methods. The third chapter of this dissertation focuses on the differential mechanisms underlying the activation of TF:FVIIa by bile acids.

Aim 2: Determine the role of hepatocyte TF in carbon tetrachloride induced liver injury. The studies in the fourth chapter examine the role of hepatocyte TF in driving coagulation after administration of CCl_4. Previously, it was documented that hepatocyte TF was required for the activation of coagulation after administration with hepatotoxic doses of APAP. However, it is unknown if hepatocyte TF is universally required to activate coagulation during hepatotoxicity. My hypothesis is that deficiency in hepatocyte TF will decrease the activation of coagulation after challenge with CCl_4.

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CHAPTER 2

Direct amplification of tissue factor:factor VIIa procoagulant activity by bile acids drives intrahepatic coagulation

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Abstract

Objective: Regulation of TF (tissue factor):FVIIa (coagulation factor VIIa) complex procoagulant activity is especially critical in tissues where plasma can contact TF-expressing cells. One example is the liver, where hepatocytes are routinely exposed to plasma because of the fenestrated sinusoidal endothelium. Although liver-associated TF contributes to coagulation, the mechanisms controlling the TF:FVIIa complex activity in this tissue are not known. **Approach and Results:** Common bile duct ligation in mice triggered rapid hepatocyte TF-dependent intrahepatic coagulation coincident with increased plasma bile acids, which occurred at a time before observable liver damage. Similarly, plasma TAT (thrombin-antithrombin) levels increased in cholestatic patients without concurrent hepatocellular injury. Pathologically relevant concentrations of the bile acid glycochenodeoxycholic acid rapidly increased hepatocyte TF-dependent procoagulant activity *in vitro*, independent of *de novo* TF synthesis and necrotic or apoptotic cell death. Glycochenodeoxycholic acid increased hepatocyte TF activity even in the presence of the phosphatidylserine-blocking protein lactadherin. Interestingly, glycochenodeoxycholic acid and taurochenodeoxycholic acid increased the procoagulant activity of the TF:FVIIa complex relipidated in unilamellar phosphatidylcholine vesicles, which was linked to an apparent decrease in the K_m for FX (coagulation factor X). Notably, the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a bile acid structural analog, did not increase relipidated TF:FVIIa activity. Bile acids directly enhanced factor X activation by recombinant soluble TF:FVIIa complex but had no effect on FVIIa alone. **Conclusions:** The results indicate that bile acids directly accelerate TF:FVIIa–driven coagulation reactions, suggesting a novel mechanism whereby elevation in a physiological mediator can directly increase TF:FVIIa procoagulant activity.

Introduction

Tissue factor (TF) is the primary activator of blood coagulation and plays a critical role in both primary hemostasis and thrombosis. Cellular expression of TF is regulated through transcriptional and translational mechanisms.[1] A cellular barrier separating extravascular TF from its plasma ligand, coagulation factor VIIa (FVIIa), prevents inappropriate intravascular coagulation.[2, 3] Procoagulant activity of the TF:FVIIa complex is also regulated by post-translational mechanisms, wherein the TF:FVIIa complex can exist in an encrypted state defined by little to no procoagulant function.[4, 5] Proposed mechanisms underlying the decryption (i.e., activation) of TF:FVIIa procoagulant activity focus on interactions with anionic phospholipids (e.g., phosphatidylserine) in the cell membrane and oxidation of extracellular disulfides on the TF molecule.[5] Discovery of these mechanisms has been enabled by chemical tools including calcium ionophores and oxidizing agents (e.g., $HgCl_2$).[6-8] However, examples of TF decryption triggered by (patho)physiologically-relevant small molecules are lacking.

Liver parenchymal cells (i.e., hepatocytes) have been shown to express low levels of TF.[3, 9] However, unlike other tissues, the fenestrated endothelium of the liver microvasculature allows plasma unrestricted access to hepatocytes. In addition, hepatocytes are the primary site of synthesis of several coagulation factors, including FVII(a). Consequently, TF expression by hepatocytes occurs in a microenvironment lacking the traditional "hemostatic envelope". Indeed, isolated hepatocytes seem to express TF complexed with FVIIa, highlighting the theoretical importance of TF:FVIIa encryption as a means to prevent inappropriate coagulation. However, the molecular mechanisms and mediators controlling procoagulant function of the TF:FVIIa complex in the liver are largely unknown. Notably, hepatocyte TF-dependent coagulation is evident in experimental settings where the concentration of plasma bile acids is increased.[10-12] Although bile acids have

well-described pro-inflammatory activity,[13, 14] the effect of these molecules on the TF:FVIIa procoagulant activity has not been examined.

We tested the hypothesis that bile acids stimulate TF:FVIIa procoagulant activity and used a combination of *in vivo* and *in vitro* approaches to determine whether this could form a novel mechanism of TF:FVIIa decryption by a (patho)physiologically-relevant molecule elevated in conditions of liver disease.

Materials and Methods

Mice and bile duct ligation: Wild-type C57Bl/6J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Littermate $TF^{flox/flox}$ mice (control mice) and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$ [hepatocyte TF-deficient] mice) backcrossed 8 generations onto a C57Bl/6J background have been described previously. [9, 11] Approximately 50 male mice between the ages of 10 and 18 weeks were used in this study. The hypothesis to be tested for mouse experiments relied on baseline knowledge obtained from the literature on the time course of bile duct ligation (BDL)–induced hepatic injury, TF-dependent coagulation, and biliary pressure after BDL in males.[11, 15-17] Thus, male mice were used for BDL experiments. Mice were housed at an ambient temperature of ≈22°C with alternating 12-hour light/12-hour dark cycles and provided purified water and normal rodent diet (Teklad Irradiated 22/5 Rodent Diet 8940, Envigo, Indianapolis, IN) ad libitum. Obstructive cholestasis was induced in mice by surgical ligation of the common bile duct, as described previously.[14] Under deep surgical isoflurane (3%–5%) anesthesia, an abdominal incision was made to reveal the common bile duct. Cotton-tipped applicators were used to visualize the bile duct, which was then separated from other tissue using forceps. The bile duct was then ligated with sutures between the gallbladder and the intestine, the incision site covered with gauze soaked

in warm sterile saline, and the mouse was kept on a warming pad under deep surgical anesthesia for 30 minutes. For sham surgeries, the same steps were performed except ligation of the bile duct. After 30 minutes, sodium citrate (3.2%) was injected at 8 mL/kg into the vena cava. Thirty seconds after injection of sodium citrate, anticoagulated whole blood was collected into the same syringe. The liver was then removed, the left lateral lobe fixed in 10% neutral-buffered formalin, and remaining lobes were snap-frozen in liquid nitrogen. Mice were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facilities at Michigan State University. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Human plasma samples: Patients admitted to the University of Kansas Hospital were enrolled into an Institutional Review Board–approved protocol by the Kansas University Liver Center Staff or University of Kansas Medical Center Staff for the isolation of plasma from patients with suspected cholestasis. The study fully adhered to the Helsinki Declaration, and informed consent was acquired from all patients before the onset of the study. The primary inclusion criterion was patients with suspected cholestasis undergoing a planned endoscopic retrograde cholangiopancreatography for diagnosis and treatment. Blood was drawn before the initial endoscopic retrograde cholangiopancreatography and stored at –80°C until use. For the purpose of this study, the patients were subdivided into 3 categories based on the clinical values obtained by the University of Kansas Hospital labs. Biochemical evidence of cholestasis or hepatocellular injury was absent in some patients, indicated by ALT (alanine aminotransferase) levels < 50 U/L and ALP (alkaline phosphatase) levels < 109 U/L (4 males and 5 females). Patients presenting with biochemical evidence of cholestasis, but without hepatocellular injury, had low ALT levels

(ALT 11-52 U/L) with an ALP > 109 U/L (7 males and 7 females). Patients with cholestatic liver injury had elevated ALT (87-1122 U/L) and cholestasis as evidenced by endoscopic retrograde cholangiopancreatography and ALP ≥109 U/L (10 males and 8 females).

Hepatocyte isolation and treatment: Rat tail collagen (Corning) was diluted in acidified sterile water (0.05 N HCl) and coated on 6-well culture plates (Greiner Bio-One, Monroe, NC) at 100 μg/mL overnight at 4°C. The plates were then washed 3× with sterile PBS and air-dried for 10 minutes. Hepatocytes from male mice were isolated by perfusion and collagenase digestion, as previously described.[9] Cell viability was determined by trypan blue exclusion, and hepatocytes with at least 80% viability were used. Hepatocytes were plated at a density of 500 000 cells per well in Williams E medium (Sigma-Aldrich) containing 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich). After 2 hours, nonadherent cells were removed and replaced with fresh media containing 10% FBS. The cells were then incubated overnight at 37°C and 5% CO_2. The next day, the cells were treated with various concentrations of glycochenodeoxycholic acid (GCDCA; CHEM-IMPEX International, Wood Dale, IL) or the vehicle HEPES buffered saline (HBS; 137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES) for 15 minutes in HBS-containing albumin before assessment of the TF procoagulant activity (see below). In select experiments, the cells were pretreated with actinomycin D (0.2 μg/mL, Sigma-Aldrich), GW4064 (10 μmol/L, Tocris, Minneapolis, MN), or caspase 3/7 inhibitor (Ac-DEVD-CHO, 50 μmol/L, Biolegend, San Diego, CA) 30 minutes before the bile acid treatment. The vehicle for each compound was dimethyl sulfoxide (DMSO), and the final concentration of DMSO in culture was 0.1%. To assess the effect of bile acid treatment on the activation of apoptosis, levels of cleaved caspase-3 were assessed by Western blot with Jo2 (anti-Fas antibody)/actinomycin D cotreatment

(0.5 µg/mL Jo2, BD, Franklin Lakes, NJ and 0.2 µg/mL actinomycin D) used as a positive control.[18]

Western blot for cleaved caspase-3: Cleaved caspase-3 levels were determined by Western blotting as described previously.[18] Hepatocyte pellets were homogenized in 1× RIPA (radioimmunopreciptation assay) buffer containing protease and phosphatase inhibitors (G-Biosciences, St. Louis, MO) and the homogenate was rotated end-over-end for 30 minutes at 4°C, spun at 10 000×*g* for 10 minutes, and the supernatants were saved. Equal amounts of protein were resolved using SDS-PAGE. Following semidry transfer, the polyvinylidene fluoride (PVDF) membrane was blocked in 5% BSA in TBST (Tris-buffer saline+Tween-20; 50 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween-20, pH 7.4) and then incubated overnight with a rabbit monoclonal anticleaved caspase-3 antibody (Asp175, clone 5A1E, 1:1000 dilution; Cell Signaling Technology, Danvers, MA) at 4°C. Next, the membrane was washed in TBST and then incubated with a goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at room temperature. The membrane was washed and then incubated with Clarity Western enhanced chemiluminescence substrate solution (BioRad, Hercules, CA) and exposed to blue autoradiography film (ISC BioExpress, Kaysville, UT). The membrane was stripped in Restore Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) and probed for GAPDH using a mouse monoclonal antibody (Proteintech, Rosemont, IL, 1:10 000 dilution) overnight at 4°C and developed as described earlier with a goat anti-mouse horseradish peroxidase–conjugated secondary antibody.

Measurement of plasma thrombin-antithrombin levels, hepatic fibrin(ogen) deposition, and liver procoagulant activity: Plasma TAT (thrombin-antithrombin) levels were determined using a commercial ELISA (Siemens Healthcare Diagnostics, Malvern, PA). Paraffin-embedded formalin-fixed livers were sectioned and stained for fibrin(ogen) using a polyclonal rabbit anti-human fibrin(ogen) antibody (Agilent, Dako, Santa Clara, CA), as described previously.[19] Labeling was performed by the Michigan State University Investigative Histopathology Laboratory, a Division of Human Pathology. Images were captured from the scanned sections obtained using an Olympus VS110 system. Liver procoagulant activity was determined using a single-stage clotting assay, as described previously.[9]

Determination of plasma bile acid concentration and hepatocyte injury *in vitro* and *in vivo*: Bile acids were measured using a commercial kit (Diazyme, Poway, CA) with the following modification in sample and reagent volume: 10 µL of plasma, 135 µL of R1, and 45 µL of R2. Plasma ALT activity was determined using a commercial reagent (Thermo Fisher Scientific, Waltham, MA). *In vitro* cytotoxicity was assessed by the ALT release into the culture medium. Absorbance changes were evaluated using an Infinite M200 plate reader (Tecan Durham, NC).

Relipidation of full-length TF into unilamellar vesicles: Full-length recombinant TF (Enzyme Research Laboratories, South Bend, IN) was relipidated in unilamellar vesicles composed of l-α-phosphatidylcholine (PC, isolated from chicken egg) and cholesterol at a 1:5 molar ratio, containing various amounts of L-α-phosphatidylserine (PS, isolated from porcine brain; Avanti Polar Lipids, Alabaster, AL), as described previously.[20] For some studies, TF-deficient vesicles composed of a molar ratio of 50:50 PC:PS were synthesized as described.[21]

Determination of TF:FVIIa procoagulant activity: TF:FVIIa activity was determined as described previously[9] by measuring conversion of human FX (coagulation factor X) to FXa. For studies involving hepatocytes, the culture medium was removed from the wells, and the cells were treated with HBS-containing albumin containing vehicle (HBS) or various concentrations (0–500 μmol/L) of GCDCA (CHEM-IMPEX International) or taurochenodeoxycholic acid (TCDCA; Matrix Science, Columbia, SC). After 15 minutes, FX (100 nmol/L final, Enzyme Research Laboratories) was added to the wells. For studies using relipidated TF, vesicles containing TF were diluted (0.1 nmol/L TF final), incubated with human FVIIa (5 pM final, Enzyme Research Laboratories) in HBS-containing albumin containing calcium (5 mmol/L final) for 5 minutes at 37°C.Various concentrations (0–125 μmol/L) of GCDCA, TCDCA, or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Pierce, Thermo Fisher Scientific) were added before addition of FX (100 nmol/L final). In some studies, relipidated TF (0.1 nmol/L final; in PC or PC [99%]:PS [1%]) was treated with GCDCA (125 μmol/L final) and FVIIa (5 pM final) as above, with the addition of lactadherin (1–40 nmol/L final; Haematologic Technologies, Inc, Essex Junction, VT) or vehicle (HBS). For kinetic studies using relipidated TF, the FX concentration was increased up to 5 μmol/L, and the reaction was stopped after 60 minutes. For studies using sTF (soluble TF; 100 nmol/L final; kindly provided by Dr James Morrissey, University of Michigan), a higher concentration of FVIIa was used (5 nmol/L), as reported previously. [22] In some experiments involving sTF:FVIIa, reactions were performed in the presence of PC:PS (50:50). FXa generation was allowed to proceed at 37°C for 15 minutes for experiments with hepatocytes and 30 minutes for experiments using relipidated TF and sTF. FXa generation was stopped with EDTA (pH 7.4, 5 mmol/L final). FXa activity in each sample was assessed using

a chromogenic substrate (RGR-Xachrom; 0.667 mmol/L final; Enzyme Research Laboratories), and the data were collected using an Infinite M200 microplate reader (Tecan).

Thrombin Generation: Thrombin generation was measured using a Fluoroskan Microplate Fluorometer (ThermoFisher Scientific, Waltham, MA) by calibrated automated thrombography, as described previously.[23-25] Briefly, 10 µL of trigger (0.5 or 2 nmol/L relipidated TF [in 100% PC vesicles] final) was added to 40 µL of platelet-poor plasma mixed with various concentrations of GCDCA (0–125 µmol/L). Reactions were initiated by adding 10 µL of FluCa solution (0.416 mmol/L ZGGR-AMC [Z-Gly-Gly-Arg-AMC] substrate, 16.6 mmol/L $CaCl_2$, 60 mg/mL BSA in 20 mmol/L HEPES, 0.02% NaN_3, pH 7.3) to the wells. Data were analyzed according to Hemker and Kremers[24] to yield parameters: lagtime (time at 6 nmol/L of thrombin), time to peak, velocity index (peak/[time to peak−lagtime]), peak, and endogenous thrombin potential (expressed as nM thrombin × minute).

Statistics: Comparisons of 2 groups were made using Student t test. Comparisons of ≥3 groups were made by 1- or 2-way ANOVA, with or without paired analysis for studies involving vesicles and sTF. Student-Newman-Keuls test was used for multiple comparisons. Datasets without a normal distribution or lacking homogeneity of variance were transformed (generally Log_{10}) before analysis. Michaelis-Menten parameters were determined using GraphPad Prism 8. All data are reported as mean+SEM, and the criterion for statistical significance was $P<0.05$.

Results

BDL triggers rapid coagulation cascade activation.

Cholestasis is associated with the activation of coagulation in patients and in experimental mice.[11, 26, 27] Hepatocyte TF has been shown to activate coagulation after BDL in mice, but most studies examine coagulation well after liver pathology (i.e., necrosis and fibrosis) has developed.[11] Biliary pressure increases within minutes after BDL,[15, 16] suggesting rapid exposure of liver parenchyma to abnormally high levels of bile. We found that BDL increased plasma bile acid concentrations within 30 minutes (Figure 5A). Although BDL ultimately causes liver damage, [17, 28] hepatocellular necrosis was not evident 30 minutes after BDL, as plasma ALT levels were not increased (Figure 5B). Despite no evidence of hepatocellular injury, plasma TAT levels were significantly increased 30 minutes after BDL, indicating activation of coagulation (Figure 5C). Similar results were observed in a small preliminary study (n=4–5 mice per group) in female mice (not shown). Hepatic fibrin(ogen) deposits also increased in BDL mice and resembled microthrombi primarily localized within the sinusoids (Figure 5D). BDL did not increase total liver procoagulant activity (Figure 5E), but the procoagulant response after BDL was significantly reduced in mice with hepatocyte TF deficiency ($HPC^{\Delta TF}$), as indicated by reduced plasma TAT levels (Figure 5F). Collectively, these results indicate that BDL triggers rapid TF-dependent coagulation before the development of liver injury.

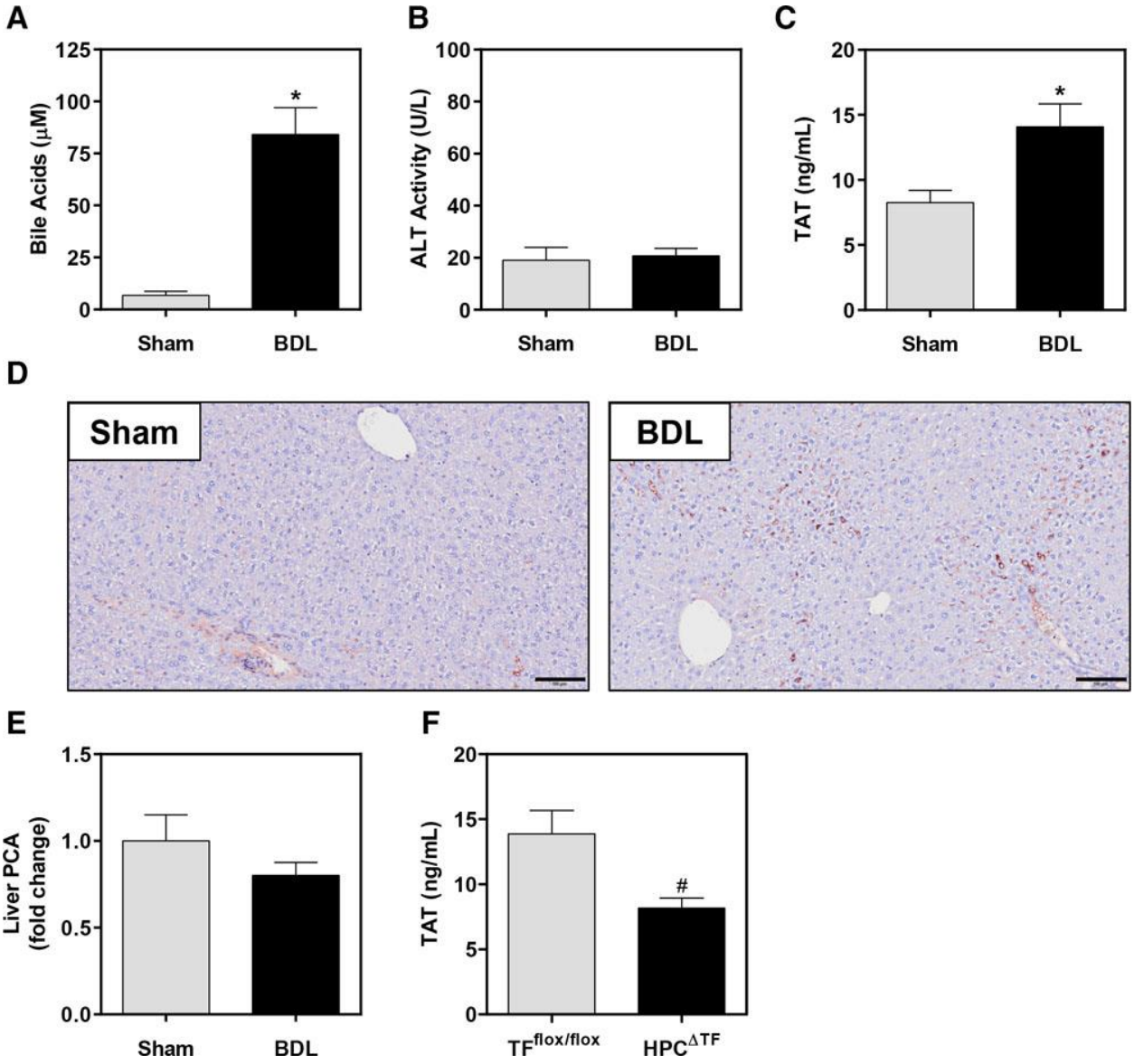

Figure 5. Obstructive cholestasis in mice triggers rapid intrahepatic coagulation. Male wild-type mice were subjected to bile duct ligation (BDL) or sham surgery for 30 min, and plasma (**A**) total bile acids, (**B**) ALT (alanine aminotransferase) activity, and (**C**) TAT (thrombin-antithrombin) were assessed. **D**, Immunohistochemical labeling of hepatic fibrin(ogen) deposition (brown, original magnification ×100) was assessed. Scale bar=100 μm. **E**, Levels of liver procoagulant activity (PCA) were determined using a single-stage clotting assay. N=6 mice for sham surgery and N=8 mice for BDL surgery. **F**, $TF^{flox/flox}$ mice (N=10) and $TF^{flox/flox}$/AlbCre ($HPC^{\Delta TF}$ [hepatocyte TF-deficient], N=9) were subjected to BDL for 30 min, and plasma TAT levels were assessed. Data are expressed as mean+SEM. TF indicates tissue factor. *P<0.05 vs sham mice. #P<0.05 vs $TF^{flox/flox}$ mice.

Association of coagulation with cholestasis in humans.

Patients with cholestasis often present with coincident hepatocellular injury, making it challenging to distinguish changes in coagulation biomarkers from hepatocellular injury itself. We examined plasma TAT levels in a group of patients referred for endoscopic retrograde cholangiopancreatography for confirmation of a diagnosis of cholestasis. These patients were separated into cohorts with biochemical evidence of cholestasis (i.e., elevation in serum ALP; Figure 6A) with or without concurrent hepatocellular injury (i.e., elevation in serum ALT activity; Figure 6B). Interestingly, plasma TAT levels were increased in cholestatic patients irrespective of biochemical indication of liver damage (Figure 6C). This suggests that, similar to experimental cholestasis in mice, clinical conditions associated with increased bile acids are associated with increased coagulation without concurrent hepatocellular injury.

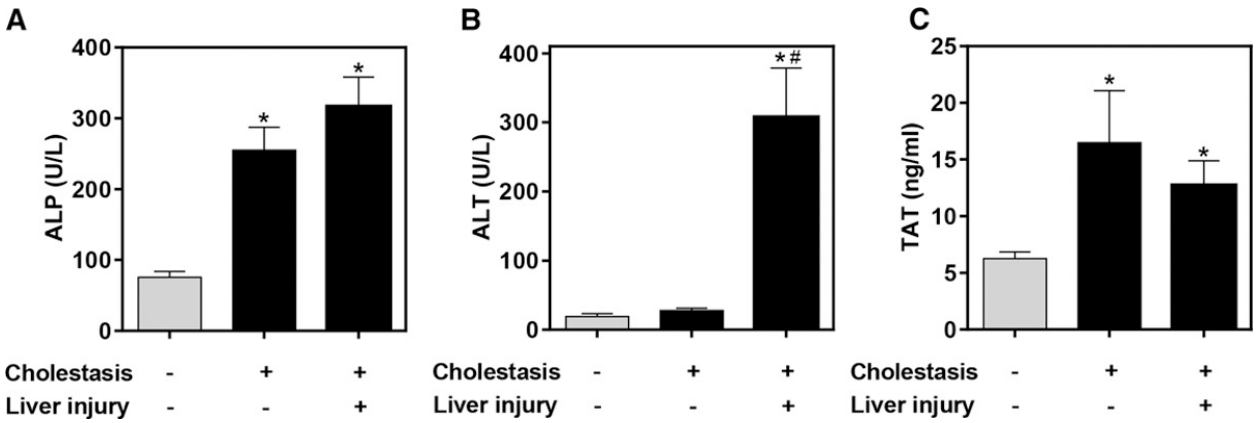

Figure 6. Coagulation activation is independent of hepatocellular injury in patients with cholestasis. Plasma samples were obtained from patients hospitalized for suspected cholestasis. Patients were defined as either uninjured (N=9; 4 males and 5 females), cholestatic without liver injury (N=14; 7 males and 7 females), or cholestatic with liver injury (N=18; 10 males and 8 females), as described in Materials and Methods. **A**, Serum ALP (alkaline phosphatase), (**B**) serum ALT (alanine aminotransferase), and (**C**) plasma TAT (thrombin-antithrombin) levels were determined. Data are expressed as mean+SEM. $^{*}P<0.05$ vs uninjured patients. $^{\#}P<0.05$ vs patients with cholestasis and without liver injury.

GCDCA increases hepatocyte TF:FVIIa procoagulant activity.

Bile acids are highly concentrated (i.e., >2 mmol/L) in bile and obstruction of bile flow causes exposure of hepatocytes to high levels of bile acids. Total plasma bile acid concentration increases in patients with various liver diseases, [29-31] with glycine-conjugated bile acids comprising the majority of bile acids in humans.[32] Because hepatocytes are likely exposed to high bile acid concentrations, we next determined whether bile acids affect hepatocyte TF:FVIIa procoagulant activity. Treatment of primary mouse hepatocytes with GCDCA for 15 minutes significantly increased TF:FVIIa procoagulant activity in a concentration-dependent manner (Figure 7A). Importantly, GCDCA treatment did not cause necrotic cell death at this time point, indicated by no significant changes in ALT levels (Figure 7B). Similarly, and consistent with prior studies,[32] GCDCA did not cause apoptosis, denoted by the lack of caspase-3 cleavage (Figure 7C). Moreover, the rapid GCDCA-mediated increase in TF:FVIIa activity was not affected by pretreatment with an inhibitor of caspase 3/7 (Ac-DEVD-CHO), suggesting that TF activity was not increased as a consequence of caspase-directed apoptosis (Figure 7D). Direct activation of the bile acid nuclear receptor FXR with GW4064 had no effect on TF:FVIIa procoagulant activity (Figure 7E) and the GCDCA-mediated increase in the TF:FVIIa activity was unaffected by pretreatment with ActD, an inhibitor of transcription (Figure 7F). Collectively, the results indicate that pathologically-relevant concentrations of GCDCA rapidly increase TF:FVIIa procoagulant activity on primary hepatocytes.

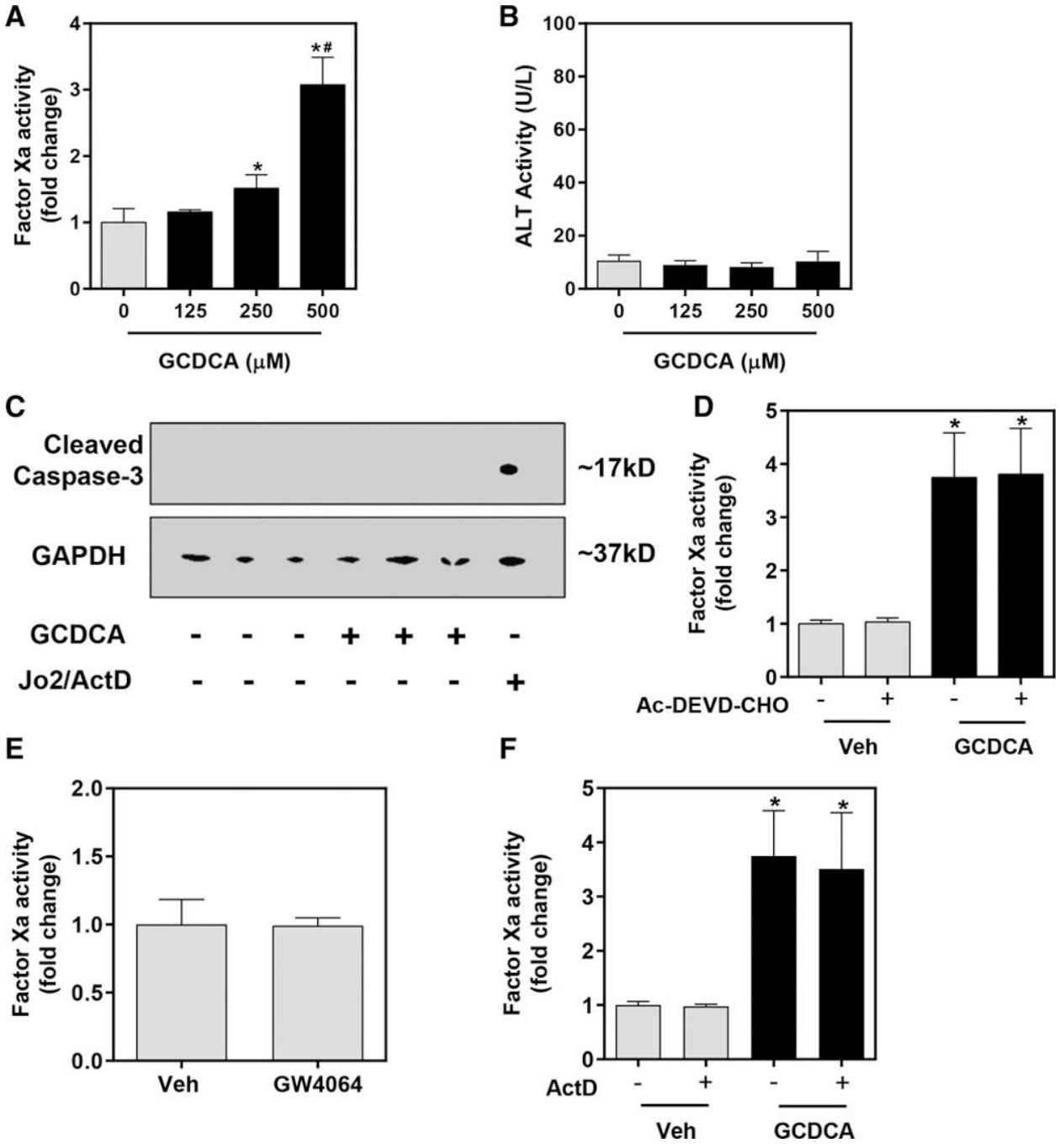

Figure 7. Glycochenodeoxycholic acid (GCDCA) increases hepatocyte TF (tissue factor):FVIIa (coagulation factor VIIa) activity independent of known mechanisms. Primary hepatocytes were isolated from male wild-type mice and treated with GCDCA or vehicle (HEPES buffered saline [HBS]) for 30 min. **A**, TF:FVIIa procoagulant activity was measured by FXa (coagulation factor Xa) generation, and (**B**) ALT (alanine aminotransferase) activity was assessed in the culture medium. **C**, Cleaved caspase-3 (marker of apoptosis) and GAPDH were determined by Western blot. Jo2 (anti-Fas antibody)/actinomycin D (ActD) treatment was used as a positive control for caspase-3 cleavage. **D**, Hepatocytes were pretreated with Ac-DEVD-CHO

Figure 7. (cont'd). (caspase-3/7 inhibitor, 50 µmol/L) or dimethyl sulfoxide (DMSO) vehicle (0.1% final) 30 min before GCDCA treatment (500 µmol/L, 15 min), and TF:FVIIa procoagulant activity was determined by FXa generation. **E**, Wild-type hepatocytes were treated with the FXR (farnesoid X receptor) agonist GW4064 (10 µmol/L) or (**F**) ActD (0.2 µg/mL) or Veh (DMSO, 0.1% final) in the presence of GCDCA (500 µmol/L) for 30 min before determination of FXa activity. Data are expressed as mean+SEM, representing experiments from at least 3 separate hepatocyte isolations. *P<0.05 vs respective vehicle treatments. #P<0.05 vs 250 µmol/L GCDCA treatment.

Direct effects of bile acids on relipidated TF:FVIIa procoagulant activity.

The rapid activation of TF:FVIIa by GCDCA in the absence of cell death and without *de novo* TF gene expression (i.e. activation occurred in the presence of an inhibitor of transcription) exhibited the hallmarks of TF decryption. We wondered if bile acids could directly enhance TF:FVIIa procoagulant activity. To explore this possibility, we relipidated recombinant, full-length human TF into PC/cholesterol unilamellar vesicles with limited procoagulant activity. In this baseline condition, GCDCA significantly increased TF:FVIIa-dependent FXa generation in a concentration-dependent manner (Figure 8A). Glycine-conjugated bile acids (e.g. GCDCA) are the predominant species in humans; [32] mice carry comparable bile acids, but conjugated largely to taurine (e.g. TCDCA). [33] Interestingly TCDCA also evoked a concentration-dependent increase in TF:FVIIa-dependent FXa generation (Figure 8B). TCDCA and GCDCA are, in principle, physiologically-relevant anionic detergents. Thus, we also performed similar studies with the non-physiological detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a zwitterionic bile acid homolog. Notably, at equimolar concentrations, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate did not increase TF:FVIIa–dependent FXa generation (Figure 8C).

In studies performed on PC vesicles containing TF, GCDCA (150 μmol/L) decreased the apparent Km for TF:FVIIa–dependent FXa generation (2.0±0.3 μmol/L to 1.0±0.1 μmol/L, $P<0.05$) without affecting the apparent Vmax (2.6±0.2 pM/s (HBS) and 2.4±0.1 pM/s (GCDCA). Prior studies have shown that PS (6%) reduces the Km for this reaction by nearly 36-fold.[34] Thus, we next determined whether bile acid enhancement of TF:FVIIa procoagulant activity would still be evident in the presence of PS, a potent activator of TF:FVIIa activity. In agreement with previous studies,[35] the addition of low levels of PS (1%) to the vesicles dramatically increased

TF:FVIIa–dependent Xa generation (Figure 8D). Interestingly, bile acid enhancement of TF:FVIIa activity was absent in the presence of PS (Figure 8E and 8F). Collectively, the results suggest that bile acids directly increase TF:FVIIa–dependent FXa generation, but this effect is mitigated by the presence of a strong activators, such as PS.

Next, we evaluated the effect of GCDCA on thrombin generation initiated by TF relipidated in PC. Importantly, relipidation of TF in vesicles without PS required a final concentration of TF much higher than traditionally applied in this assay (i.e., low nmol/L) to initiate thrombin generation. Relipidated TF (in PC only) was used to trigger coagulation in normal human plasma containing various concentrations of GCDCA. Interestingly, the addition of GCDCA to plasma subtly enhanced thrombin generation (Figure 11 in appendix). Although effects were modest, analysis by ANOVA suggested a significant concentration-dependent effect of GCDCA on time to peak, peak, and velocity of thrombin generation. These results are consistent with the effect of bile acids on TF:FVIIa activity and the concept that GCDCA may amplify the procoagulant response *in vivo*, suggested by our observations in both humans and mice with cholestasis.

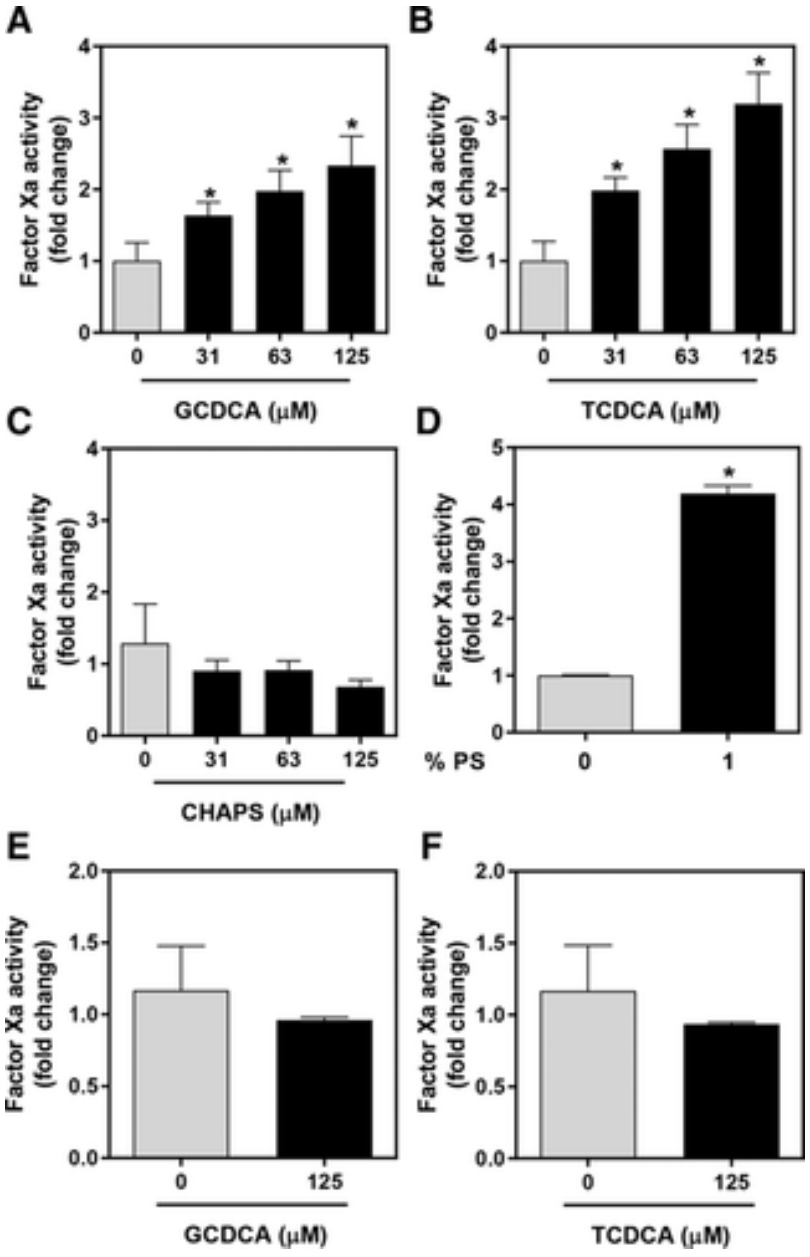

Figure 8. Direct effect of bile acids on relipidated TF (tissue factor):FVIIa (coagulation factor VIIa) procoagulant activity. TF relipidated in phosphatidylcholine (PC)/cholesterol (see Methods) was incubated with FVIIa (5 pM final) for 5 min and then incubated with FX (coagulation factor X; 100 nmol/L), vehicle (HEPES buffered saline [HBS]) or various concentrations of (**A**) glycochenodeoxycholic acid (GCDCA), (**B**) taurochenodeoxycholic acid (TCDCA), (**C**) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) before **Figure 8.** (cont'd). assessment of FXa generation 30 min later. **D**, FXa generation by TF relipidated in vesicles containing PC and cholesterol or vesicles containing 1% phosphatidylserine (PS). **E** and **F**, Vesicles containing 1% PS were treated with FVIIa (5 pM final) for 5 min and then treated with vehicle (HBS), GCDCA or TCDCA for 30 min before FXa generation was assessed. Data are expressed as mean+SEM, representing data from at least 3 separate experiments. *P<0.05 vs vehicle.

Direct effects of bile acids on soluble TF:FVIIa procoagulant activity.

Next, we determined whether bile acids had direct effects on FVIIa catalytic activity. In the absence of TF, high levels of FVIIa have been shown to convert FX to FXa in the presence of phospholipids.[20, 21] Indeed, as a positive control, we found that vesicles composed of equal amounts of PC:PS increased TF-independent activation of FX by FVIIa (Figure 9A). The addition of sTF to the reaction further increased FX activation in the presence of phospholipids (Figure 9A). In contrast to phospholipids, neither TCDCA nor GCDCA had any effect on TF-independent activation of FX by FVIIa (Figure 5B). Remarkably, both TCDCA and GCDCA caused a significant and concentration-dependent increase in FX activation by the sTF:FVIIa complex (Figure 9C and 9D). The results suggest that the amplification of FXa generation by bile acids requires the TF:FVIIa complex.

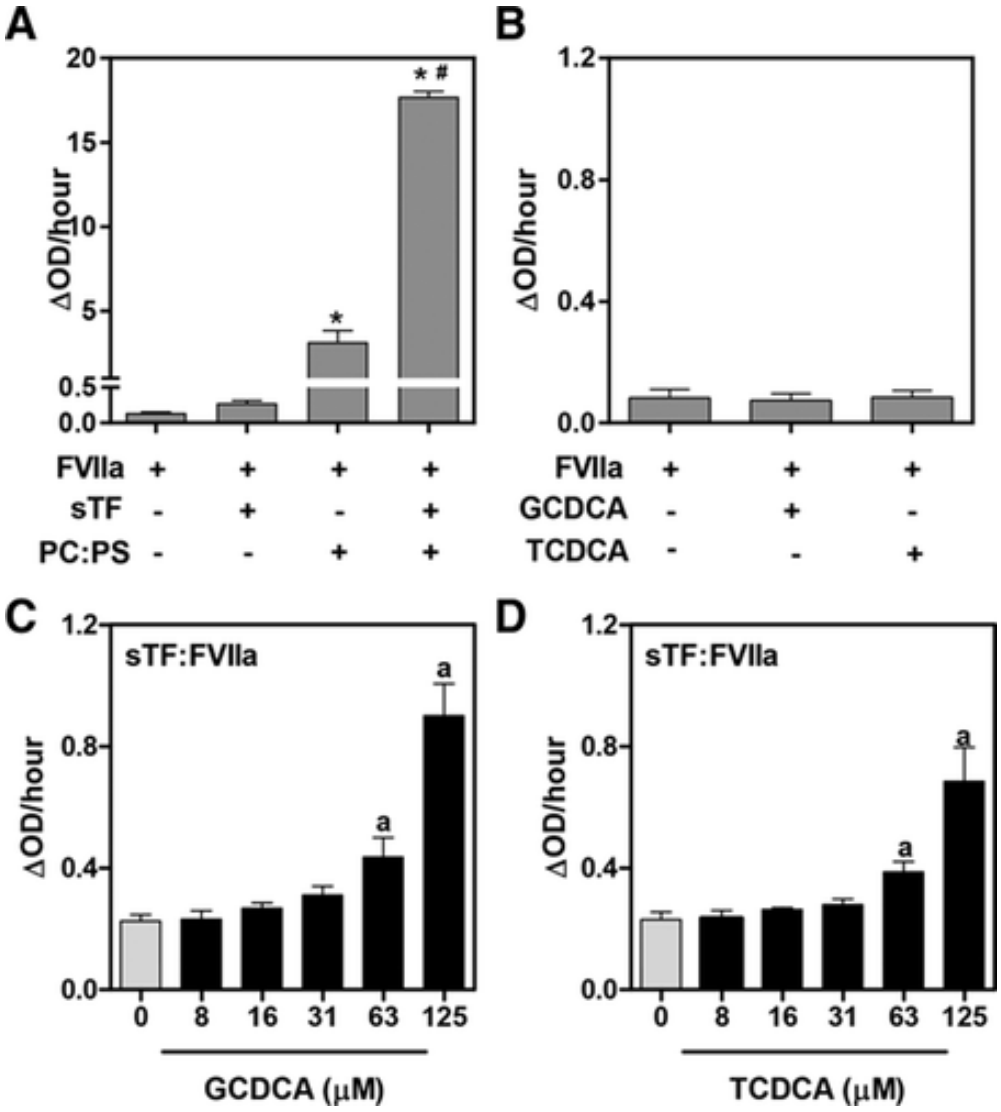

Figure 9. Direct effect of bile acids on soluble TF (tissue factor):FVIIa (coagulation factor VIIa) procoagulant activity. **A**, FX (coagulation factor X; 100 nmol/L) was incubated with FVIIa (5 nmol/L) in the presence or absence of soluble TF (sTF, 100 nmol/L) with or without unilamellar vesicles (phosphatidylcholine [PC]:phosphatidylserine [PS] 50:50, 100 μmol/L) or respective vehicle (HEPES buffered saline), and FXa generated for 30 min. **B**, The effect of bile acids (125 μmol/L) on TF-independent FVIIa (5 nM)-mediated FX activation. **C** and **D**, sTF (soluble TF):FVIIa complex (100 nmol/L sTF, 5 nM FVIIa) was incubated with various concentrations of glycochenodeoxycholic acid (GCDCA) or taurochenodeoxycholic acid (TCDCA). FXa generation was assessed in all experiments and is represented as a change in absorbance (i.e., optical density) over time (OD/hour). Data are expressed as mean+SEM from at least 3 separate experiments. *P<0.05 vs FVIIa group. #P<0.05 vs respective treatment without sTF. aP<0.05 vs vehicle.

Direct effects of GCDCA independent of phosphatidylserine. The effect of GCDCA on relipidated and sTF:FVIIa activity suggests a direct effect on procoagulant activity. To distinguish this potential direct mechanism from the effects of PS exposure, we used the PS-binding protein lactadherin. Lactadherin binds to PS and inhibits the PS-dependent enhancement of TF:FVIIa activity.[36] In a concentration-dependent manner, lactadherin reduced the activity of TF:VIIa relipidated in PS:PC (1:99%) vesicles but had no effect on TF relipidated solely in PC (Figure 10A). There was also no effect of lactadherin on GCDCA-stimulated TF:FVIIa activity (Figure 10B), allowing us to apply lactadherin to selectively inhibit PS in cultured hepatocytes. We selected the lowest concentration of lactadherin that inhibited PS-dependent TF:FVIIa activity by ≈50%. Lactadherin (20 nmol/L) significantly reduced hepatocyte TF procoagulant activity, even in the absence of GCDCA, implying baseline PS exposure in cultured hepatocytes (Figure 10C). Remarkably, GCDCA treatment increased hepatocyte TF procoagulant activity even in the presence of lactadherin (Figure 10C). Together, the results suggest that PS exposure is not a vital component of the mechanism whereby GDCCA increases hepatocyte TF procoagulant activity.

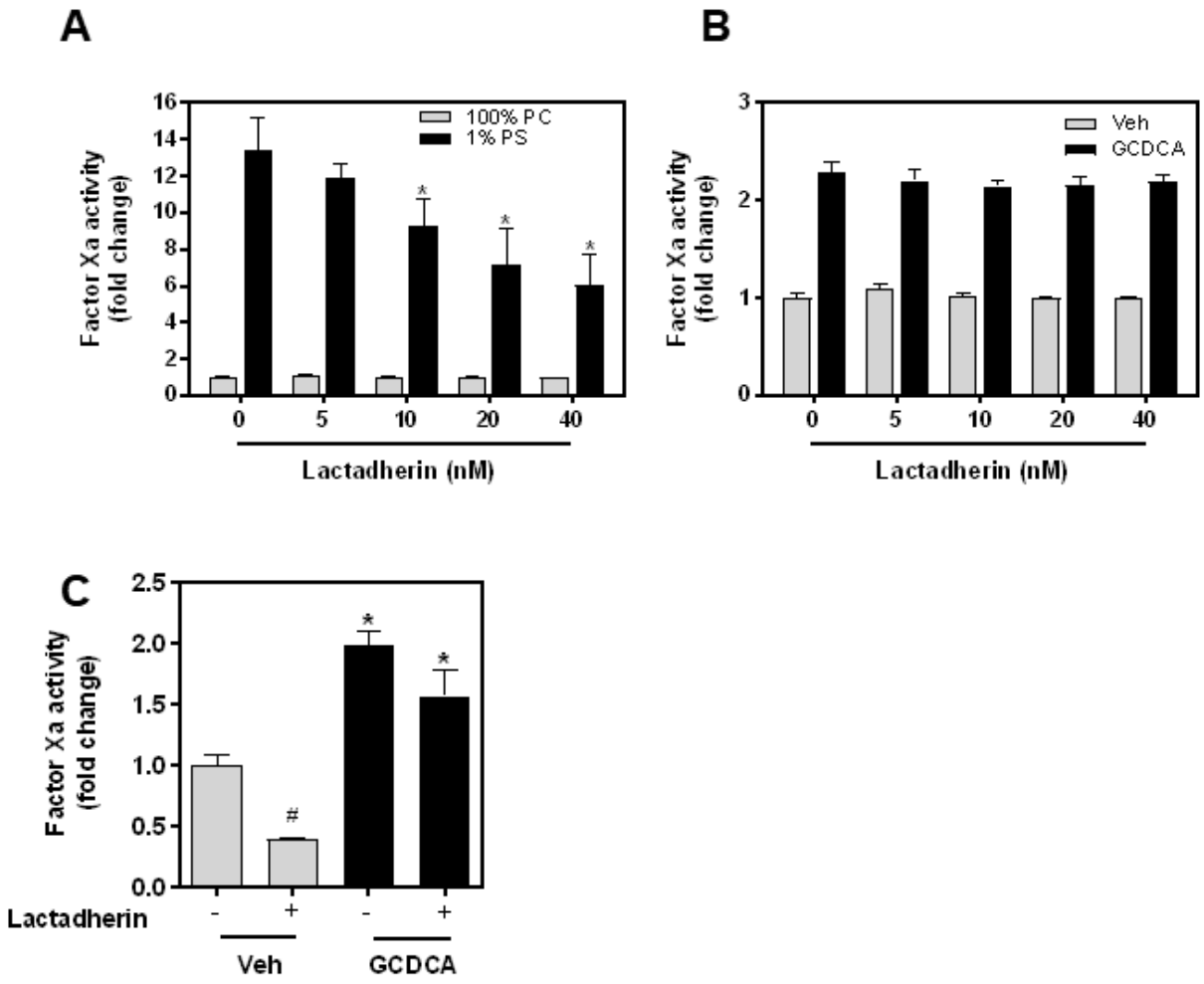

Figure 10. Effect of blocking phosphatidylserine (PS) on bile acid–driven relipidated and hepatocyte TF (tissue factor) procoagulant activity. **A**, TF relipidated (0.1 nmol/L final) in vesicles of various phospholipid composition were incubated with FVIIa (coagulation factor VIIa; 5 pM final) for 5 min. For **B**, TF relipidated in phosphatidylcholine (PC) vesicles was treated with glycochenodeoxycholic acid (GCDCA; 125 µmol/L final) or vehicle (HEPES buffered saline [HBS]). For both **A** and **B**, 100 nmol/L FX (coagulation factor X) was added in the presence or absence of various concentrations of lactadherin, and FXa generation was measured 60 min later. **C**, Primary mouse hepatocytes were isolated from wild-type male mice and treated with GCDCA (500 µmol/L) or vehicle (HBS; vehicle [Veh]) for 15 min in the presence or absence of lactadherin (20 nM). FXa generation was assessed 30 min later. Data are expressed as mean+SEM, representing experiments from at least 3 separate hepatocyte isolations and 4 separate vesicle experiments. For **A**, *P<0.05 vs 1% PS vesicles without lactadherin. For **C**, *P<0.05 compared with respective Veh-treated cells. #P<0.05 compared with respective group without lactadherin.

Discussion

Mechanisms of TF decryption have been discovered using non-physiological chemical triggers (e.g., calcium ionophores, $HgCl_2$, etc) of TF:FVIIa procoagulant activity.[6-8] To identify new pathologically relevant mediators of TF:FVIIa decryption, we considered TF expressed by liver parenchymal cells. Because the liver lacks a traditional hemostatic envelope, regulation of TF:FVIIa by encryption seems essential to restrict intrahepatic coagulation. We demonstrate here that bile acids can increase hepatocyte TF:FVIIa procoagulant activity. Notably, bile acid activation of hepatocyte TF:FVIIa procoagulant activity occurred rapidly, in the absence of apoptotic or necrotic cell death, and did not require de novo transcription, which are hallmarks of direct TF:FVIIa decryption.[5] Paired with the finding that bile acids amplify TF-driven thrombin generation in plasma, rapid intrahepatic coagulation in experimental BDL, and evidence of injury-independent coagulation activation in small group of cholestatic patients, these results strongly suggest bile acids are a pathologically relevant amplifier of the TF-driven coagulation. Collectively, the results reveal a novel connection between a physiological mediator elevated in disease and direct amplification of TF:FVIIa procoagulant activity.

Mechanisms underlying the decryption of TF:FVIIa activity have focused on changes in cellular membrane composition because PS externalization causes robust increases in TF:FVIIa activity.[37-40] Additionally, prior studies have shown that apoptotic hepatocytes are highly procoagulant.[18] However, apoptosis does not contribute to liver injury after BDL,[41] making it unlikely to contribute to rapid coagulation in this model, nor is apoptosis a primary lesion in clinical obstructive cholestasis.[32] Although GCDCA causes hepatocyte necrosis over time,[32] we observed increased TF:FVIIa activity before cell injury. In agreement with prior reports,[32] we did not observe apoptosis in the GCDCA-treated hepatocytes, and a caspase inhibitor had no effect on

GCDCA-driven TF:FVIIa activity. These results correspond with our observation that coagulation is activated in the absence of detectable liver injury after BDL in mice and in cholestatic patients. Overall, it seems unlikely that the rapid bile acid–mediated increase in the TF:FVIIa activity we observed is simply driven by cell death.

Multiple mechanisms have been reported to influence TF:FVIIa activity.[42] The most studied are isomerization of TF extracellular disulfides by protein disulfide isomerase and interactions with exposed anionic phospholipids, such as PS. Although it is difficult to exclude a contribution of these processes to the effect of bile acids on TF:FVIIa activity, our in vitro studies suggest neither mechanism is essential. Specifically, we found that addition of the inhibitor anti-PDI antibody (protein disulfide isomerase; RL-90)[43] had no effect on GCDCA-induced hepatocyte TF procoagulant activity (data not shown). Moreover, although hepatocyte apoptosis drives PS exposure,[18] in preliminary studies, we were not able to detect an increase in cell-surface PS in GCDCA-treated cells using annexin V labeling and flow cytometry. However, this assay could overlook small changes in PS that could affect coagulation reactions. Notably, GCDCA treatment increased hepatocyte TF activity even in the presence of the PS-binding protein lactadherin. Viewed in the context of direct effects of GCDCA on relipidated and sTF:FVIIa activity suggests a direct mechanism not requiring reorganization of membrane phospholipids, such as PS.

The catalytic activity of FVIIa alone was not affected by GCDCA nor TCDCA, implying formation of the TF:FVIIa complex is required for the bile acid effect. Interestingly, we found that GCDCA reduced the apparent K_m for TF:FVIIa–driven FX activation. One possibility is that GCDCA increases TF:FVIIa activity by a mechanism analogous to that of PS, allowing bile acids to substitute for membrane phospholipids as drivers of TF:FVIIa activity, albeit less efficiently based on the more robust reduction in K_m by PS.[34] This hypothesis is consistent with bile acids

directly increasing sTF:FVIIa activity and our related observation that modest amounts of PS limit the effect of bile acids on relipidated TF. Even in light of these direct effects, our studies do not exclude a mechanism whereby bile acids increase TF activity through changes in components of the plasma membrane. Indeed, at physiological pH, GCDCA and TCDCA are essentially anionic detergents, a property critical for their role in digestion.[44] However, the concentrations used in this study are well below their critical micelle concentration in physiological solutions.[45, 46] Moreover, the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, a bile acid structural homolog, did not increase TF:FVIIa activity. This result, combined with the lack of effect on markers of membrane permeability (i.e., ALT release), is inconsistent with a nonspecific detergent-like effect being a primary mechanism. Overall, our studies suggest a direct, perhaps PS-like mechanism whereby GCDCA and other bile acids alter TF:FVIIa activity.

In summary, we discovered that bile acids directly increase TF:FVIIa procoagulant activity, displaying hallmarks of TF:FVIIa decryption. This may be particularly relevant to the liver, as subtle changes in TF:FVIIa activity in the leaky sinusoidal vasculature may be sufficient to drive pathological coagulation. Indeed, we report evidence of rapid intrahepatic coagulation without concurrent liver injury in experimental cholestasis in mice and increased coagulation in human patients with cholestasis. Importantly, plasma and hepatic bile acid levels are increased in patients with the most common forms of liver disease,[31, 47, 48] and our results suggest that bile acids may be coupled to intrahepatic activation of coagulation or hypercoagulability in these patients. Overall, these studies advance the field by pinpointing mechanisms of TF decryption driven by pathologic elevation of a physiologically relevant mediator. Identification of bile acids as a trigger of coagulation suggests a novel stimulus capable of disrupting the delicate hemostatic balance in liver disease, leading to pathological intrahepatic coagulation.

APPENDIX

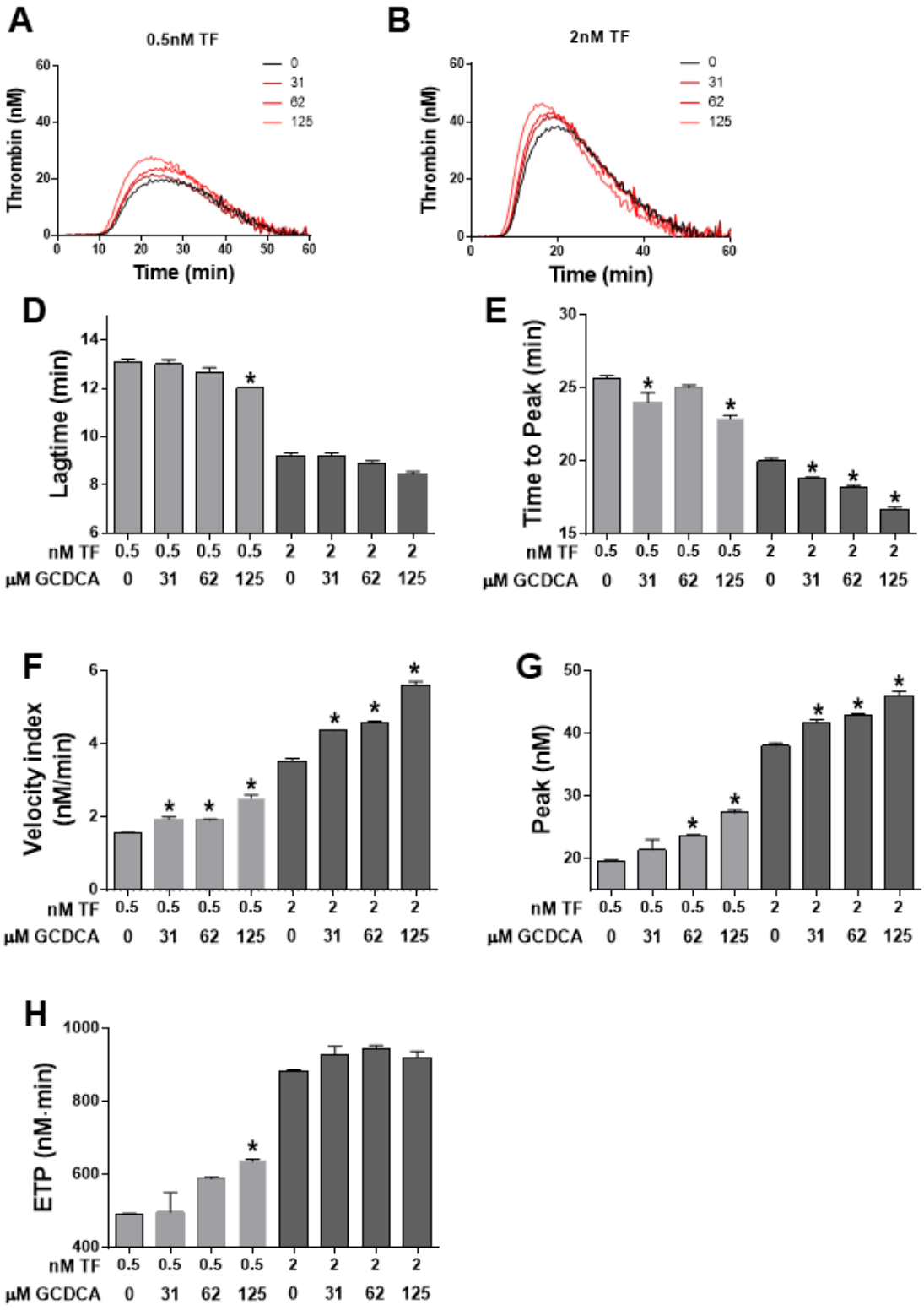

Figure 11. Effect of GCDCA on thrombin generation. Thrombin generation in normal human plasma containing various concentrations of GCDCA was triggered with TF relipidated in PC at a final concentration of (**A**) 0.5 nM TF or (**B**) 2 nM TF. (**D**) lagtime, (**E**) time to peak (F) peak, (**G**) velocity index, and (**H**) endogenous thrombin potential (ETP) were determined.

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CHAPTER 3

Taurocholic acid increases hepatocyte tissue factor procoagulant activity through non-apoptotic phosphatidylserine externalization.

Kevin S. Baker, Anna K. Kopec, Holly Cline-Fedewa, and James P. Luyendyk

Abstract

Objective: Blood coagulation is initiated by the tissue factor (TF):coagulation factor VIIa (FVIIa) complex. The TF:FVIIa expressed by liver parenchymal cells (i.e., hepatocytes) normally lacks procoagulant activity, but is activated in disease states to trigger coagulation. Prior studies showed that certain bile acids, which are elevated in patients with liver disease, directly increase TF:FVIIa procoagulant activity. Here, we sought to determine whether this effect was shared by structurally related bile acids, particularly those elevated in experimental cholestasis. **Results:** Treatment of hepatocytes with taurocholic acid (TCA), a bile acid highly elevated in mice after bile duct ligation, significantly increased hepatocyte TF:FVIIa procoagulant activity. However, unlike the closely related bile acid taurochenodeoxycholic acid, TCA did not directly increase relipidated TF:FVIIa procoagulant activity. Moreover, the effect of TCA treatment on hepatocyte TF:FVIIa activity was retained even after TCA was removed from the culture medium. These studies suggested TCA treatment indirectly increased TF:FVIIa procoagulant activity. Thus, we tested the hypothesis that TCA increased hepatocyte procoagulant potential by increasing surface phosphatidylserine levels. TCA treatment increased hepatocyte prothrombinase activity even in the presence of a caspase inhibitor. Addition of the phosphatidylserine-binding protein lactadherin attenuated the TCA driven increase in TF:FVIIa procoagulant activity. **Conclusion:** The results indicate that TCA indirectly increases hepatocyte TF:FVIIa procoagulant activity by driving non-apoptotic phosphatidylserine externalization. These studies suggest that not all bile acids directly increase TF:FVIIa procoagulant activity; moreover, these studies also highlight that some bile acids may generally amplify coagulation reactions by alternate mechanisms.

Introduction

Tissue factor (TF) is the primary activator of blood coagulation and the transmembrane receptor for coagulation factor VIIa (FVIIa). The TF:FVIIa complex triggers coagulation by activating coagulation factors IX and X.[1, 2] TF:FVIIa procoagulant activity is controlled in part by restricting TF expression to extravascular cells, separating TF from FVIIa in the blood.[3, 4] Procoagulant activity of the TF:FVIIa complex is also regulated by post-translational mechanisms, wherein procoagulant activity of TF:FVIIa is very low (i.e., encrypted).[5] This level of TF:FVIIa regulation is presumed to be critical in several tissues, such as the liver, where the fenestrated sinusoidal endothelium and locally high FVIIa expression by hepatocytes make for a precarious site for TF expression. Indeed, TF:FVIIa expressed on liver parenchymal cells (i.e., hepatocytes) normally resides in a non-procoagulant state.[6] Notably, hepatocyte TF has been shown to drive coagulation in the context of liver disease, but the mechanisms controlling TF:FVIIa activation in the liver are not fully understood.[6, 7]

The procoagulant activity of TF:FVIIa is dramatically increased in response to numerous stimuli,[8-10] and several mechanisms underlying this change have been described.[11, 12] Most notably, externalization of anionic phospholipids (e.g., phosphatidylserine) to the outer leaflet of the plasma membrane dramatically increases TF:FVIIa procoagulant activity.[9] Indeed, phosphatidylserine exposure increases hepatocyte TF:FVIIa procoagulant activity and hepatocyte apoptosis drives coagulation *in vivo*.[13, 14] In addition, there may be mechanisms of TF:FVIIa activation that are unique to liver diseases. For example, we recently showed that certain bile acids, cholesterol metabolites produced in the liver that are elevated in patients with liver disease, can increase TF:FVIIa activity.[15] Indeed, both glycine- and taurine-conjugated chenodeoxycholic acid (GCDCA and TCDCA, respectively) directly increased the procoagulant activity of relipidated

TF:FVIIa, an effect recapitulated in cultured hepatocytes.[15] Notably, bile acids are a diverse series of molecules, originating from two primary bile acids, cholic acid and chenodeoxycholic acid.[16] While these two primary bile acids differ by only a hydroxyl group, it is unclear if derivatives of each bile acid affect TF:FVIIa procoagulant activity.

We sought to directly compare the effect of bile acids with subtle structural diversity (i.e., TCDCA and taurocholic acid; TCA) on TF:FVIIa activity, and determine whether TCA, a bile acid dramatically increased in obstructive cholestasis in mice,[17] increased hepatocyte TF:FVIIa activity.

Materials and Methods

Mice: Wild-type C57Bl/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Additionally, littermate $TF^{flox/flox}$ mice (control mice) and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$) backcrossed 8 generations onto a C57Bl/6J background were used for some experiments and have been described previously.[18] Mice were housed at an ambient temperature of approximately 22°C with alternating 12-hour light/12-hour dark cycles and were provided purified water and rodent diet (Teklad Irradiated 22/5 Rodent Diet 8940, Envigo, Indianapolis, IN) *ad libitum* before study initiation. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility at Michigan State University. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Murine hepatocyte isolation: Primary mouse hepatocytes were isolated from wild-type, $TF^{flox/flox}$, and $HPC^{\Delta TF}$ mice by perfusion and collagenase digestion, as previously described.[6] Cell viability

was determined by trypan blue exclusion and hepatocytes from isolations with at least 80% initial viability were used. Hepatocytes were plated on 6-well culture plates (BD Falcon, Franklin Lakes, NJ) at a density of 500,000 cells per well in Williams E medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Seradigm, VWR International LLC, Radnor, PA) and 1% penicillin/streptomycin (Sigma-Aldrich). After 2 hours, non-adherent cells were removed and fresh medium was added. The cells were then incubated overnight at 37°C and 5% CO_2 prior to bile acid treatment.

***In vitro* treatments:** In experiments where the TCA treatment was removed, the hepatocytes were treated with various concentrations of sodium taurocholate (TCA; Sigma-Aldrich) in FBS-free Williams E medium. After 15-minute treatment with TCA, the medium was aspirated off the cells and HEPES buffered saline (HBS; 137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]) containing 0.1% albumin (HBSA) was added before hepatocyte TF activity was determined (see below). In select experiments, cells were pretreated with actinomycin D (0.2 µg/mL, Sigma-Aldrich), wortmannin (100 nM final, Tocris, Minneapolis, MN), PD98059 (20 µM final, Tocris), SB203580 (10 µM final, Tocris), Go6983 (100 nM final, Cayman Chemical, Ann Arbor, MI), Ac-DEVD-CHO (50 µM final, Biolegend), or DMSO vehicle (0.1% final) for 30 minutes before TCA treatment (1 mM final). In select experiments, cells were treated with an anti-protein disulfide isomerase (PDI) antibody (50 µg/mL final, RL-90; Scripps Research Institute, Antibody Production Core) or an isotype control (IgG) 30 minutes before treatment with TCA (1 mM final). Additionally, in select experiments, TCA-treated (1 mM final) hepatocytes were incubated with lactadherin for 15 minutes (105 nM final, Haematologic Technologies Inc., Essex Junction, VT) before hepatocyte

TF activity was assessed. In one set of experiments, a time course was performed before hepatocyte TF activity was determined. In experiments where the TCA treatment was kept on during the determination of TF activity, the cells were treated with TCA in HBSA. After 15-minute treatment with TCA, hepatocyte TF activity was determined.

Determination of phosphatidylserine externalization in cultured mouse hepatocytes: Primary mouse hepatocytes were isolated, cultured, and treated with TCA or vehicle as described above. Externalization of phosphatidylserine was determined using a prothrombinase assay as previously described.[9] In brief, treated hepatocytes were incubated with human coagulation factor Va and factor Xa (0.5 nM and 0.1 nM final respectively, Enzyme Research Laboratories, South Bend, IN) for 5 minutes at 37°C in HBSA containing $CaCl_2$ (2.5 mM final). HBSA containing human prothrombin (0.2 μM final, Enzyme Research Laboratories) was then added to the cells and the plate was incubated for 15 minutes at 37°C. The reaction was then stopped by addition of EDTA (pH 7.4, final concentration 5 mM). Thrombin activity was determined using a chromogenic substrate (Z-D-Arg-Gly-Arg-pNA; 0.667 mM; Bachem, Torrance, CA) and the results were expressed as change in absorbance at 405 nm. In select studies, a caspase 3/7 inhibitor (50 μM final, Ac-DEVD-CHO, Cayman Chemical) or DMSO (0.1% final) were added for 30 minutes prior to TCA treatment.

Measurement of cytotoxicity: Alanine aminotransferase (ALT) release into the culture medium was determined using a commercially available reagent (Thermo Fisher, Waltham, MA). After 15-minute treatment with vehicle or TCA (0.2 and 1 mM final), the cell culture supernatant was collected, and the cells were lysed in PBS containing 0.1% Triton X-100. The culture supernatant

and lysed cells were spun down at 500 x g for 2 minutes. ALT activity was determined in cleared supernatant and cell lysate and expressed as percent ALT released into the supernatant.

TF relipidation: Full-length recombinant tissue factor (Haematologic Technologies Inc.) was relipidated in unilamellar vesicles composed of l-α-phosphatidylcholine (isolated from chicken egg, Avanti Polar Lipids, Alabaster, AL), as described previously.[15, 19]

Determination of TF:FVIIa procoagulant activity: Hepatocyte TF:FVIIa activity was assessed by measuring the capacity of mouse hepatocytes to convert coagulation factor X (FX) to its active form FXa. Human FX (100 nM final, Enzyme Research Laboratories) in pre-warmed sterile HBSA containing $CaCl_2$ (2.5 mM final) was added to each well. After 15 minutes at 37°C, the reaction was stopped by addition of EDTA (pH 7.4, 5 mM final). FXa activity in each sample was determined using a chromogenic substrate (H-D-Phe-Homopro-Arg-pNA, 0.667 mmol/L final, Bachem),compared to a standard curve generated using human FXa (Enzyme Research Laboratories), and expressed as fold change. For studies using relipidated TF, vesicles containing TF were diluted (0.1 nM TF final) and incubated with human FVIIa (5 pM final, Enzyme Research Laboratories) in HBSA containing calcium (2.5 mM final) for 5 minutes at 37°C. Various concentrations of bile acid and human FX (100 nM final, Enzyme Research Laboratories) were then added. After 30 minutes, FXa generation was stopped with EDTA (pH 7.4, 5 mM final) and FXa activity was assessed as described above.

Statistics: Comparison of two groups was performed using Student's t-test and comparison of 3 or more groups was performed by ANOVA with Student-Newman-Keuls post-hoc test. The criterion for statistical significance was $P<0.05$.

Results

Taurocholic acid (TCA) increases hepatocyte TF:FVIIa activity but does not directly activate the TF:FVIIa complex.

Bile contains a myriad of different bile acids that are elevated in both experimental and clinical settings of liver disease.[17, 20] Interestingly, bile acids increased in mice after obstructive cholestasis differ from those previously identified as direct activators of the TF:FVIIa complex (i.e., GCDCA, TCDCA).[15, 17] Treatment of primary mouse hepatocytes with a pathologically relevant concentration of TCA (1 mM)[17] significantly increased hepatocyte TF:FVIIa activity (Figure 12A), assessed by the ability of mouse hepatocytes to convert FX to FXa. To determine if this increase was due to direct activation of the TF:FVIIa complex, we determined the effect of TCA on TF:FVIIa relipidated in phosphatidylcholine vesicles. Surprisingly, there was no evidence of a concentration-dependent effect of TCA on TF:FVIIa activity (Figure 12B). However, TCDCA increased relipidated TF:FVIIa activity in a concentration-dependent manner, in agreement with prior studies (Figure 12B).[15] Even pathologically relevant concentrations of TCA (1 mM) did not directly increase TF:FVIIa activity (not shown). The results indicate that TCA increases hepatocyte TF:FVIIa procoagulant activity, but unlike TCDCA, does not directly increase TF:FVIIa activity.

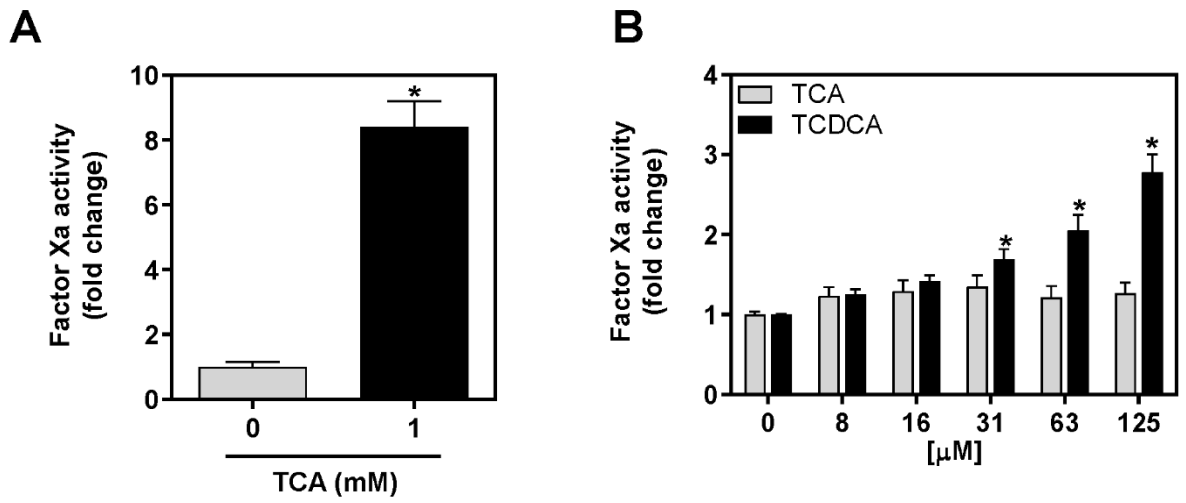

Figure 12. Effect of TCA on hepatocyte and relipidated TF activity. **(A)** Primary mouse hepatocytes were isolated from wild-type mice and cultured overnight. Culture medium was exchanged for HEPES buffered saline containing albumin (HBSA) and cells were treated with TCA (1 mM final) or vehicle (HBS) 15 minutes prior to adding FX (100 nM final) (N = 3 mice). FXa generation was measured 15 minutes after FX was added. The data are expressed as mean + SEM. **(B)** TF-containing (0.1 nM final) unilamellar phosphatidylcholine vesicles were incubated with FVIIa (coagulation factor VIIa; 5 pM final) for 5 minutes, then treated with various concentrations of TCA or TCDCA (8-125 μM final) or vehicle (HBS) and FX (100 nM final) for 30 minutes prior to measurement of FXa generation. Individual assays were performed in triplicate. Data represent fold change compared to vehicle-treated vesicles for 6 independent assays (two batches of relipidated TF-containing vesicles) and the results are presented as mean + SEM. *$p<0.05$ compared to respective vehicle treatments.

TCA induces a lasting increase in hepatocyte TF:FVIIa activity.

Because TCA significantly increased hepatocyte TF:FVIIa procoagulant activity, but did not increase the activity of relipidated TF:FVIIa, we explored the possibility that TCA indirectly modified hepatocyte TF:FVIIa activity. To test this, mouse hepatocytes were treated with TCA for various times and TCA was removed prior to FXa generation. TCA (1 mM) induced a time-dependent increase in FXa generation, even when removed prior to the determination of TF:FVIIa activity (Figure 13A). This increase in TF activity was not a consequence of bile acid toxicity, as there was no evidence of ALT release from TCA-treated hepatocytes (Figure 13B). FXa generation by TCA-treated hepatocytes was abolished in hepatocytes isolated from mice with liver TF deficiency ($HPC^{\Delta TF}$), confirming specificity (Figure 13C). Interestingly, prolonged treatment (24 hours) of hepatocytes with 200 μM TCA caused a slight but significant increase in TF procoagulant activity (Figure 13D). Collectively, the results suggest that TCA causes a concentration and time-dependent increase in hepatocyte TF:FVIIa activity via an indirect mechanism.

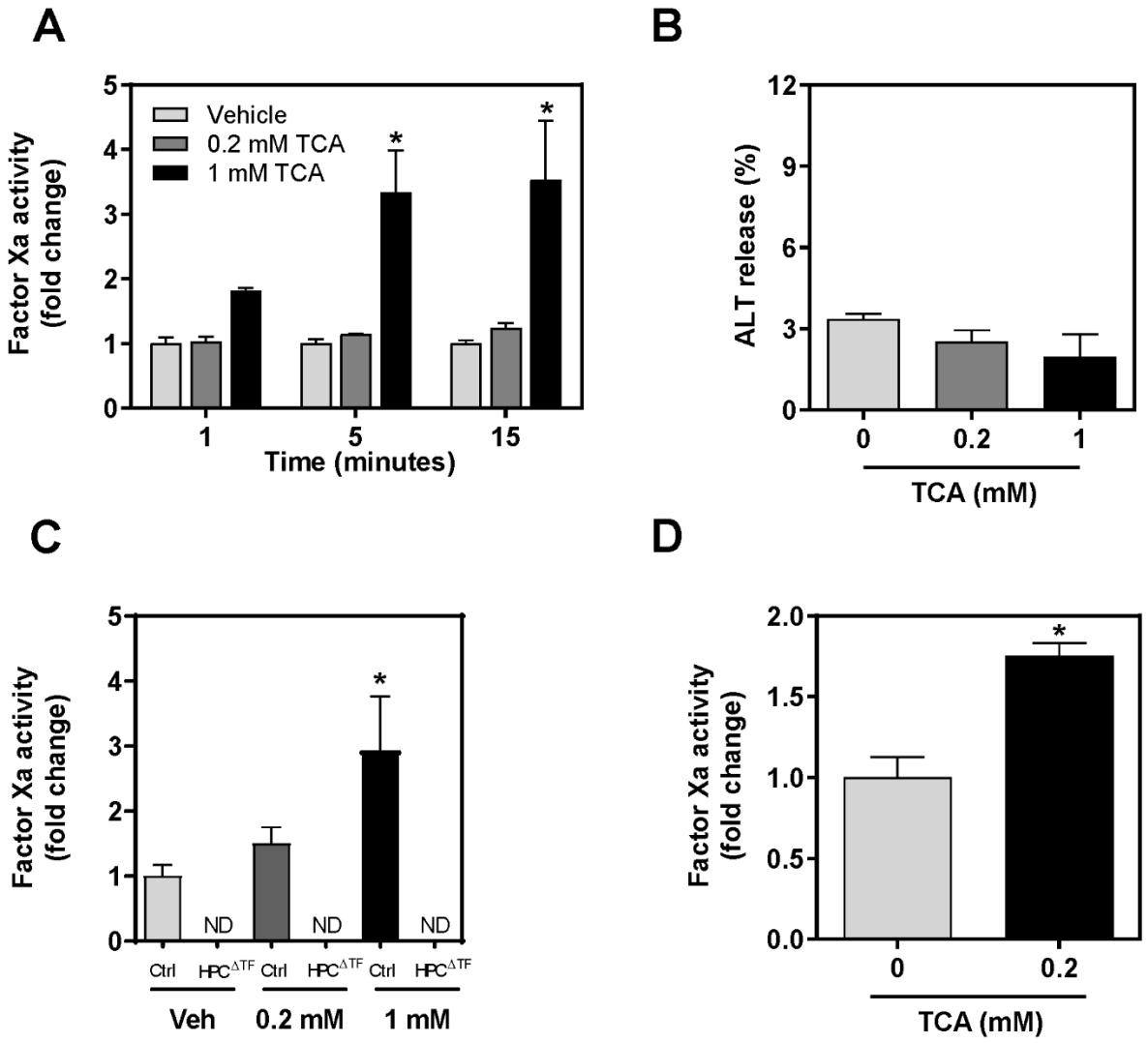

Figure 13. The effect of transient TCA treatment on hepatocyte TF activity. **(A)** Primary mouse wild-type hepatocytes were treated with vehicle (PBS) or various concentrations of TCA for the indicated times in culture medium (N = 3 mice). FXa generation was determined 15 minutes later. **(B)** Alanine aminotransferase (ALT) release was determined 15 minutes after treatment with TCA (0.2 and 1 mM) or vehicle (PBS) and expressed as a % of total (N = 3 mice). **(C)** Primary mouse $TF^{flox/flox}$ (Ctrl) and TF deficient ($HPC^{\Delta TF}$) hepatocytes were treated with TCA (0.2 and 1 mM final) or vehicle (Veh; PBS) for 15 minutes, and FXa generation was determined 15 minutes later (N = 3 mice). **(D)** Primary mouse wild-type hepatocytes were treated with TCA (0.2 mM final) or vehicle (PBS) for 24 hours and then FXa generation was determined (N = 6 mice). For these experiments, the culture medium containing treatment was replaced with HBSA before FXa generation was determined as described in the materials and methods section. The data are expressed as mean + SEM. *$p<0.05$ compared to respective vehicle treatments.

TCA increases hepatocyte TF activity independent of transcription.

Because TCA increased hepatocyte TF:FVIIa activity indirectly, we considered the possibility that rapid activation of intracellular signaling and expression of TF occurred after TCA treatment. Indeed, bile acids have been shown to activate several intracellular pathways,[21] many of which have been shown to modify TF expression and activity.[22] However, pretreatment of hepatocytes with pharmacologic inhibitors of phosphoinositide 3-kinase (PI3K; wortmannin), mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK; PD98059), p38 mitogen-activated protein kinases (SB203580), or protein kinase C (PKC; Go6983) had no effect on TF:FVIIa activity 15 minutes after treatment of hepatocytes with TCA (1 mM) (Figure 14A-D). Prior studies document efficacy of each inhibitor at the selected concentration.[23-26] Moreover, pretreatment of hepatocytes with the transcriptional inhibitor actinomycin D did not affect TF:FVIIa activity in either vehicle or TCA-treated hepatocytes (Figure 14E). The results suggest that frequently implicated signaling pathways and transcription are not be required for TCA to increase hepatocyte TF:FVIIa procoagulant activity.

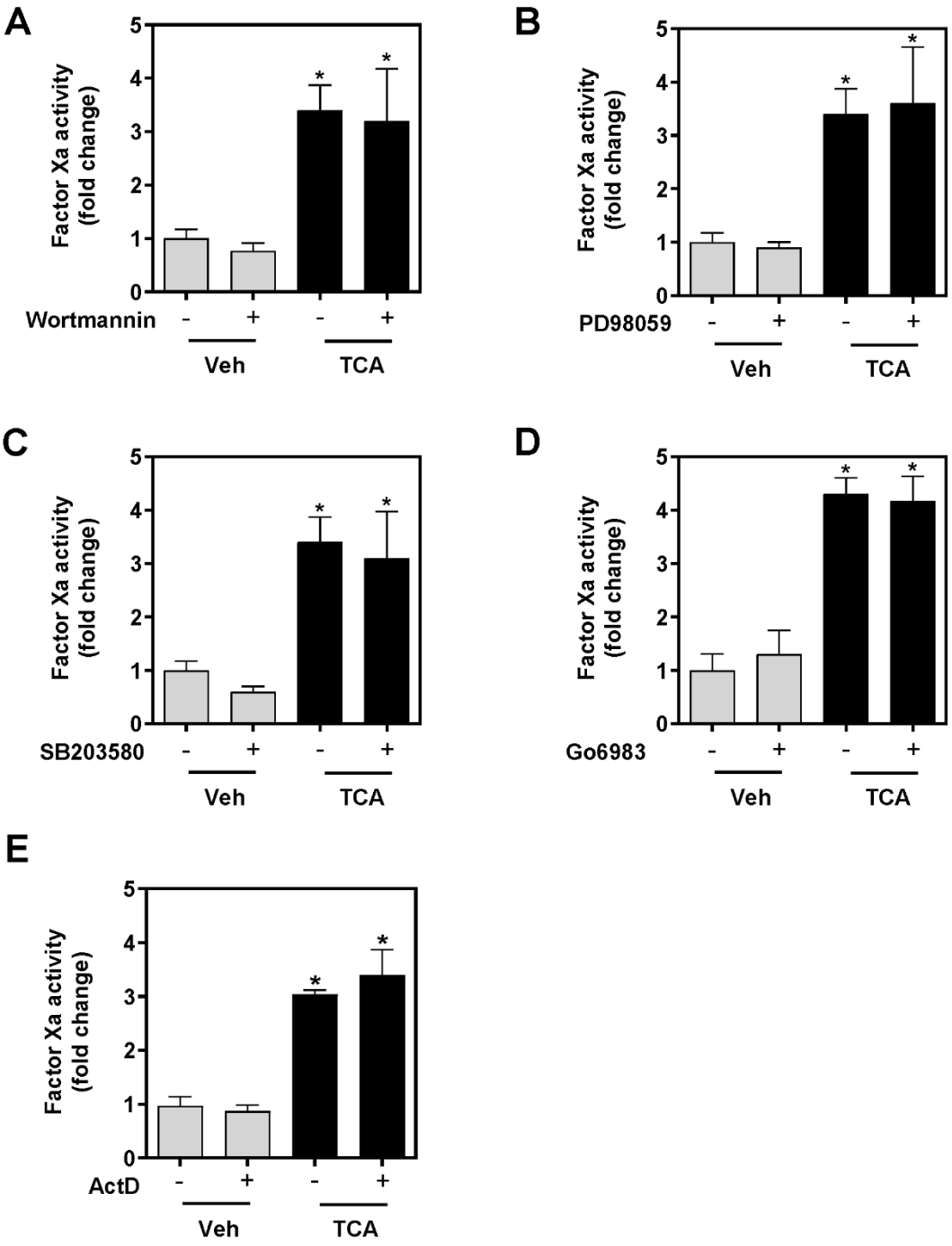

Figure 14. Bile acid signaling pathways and transcription are not required for TCA-driven hepatocyte TF activity. Primary mouse wild-type hepatocytes were pretreated for 30 minutes with **(A)** wortmannin (PI3K inhibitor; 100 nM final), **(B)** PD98059 (MEK/ERK inhibitor; 20 µM final), **(C)** SB203580 (p38 inhibitor; 10 µM final), **(D)** Go6983 (PKC inhibitor; 100 nM final), **(E)** actinomycin D (0.2 µg/mL final), or DMSO vehicle (0.1% final), then treated with TCA (1 mM final) or PBS vehicle (Veh) for 15 minutes (N = 3 mice). For these experiments, culture medium

Figure 14. (cont'd). containing the treatment was then replaced HBSA and FXa generation was determined as described in the materials and methods section. The data are expressed as mean + SEM. *$p<0.05$ compared to respective vehicle-treated group.

TCA increases TF:FVIIa procoagulant activity by externalizing phosphatidylserine

Next, we examined the potential involvement of two mechanisms shown previously to activate the TF:FVIIa complex in other experimental systems, protein disulfide isomerase (PDI) and phosphatidylserine externalization.[27] Through modulation of extracellular disulfides in the TF protein, PDI has been suggested to be involved with the activation of TF:FVIIa procoagulant activity.[28] However, inhibition of PDI activity with a monoclonal antibody (anti-PDI; RL-90), shown previously to decrease TF procoagulant activity,[29, 30] did not affect baseline or TCA driven hepatocyte TF:FVIIa activity. (Figure 15A). Next, we determined the effect of TCA treatment on externalization of phosphatidylserine on the cell membrane.[31] Interestingly, TCA significantly increased prothrombinase activity within 15 minutes, indicating externalization of phosphatidylserine (Figure 15B). Although prior studies have shown that phosphatidylserine externalization and increased TF activity are hallmarks of hepatocyte apoptosis and prevented by caspase inhibitors,[13] inhibition of caspases did not prevent TCA-mediated increases in prothrombinase activity and TF:FVIIa activity (Figure 15C-D). This suggests that TCA treatment drives a rapid non-apoptotic externalization of phosphatidylserine in cultured hepatocytes. To determine whether phosphatidylserine externalization contributed to the TCA-mediated increase in FXa activity, we used lactadherin, a high affinity phosphatidylserine-binding protein.[32] Notably, the addition of lactadherin attenuated both baseline and TCA-induced TF procoagulant activity in hepatocytes (Figure 15E). Collectively, the results suggest that TCA increases hepatocyte TF:FVIIa procoagulant activity by externalizing phosphatidylserine in the absence of apoptosis.

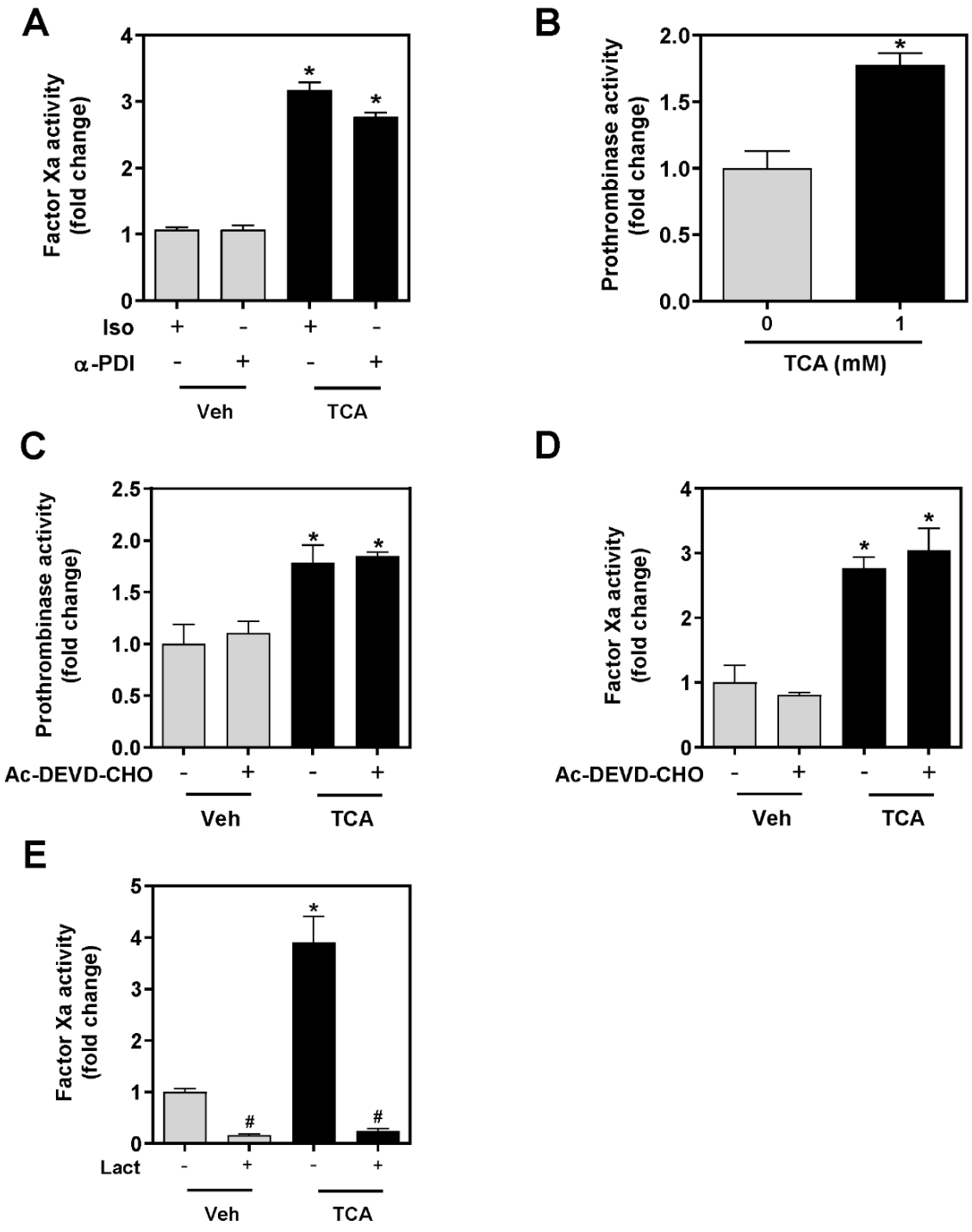

Figure 15. TCA drives TF:FVIIa procoagulant activity by increasing hepatocyte phosphatidylserine externalization. (A) Primary mouse wild-type hepatocytes were pretreated with an anti-protein disulfide isomerase antibody (50 µg/mL final, α-PDI, RL-90) or isotype control antibody (Iso) for 30 minutes prior to 15-minute treatment with TCA (1 mM final) or

Figure 15. (cont'd). vehicle (Veh; PBS), after which FXa generation was determined (N = 3). **(B)** Primary mouse wild-type hepatocytes were treated with TCA (1 mM final) or vehicle for 15 minutes (PBS) and prothrombinase activity was determined (N = 3 mice). For panels C-D, primary mouse wild-type hepatocytes were pretreated with Ac-DEVD-CHO (Caspase 3/7 Inhibitor, 50 μM final) or vehicle (DMSO, 0.1% final) 30 minutes prior to treatment with TCA (1 mM final) or vehicle (Veh; PBS) (N = 3 mice). **(C)** Prothrombinase activity and **(D)** factor Xa generation were determined 15 minutes after TCA treatment. **(E)** Primary mouse wild-type hepatocytes were treated with TCA (1 mM final) or vehicle (Veh, PBS) for 15 minutes, then the medium containing the treatment was removed, and the cells were incubated with lactadherin (Lact; 105 nM) for 15 minutes prior to measuring FXa generation (N = 3 mice). For these experiments, culture medium containing the treatment was then replaced with HBSA before FXa or prothrombinase generation was determined as described in the materials and methods section. The data are expressed as mean + SEM. *$p<0.05$ compared to respective vehicle-treated cells. #$p<0.05$ compared to cells of the same treatment in the absence of lactadherin.

Discussion

Our prior studies presented evidence in support of the hypothesis that bile acids may promote coagulation activation by decrypting procoagulant activity of the TF:FVIIa complex.[15] TCDCA and GCDCA, both amino acid conjugates of the primary bile acid chenodeoxycholic acid, directly increased activity of TF:FVIIa complex relipidated in phosphatidylcholine. Noting the structural diversity between bile acids, we sought to determine whether TCA, a bile acid highly elevated in liver and plasma of mice after BDL, also increased TF:FVIIa activity. Although TCA increased hepatocyte TF procoagulant activity at pathologically relevant levels, TCA did not directly increase TF:FVIIa procoagulant activity. The results suggest that TCA can drive rapid changes in coagulation through indirect effects on the plasma membrane. Moreover, the results add weight of evidence to the hypothesis that bile acids contribute to coagulation cascade activation, with the novel addition that bile acid structure may direct the mechanism whereby this occurs.

TCA increased TF:FVIIa procoagulant activity in cultured hepatocytes, but unlike TCDCA, did not increase the activity of relipidated TF:FVIIa. This suggests a connection between bile acid structure and the potential to directly increase TF:FVIIa activity. Although both bile acids are conjugated to taurine, TCDCA does not have a hydroxyl group on the cholesterol backbone. This subtle difference appears to enable new functionality of TCDCA, which is analogous to effects of bile acid structure on other signaling functions (e.g., receptor binding).[33-35] Although the precise basis for structural changes in bile acids driving direct TF:FVIIa activity will require in depth study, it is worth noting that the absence of the hydroxyl group in TCDCA is associated with an increase in bile acid hydrophobicity.[36] It is conceivable that this change in chemistry may affect interactions of the bile acids with plasma membrane proteins (e.g., TF) or the membrane itself, each altering

the potential to increase procoagulant activity. Further study using approaches to assess direct interactions between bile acids and the TF:FVIIa complex (e.g., surface plasmon resonance) or perhaps molecular dynamic simulation may reveal such interactions. Moreover, it seems plausible that individual bile acids affect coagulation reactions through multiple mechanisms, including direct and indirect effects on TF:FVIIa.

Externalized phosphatidylserine on the plasma membrane dramatically increases TF:FVIIa procoagulant activity.[9] Moreover, phosphatidylserine supports activation of the prothrombinase complex, enabling detection of phosphatidylserine externalization in cultured cells.[9] Interestingly, TCA treatment significantly increased prothrombinase activity in cultured hepatocytes. Because the phosphatidylserine-binding protein lactadherin prevented the TCA-dependent increase in TF:FVIIa activity, it appears that TCA drives TF:FVIIa activity by externalizing phosphatidylserine. Phosphatidylserine externalization and TF procoagulant activity increase in apoptotic hepatocytes.[13] Although some bile acids have been shown to induce apoptosis, there are concerns on the relevance of cell lines used in these studies and questions on the relevance of the concentrations of specific toxic bile acids used.[20] TCA is a relatively nontoxic bile acid, and our studies indicate that the increase in prothrombinase and TF:FVIIa activity are unaffected by caspase inhibition, indicating a mechanism independent of apoptosis.

The mechanism whereby TCA externalizes phosphatidylserine on hepatocytes is not known. Bile acids trigger intracellular signaling and gene induction by activating multiple types of extracellular and nuclear receptors,[21] the most classic of which is the nuclear receptor farnesoid X receptor (FXR).[33, 37, 38] Previously we determined that direct pharmacological activation of FXR did not rapidly increase TF:FVIIa activity,[15] making it unlikely that externalization of phosphatidylserine is driven by an FXR-dependent mechanism. Likewise, we found that prolonged

activation of FXR (24 hours) with an FXR agonist (GW4064) did not increase hepatocyte TF expression or TF:FVIIa activity (not shown). Moreover, common intracellular pathways activated by bile acids may not be required for TCA to increase TF:FVIIa activity. Interestingly, in initial studies, we also found that sodium free conditions, which dramatically reduces Na+-dependent bile acid transport, had no effect on TCA-mediated increase in TF:FVIIa activity (data not shown). This suggests the potential for TCA, and perhaps other bile acids, to have direct effects on the hepatocyte membrane. This effect is not likely a consequence of membrane damage by detergent-like actions of TCA, because we did not observe an increase in ALT release as an indicator of membrane leakiness. It is possible that TCA disrupts phospholipid asymmetry through another mechanism. For example, TCA has been shown to increase the activity of phospholipid floppases (e.g., ABCB4, ABCA1), enzymes which move membrane components (e.g., phosphatidylcholine, cholesterol) from the inner to the outer leaflet of the cell membrane.[39-41] It is also possible that noncytotoxic effects of TCA on the membrane can impact phosphatidylserine externalization or TF activity, such as altering sphingomyelin or lipid rafts.[42, 43] Future studies should determine how bile acids impact phosphatidylserine externalization.

Bile contains numerous chemically distinct bile acids synthesized from cholesterol by various liver enzymes.[16] The two primary bile acids, cholic acid and chenodeoxycholic acid, are amino acid conjugated before being transported into bile, and further diversity is imposed by metabolism by intestinal flora.[44, 45] In humans, bile acids are primarily glycine-conjugated (e.g., glycochenodeoxycholic acid).[46] In contrast, taurine-conjugated bile acids predominate in mice, with TCA and tauro-β-muricholic acid being the most concentrated in bile.[17] Exposure of the liver to high concentrations of these major bile acid species is likely in patients with liver disease, and in mice this has been fully documented.[17, 20, 46] Defining how this mixture of different bile acids

contributes to coagulation *in vivo* is challenging, particularly as our studies highlight diverse mechanisms whereby bile acids may affect TF:FVIIa activity. Moreover, our studies have focused on the immediate effects of specific bile acids. It is entirely plausible that more long-term exposure of cells to pathologic bile acid concentrations evokes relevant transcriptional changes in TF or other hemostatic factors, with species-dependent differences in regulation of genes further complicating interpretation. For instance, fibrinogen can be regulated by bile acids through the nuclear receptor FXR in human cell lines, but this was not evident in mice.[47]

In summary, we found that in comparison to TCDCA, TCA did not directly increase the procoagulant activity of the TF:FVIIa complex. Surprisingly, TCA treatment increased hepatocyte TF:FVIIa activity indirectly by promoting non-apoptotic externalization of phosphatidylserine. The results provide further evidence in support of bile acids as a relevant regulator of coagulation activation, but suggest that the mechanisms connecting bile acids to TF:FVIIa activity may be varied. Further studies examining the connection between bile acid structure and interaction with the TF:FVIIa complex may be revealing.

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CHAPTER 4

Role of hepatocyte tissue factor in carbon tetrachloride induced liver injury.

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Abstract

Objective: Tissue factor (TF) is the primary activator of blood coagulation and expression of TF by liver parenchymal cells (i.e., hepatocytes) triggers coagulation in mice challenged with the hepatotoxicant acetaminophen. However, at present it is unclear if the role of hepatocyte TF in coagulation activation is a universal feature of liver injury. We tested the hypothesis that hepatocyte TF triggers coagulation in mice challenged with carbon tetrachloride (CCl_4) and determined the impact of hepatocyte TF deficiency on acute CCl_4 induced liver injury and hepatic fibrosis. **Approach and results:** Mice with TF-deficient hepatocytes ($TF^{flox/flox}$/AlbCre) and control mice ($TF^{flox/flox}$)were challenged with CCl_4 (1 mL/kg i.p.), and the impact of hepatocyte TF deficiency on acute and chronic liver injury was determined. Surprisingly, in contrast to acetaminophen-induced liver injury, hepatocyte TF deficiency had no effect on coagulation activation or hepatocellular necrosis after acute CCl_4 challenge, indicated by plasma thrombin-antithrombin (TAT) and alanine aminotransferase activity, respectively. Next, we wanted to determine the role of hepatocyte TF in the development of hepatic fibrosis. Chronic challenge with CCl_4 (twice weekly for 6 weeks) significantly increased hepatic expression of profibrogenic genes and increased hepatic collagen deposition in control mice. Notably, hepatocyte TF deficiency had no effect on hepatic fibrosis, and similar to acute CCl_4 challenge, there was no obvious difference in coagulation activation between control mice and mice with TF-deficient hepatocytes, as indicated by plasma TAT. **Conclusion:** The results indicate that in contrast to acetaminophen-induced liver injury, hepatocyte TF does not drive coagulation in CCl_4-induced liver injury. This suggests that hepatocyte TF does not participate in the activation of coagulation in all liver injury models

Introduction

Tissue factor (TF) is the transmembrane receptor for coagulation factor VIIa (FVIIa) and the primary activator of the blood coagulation cascade.[1] It was previously found that there was a reduction in liver procoagulant activity in mice that express low levels of TF.[2, 3] Moreover, prior studies have found that TF plays an important role in activating blood coagulation in certain experimental settings of acute hepatotoxicity. [2, 4-6] Multiple cell types in the liver express TF (e.g. cholangiocytes),[2] but studies have shown that the vast majority of TF expressed in the liver is from parenchymal cells (i.e., hepatocytes).[5] Additionally, hepatocyte TF was found to be required for activating coagulation after acetaminophen-induced liver injury.[5] However, it is unclear if hepatocyte TF is universally required in models of liver injury.

Experimental settings of chemical-induced liver injury are routinely used to define mechanisms linking coagulation to liver injury.[7-11] Both carbon tetrachloride (CCl_4) and acetaminophen are converted into toxic metabolites by hepatic cytochrome P450s, which causes liver injury and centrilobular necrosis.[12, 13] Because the activation of coagulation is so connected with injury, and both toxicants injure hepatocytes, which express TF, we considered the possibility that hepatocyte TF would activate coagulation after challenge with CCl_4. A prior study utilized an antisense oligonucleotide to knockdown the expression of TF in mice before CCl_4 administration, and it was found that TF was required for CCl_4-mediated injury.[14] Although this suggests the injury required TF, the role of hepatocyte TF in activating coagulation after CCl_4 challenge has not been determined. Furthermore, chronic challenge with CCl_4 has been used to cause fibrosis, a consequence of chronic liver damage characterized by excess collagen deposition. [15, 16] Therefore, CCl_4-induced liver injury provides opportunity to examine the role of hepatocyte TF activation in chronic toxicity associated with fibrosis.

We tested the hypothesis is that deficiency in hepatocyte TF decreases coagulation and liver injury after CCl_4 challenge. We determined the role of hepatocyte TF in activating coagulation in an established model of liver injury induced by CCl_4.

Materials and methods

Mice and CCl_4 challenge: Littermate $TF^{flox/flox}$ mice (control mice) and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$ mice) backcrossed 8 generations onto a C57Bl/6J background have been described previously.[5] Roughly age-matched groups of male mice between the ages of 12-31 weeks were used for this study. Mice were housed at an ambient temperature of approximately 22°C with alternating 12-hour light/12-hour dark cycles and provided purified water and normal rodent chow (Teklad Irradiated 22/5 Rodent Diet 8940, Envigo) ad libitum. Mice were challenged with corn oil (vehicle) or 10% CCl_4 dissolved in corn oil by intraperitoneal injection (10 mL/kg). In studies involving chronic challenge with CCl_4, mice were injected twice weekly (i.e., Monday and Thursday) for 6 weeks, and samples were collected 3 days after the final injection. In studies involving acute challenge, samples were collected after 6 or 24 hours. Under isoflurane anesthesia, the liver was removed and rinsed in phosphate-buffered saline. The left lateral lobe was fixed in 10% neutral-buffered formalin for around 96 hours and processed for routine histopathological analysis. The remaining lobes were snap-frozen in liquid nitrogen for other endpoint analyses. Mice were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facilities at Michigan State University. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

Collagen staining and quantification: Paraffin-embedded, formalin-fixed liver were sectioned at 5 μM and stained with Sirius red by the Michigan State University Investigative Histopathology Laboratory, a Division of Human Pathology. For quantification of the total area of Sirius red staining, slides were first scanned by use of a Virtual Slide System VS110 (Olympus, Waltham, MA, USA) with a 20× objective, and sample images were then digitally captured from the entire left lateral lobe (Visiopharm, Broomfield, CO, USA) as described previously.[5, 15]

Measurement of activation of coagulation and hepatocellular injury: Plasma thrombin-antithrombin (TAT) levels were determined using a commercial ELISA (Siemens Healthcare Diagnostics, Malvern, PA). Plasma alanine aminotransferase (ALT) activity was determined using a commercial reagent (Thermo Fisher Scientific).

RNA isolation, cDNA synthesis, and real-time PCR: Total RNA was isolated from snap-frozen liver with TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH, USA). One microgram of total RNA was used to synthesize cDNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA) in a C1000 Thermal Cycler (BioRad, Hercules, CA). SYBR Green quantitative real-time PCR amplification was performed by use of a CFX Connect thermal cycler (Bio-Rad) with primers purchased from Integrated DNA Technologies (Coralville, IA, USA) and Perfecta SYBR Green SuperMix (Quanta Biosciences, Beverly, MA, USA). The expression of each gene was normalized to the geometric mean Ct of individual housekeeping genes (*Hprt* and *Gapdh*), and relative fold change was determined with the ΔΔCt method. The primer sequences used have been previously described.[5, 17]

Statistics: Statistical significance was determined using Prism GraphPad version 8.2. Comparison of two groups was performed using Student's t-test and comparison of 3 or more groups was performed by ANOVA with Dunnett post-hoc test. The criterion for statistical significance was $P<0.05$.

Results

Hepatocyte TF does not contribute to the activation of coagulation or liver injury after acute challenge with CCl_4

Studies have documented TF-dependent activation of coagulation after acute challenge with hepatotoxicants.[2, 4] However, it is unclear whether hepatocyte TF contributes to coagulation in all models of liver injury. Twenty-four hours after CCl_4 challenge (1 mL/kg, intraperitoneal), there was an increase in hepatocellular injury as indicated by increased serum alanine aminotransferase (ALT) (Figure 16A). Additionally, CCl_4 challenge caused coagulation cascade activation, as indicated by increased plasma thrombin-antithrombin (TAT) levels (Figure 16B). However, there was no difference in hepatocellular injury or activation of coagulation between control and mice with TF-deficient hepatocytes (Figure 16A & B). We then considered the possibility hepatocyte TF contributes to coagulation at an earlier time after CCl_4 challenge. 6 hours after challenge with CCl_4, there was an increase in levels of ALT above normal controls levels (i.e., <80 U/L), but there was no difference in hepatocellular injury between control and mice with TF deficient hepatocytes. (Figure 16C). Surprisingly, there was no difference in plasma TAT levels 6 hours after CCl_4 challenge between control mice and mice with TF deficient hepatocytes (Figure 16D). Moreover, the levels of plasma TAT were not above typical control levels (i.e., TAT

<8 ng/mL). The results suggest that hepatocyte TF deficiency does not affect coagulation cascade activation after acute challenge with CCl_4.

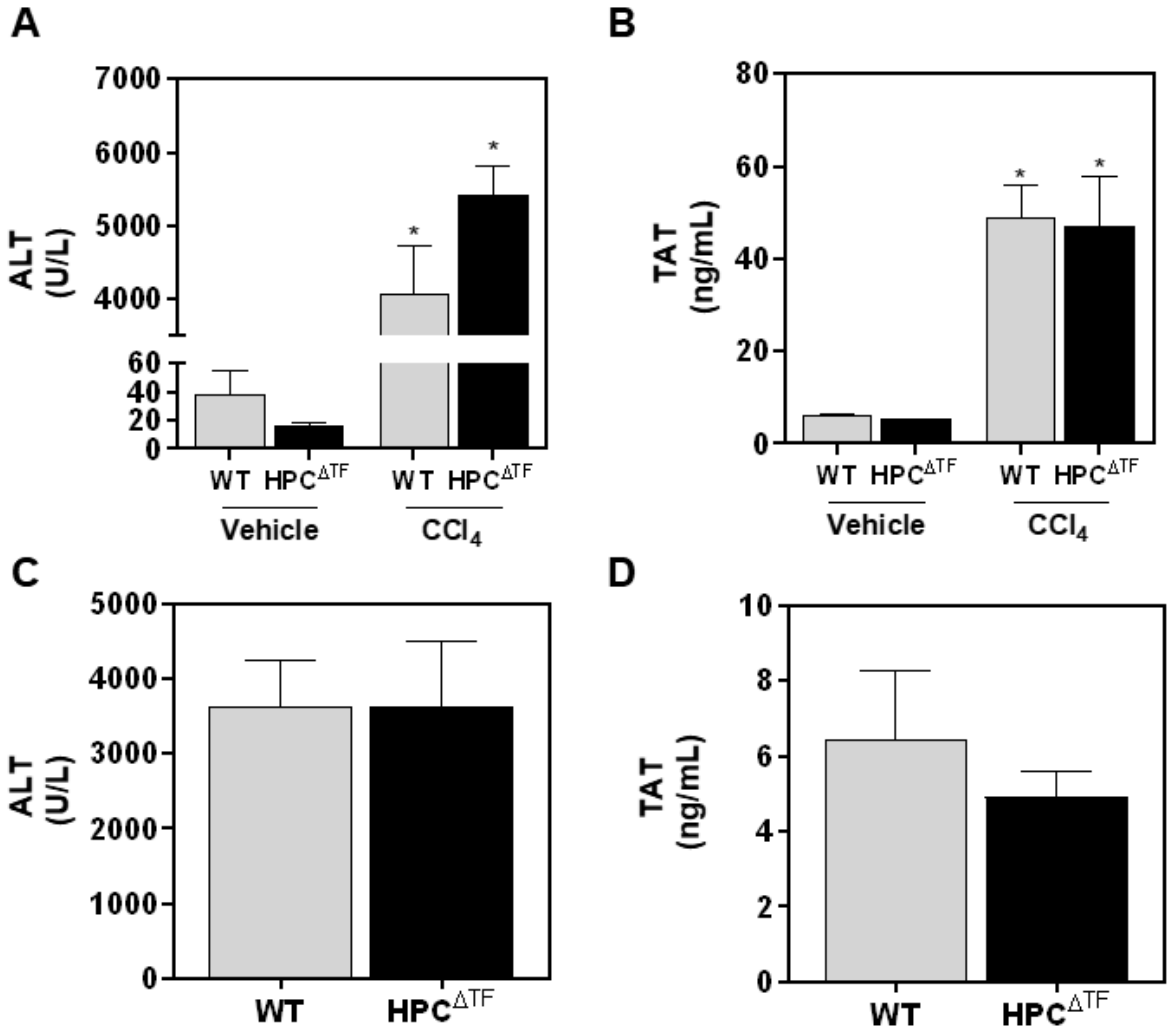

Figure 16. Deficiency in hepatocyte tissue factor had no effect on coagulation and injury after acute exposure to CCl_4. Wildtype and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$ mice) were given vehicle or carbon tetrachloride (CCl_4) for 6 or 24 hours. After 24-hour and 6-hour exposure, respectively, (A & C) alanine aminotransferase (ALT) activity and (B & D) thrombin anti-thrombin (TAT) were determined. Data are expressed as mean + SEM (6-hours, 3 mice per group; 24 hours, 3-9 mice per group). *P < 0.05 compared to vehicle treated wild-type mice.

Hepatocyte TF does not drive coagulation after chronic challenge with CCl_4

Chronic challenge with CCl_4 is often used to explore the mechanisms whereby coagulation affects disease progression (i.e., fibrosis). Because of this, we sought to determine the contribution of hepatocyte TF in activating coagulation after chronic CCl_4 challenge. First, we wanted to determine whether TF expression increases after chronic challenge with CCl_4. In agreement with prior findings, selective deletion of hepatocyte TF decreased TF mRNA in the liver (Figure 17A).[5] After chronic challenge with CCl_4, there was no significant increase in hepatocellular injury as measured by serum ALT compared to vehicle controls (Figure 17B). Additionally, we did not detect a significant increase in plasma TAT complexes after treatment with CCl_4 compared to vehicle controls (Figure 17C), making it challenging to determine the role of hepatocyte TF in coagulation after chronic CCl_4 challenge.

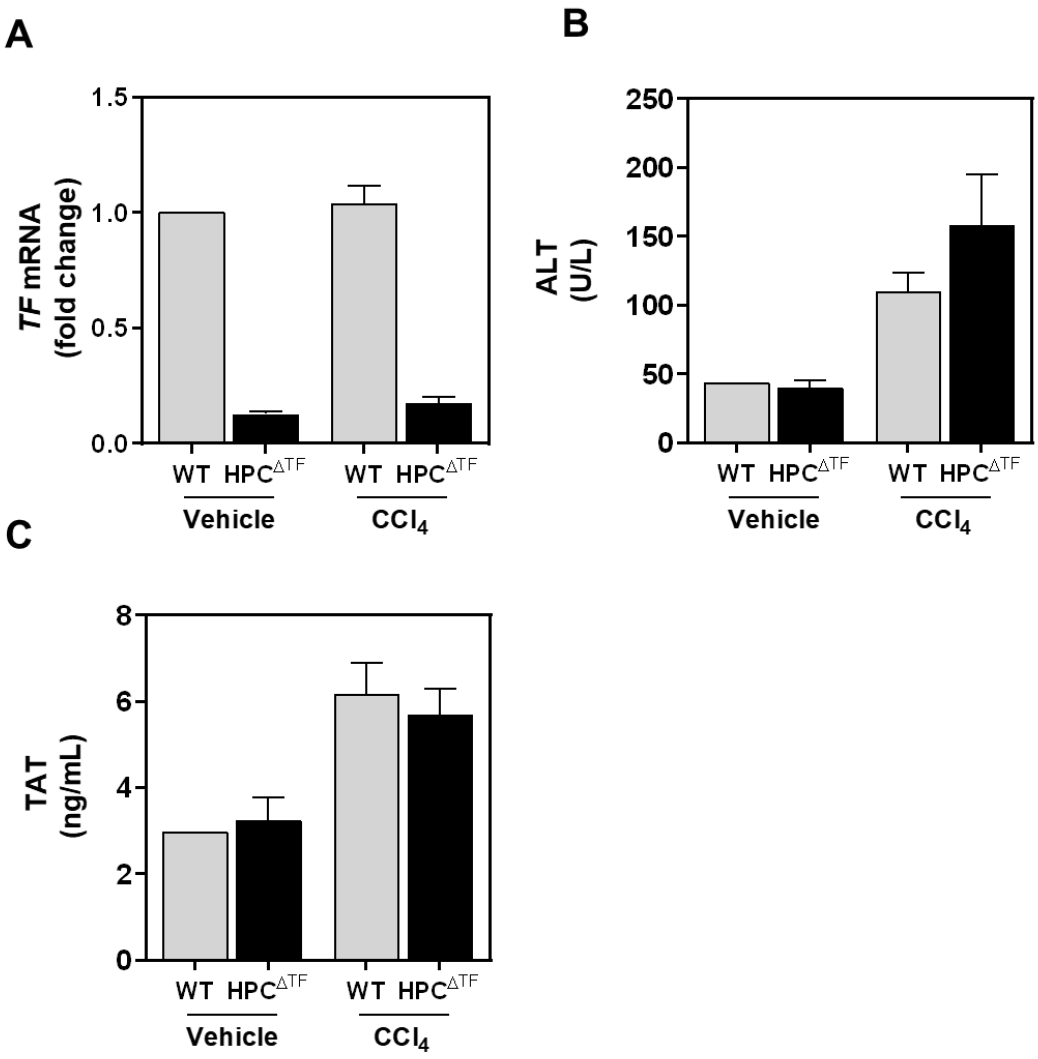

Figure 17. Deficiency in hepatocyte tissue factor had no effect on coagulation and injury after chronic exposure to CCl_4. Wildtype and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$) were given vehicle or carbon tetrachloride (CCl_4) for 6 weeks. (A) tissue factor (TF) expression, (B) serum alanine aminotransferase (ALT) activity, and (C) plasma thrombin anti-thrombin (TAT) were determined. Data are expressed as mean + SEM (2-13 mice per group).

Hepatocyte TF deficiency does not affect liver fibrosis induced by chronic CCl_4 challenge

Previous studies have identified potential pathways linking coagulation to the progression of liver fibrosis. [7, 8, 18, 19] Although there was an increase in *Col1a1* expression compared to vehicle controls, there was no difference in *Col1a1* expression between wild-type and mice with TF deficient hepatocytes (Figure 18A). In agreement with the increase of *Col1a1* expression, we observed an increase in the deposition of collagen within the liver after 6-week CCl_4 challenge (Figure 18B & C). However, there was no effect of hepatocyte TF deficiency on collagen deposition. Collectively, these results indicate that hepatocyte TF does not contribute to CCl_4-induced fibrosis.

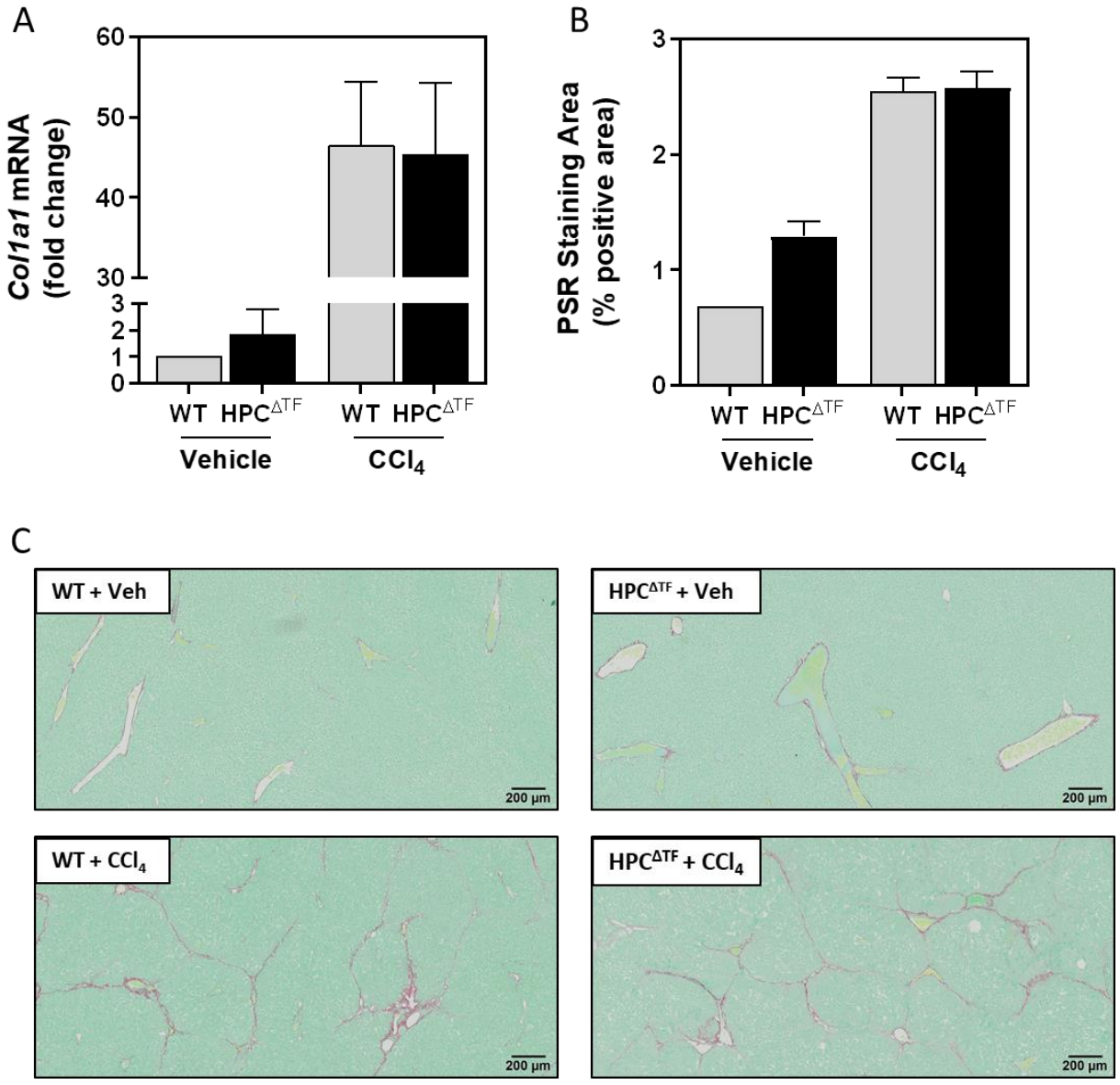

Figure 18. Deficiency in hepatocyte tissue factor had no effect on hepatic collagen deposition and expression. Wildtype and $TF^{flox/flox}$/AlbCre mice ($HPC^{\Delta TF}$) were given vehicle or carbon tetrachloride (CCl_4) for 6 weeks. (A) Gene induction of *Col1a1* in the liver was determined by qPCR. (B & C) Representative photomicrographs of picrosirius red-stained paraffin embedded liver sections viewed under polarized light (200x magnification), and positive area of picrosirius red was quantified. Data are expressed as mean + SEM (2-13 mice per group) *P < 0.05 compared to vehicle treated wild-type mice.

Discussion

Hepatocytes express the majority of TF within in liver, and as such, several studies have focused on the role of hepatocyte TF in activating coagulation in various settings of liver injury.[3, 5] However, it is unclear if hepatocyte TF is the universal trigger of coagulation in models of hepatotoxicity. Surprisingly, and in contrast to acetaminophen hepatotoxicity, hepatocyte TF deficiency did not reduce coagulation after acute CCl_4 challenge. Additionally, there was no easily discernable impact of hepatocyte TF deficiency on activation of coagulation, hepatocellular injury, or hepatic fibrosis after 6-week challenge with CCl_4. Overall, the results suggest that hepatocyte TF does not play a major role in activation of coagulation or the development of fibrosis during CCl_4-induced liver injury. This is a unique finding, because it suggests the involvement of hepatocyte TF in coagulation cascade activation is hepatotoxicant-specific.

Acetaminophen and CCl_4 hepatotoxicity share some similarity and are often broadly equated as similar models of acute hepatotoxicity. This led us to test the hypothesis that hepatocyte TF contributes to coagulation in CCl_4-mediated liver injury. However, there are key differences in the mechanisms of hepatotoxicity between these chemicals that might explain the observed difference in role of hepatocyte TF. Both acetaminophen and CCl_4 are metabolized in the liver, but a majority of the administered dose of CCl_4 is cleared through the airways.[20] This suggests the possibility that CCl4 activates TF expressed on different cell types. Following this, it is also possible that the dose of CCl_4 used may have activated coagulation in another tissue masking the contribution of liver TF to coagulation cascade activation. Of interest, after an acute hepatotoxic dose of acetaminophen, activation of coagulation occurred at the onset of hepatocyte toxicity.[4] In contrast, 6 hours after CCl_4 challenge, there was an obvious increase in hepatocellular injury not accompanied by a corresponding increase in TAT above typical control levels (i.e., TAT <8 ng/mL). Although this initial study had a small number of animals (n=3 per group), this suggests

that hepatotoxicity is not necessarily sufficient to increase coagulation after CCl_4 challenge. Notably, there was an increase in plasma TAT levels 24-hours after challenge with CCl_4 compared to vehicle challenge, but there was no difference between wild-type mice and mice with TF deficient hepatocytes. This suggests that that hepatocyte TF does not drive coagulation 24 hours after challenge with CCl_4.

Our rationale for this study was that as a primary TF-expressing cell type in the liver, if hepatocytes were injured by CCl_4 challenge, hepatocyte TF deficiency would be anticipated to reduce coagulation in experimental CCl_4-induced liver injury. Surprisingly, there was no effect of hepatocyte TF deficiency on coagulation or injury after CCl_4 challenge. It is possible that coagulation is driven by a TF-expressing non-parenchymal cell within the liver after challenge with CCl_4. For example, stellate cells are activated after challenge with acute and chronic CCl_4 and are largely responsible for depositing collagen within the liver.[21, 22] Interestingly, a prior study documented TF activity on activated stellate cells *in vitro*.[23] Another possibility is that TF-expressing inflammatory cells (e.g. monocytes) accumulate within the liver after challenge with CCl_4. Expression of TF can be induced on monocytes and macrophages,[24] and a prior study found that monocyte TF expression is increased in patients with chronic liver disease (i.e., cirrhosis).[25] Taken together, this suggests that inflammatory cell TF could be involved in activating coagulation after CCl_4 challenge. The results presented in our study suggest the possibility that a non-parenchymal source of TF could drive coagulation even in a model most associated with hepatocellular injury. Future studies identifying the role of non-parenchymal TF (e.g., monocyte, stellate cell) in the activation of coagulation after CCl_4 would be informative.

It is important to identify the mechanisms underlying the activation of coagulation in the injured liver, because coagulation has been shown to affect the progression of fibrosis.[26-28] Prior

studies have documented that the pharmacological inhibition of coagulation proteases (e.g., thrombin and FXa) attenuates liver pathology (e.g., hepatic collagen deposition).[7, 9] Although hepatocyte TF did not drive coagulation after acute challenge with CCl_4, it is possible that hepatocyte TF could participate in the development of fibrosis after chronic challenge with CCl_4 through a mechanism independent of coagulation. For example, a prior study documented that mice deficient in the cytoplasmic domain of TF, which disables multiple cell signaling functions of TF, had a reduction in liver fibrosis after chronic challenge with CCl_4.[29] Therefore, we sought to determine impact of hepatocyte TF deficiency on the development of fibrosis. In agreement with prior studies, chronic CCl_4 challenge increased collagen mRNA and subsequent collagen deposition in the livers of mice. Although there were challenges detecting an increase in the activation of coagulation, it is still clear that hepatocyte TF deficiency had no effect on fibrosis. Interestingly, a previous study found that hepatocyte TF drives coagulation after chronic bile duct ligation but did not affect the development of fibrosis.[3] Taken together, this suggests that hepatocyte TF contributes to coagulation in model-specific manner but does not contribute to the development of fibrosis after bile duct ligation or challenge with CCl_4.

When coagulation becomes activated, the serine protease thrombin is produced. Thrombin has a short half-life as it rapidly complexes with antithrombin to form TAT. Plasma TAT is often used as a biomarker for coagulation activity. However, one of the primary limitations of interpreting plasma TAT levels as a readout of coagulation activity is that TAT is measured systematically, and although it can reflect local changes in coagulation, it is difficult to distinguish an individual tissue's contribution to changes in TAT levels. Another limitation to using TAT as a biomarker is that TAT is cleared by hepatocytes, and hepatic clearance of proteins is disrupted during liver injury. [30-32] Therefore, in the context of liver injury, if TAT levels are increased, they

may remain elevated as clearance is impaired. However, we found that TAT was not elevated after chronic challenge with CCl_4. This might be explained by the time point at which samples were collected, 3 days after the final injection of CCl_4, which complicates interpretation as this gives the liver more time to clear TAT. We chose 72 hours, because we wanted to determine the effect of hepatocyte TF deficiency on fibrosis, and stable collagen formation occurs 72 hours after challenge with carbon tetrachloride. However, this time point may not have been ideal for capturing changes in TAT. Overall, TAT has limitations as a biomarker for coagulation, and in order to determine the role of hepatocyte TF in the activation of coagulation, a more tissue-specific biomarker of coagulation would be beneficial. Intrahepatic fibrin(ogen) deposition is often considered to be indicative of activation of coagulation within the liver.[2, 33] Notably, thrombin cleaves soluble fibrinogen into insoluble fibrin(ogen) that is then deposited in tissue. However, thrombin independent intrahepatic fibrin(ogen) deposition occurs after challenge with CCl_4. [15] To determine whether hepatocyte TF drives coagulation after challenge with CCl_4, the ideal biomarker would be liver-specific and quantifiable. Indeed, techniques exist that utilize fluorescent substrates of thrombin and *in vivo* imaging to determine whether coagulation is occurring within the liver.[34] Future studies should focus on techniques that can detect coagulation within the liver to determine the contribution of intrahepatic TF in activating coagulation after CCl_4-induced liver injury.

In summary, we found no difference in plasma TAT levels between wild-type mice and mice with TF deficient hepatocytes after acute challenge with CCl_4, suggesting hepatocyte TF does not drive coagulation after acute challenge with CCl_4. With the noted limitations of TAT as a biomarker of coagulation cascade activation, this observation challenges the notion that hepatocyte TF is the primary driver of coagulation in all settings of liver injury. Overall, these studies advance our knowledge of how coagulation is activated after challenge with CCl_4. Moreover, this study

highlights the importance of pursing the role of extrahepatic sources of TF during CCl_4-mediated injury.

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CHAPTER 5

Significance, Summary, and Future Directions

Significance

Strong evidence links the activation of coagulation to the progression of liver disease; however, there is little known about the mechanisms whereby intrahepatic coagulation becomes activated during liver disease. In this dissertation, I document bile acids as activators of TF:FVIIa, and define the mechanisms underlying this activation. This finding is significant as it is one of the first documented cases of a physiologically relevant activator of TF:FVIIa. Moreover, the studies in this dissertation provide a potential mechanism whereby coagulation is activated during liver disease. Additionally, these studies documented the novel finding that coagulation activation occurred prior to a time at which liver damage is detected by standard biomarkers. If further studies validated these findings and identified the precise mechanisms whereby bile acids activate coagulation *in vivo*, then a potentially translational finding from these studies is the possibility of bile acids as a therapeutic target to prevent pathologic coagulation during liver disease. Furthermore, the finding that subtle structural differences in bile acids (i.e., the presence of a hydroxyl group) affects the mechanism whereby they activate TF:FVIIa (i.e., direct activation and phosphatidylserine externalization) is particularly significant. This finding suggests that there may be critical structure-function relationships between bile acids and their potential to activate TF:FVIIa, which builds the foundation for more in-depth studies looking at the potential role of numerous bile acids in the activation of coagulation during liver disease.

Summary and future directions

The precise mechanisms of coagulation activation in liver disease are not known.. It is thought that tissue injury is coupled to the activation of coagulation, but this does not exclude the possibility of additional mechanisms independent of liver injury. Therefore, I wanted to determine if coagulation could be activated before evidence of hepatocellular injury in the context of bile duct ligation. In the bile duct ligation study (Chapter 2), a 30-minute timepoint was chosen for two reasons, 1.) biliary pressure builds within minutes simulating cholestasis[1, 2] and 2.) this pressure occurs before the onset of hepatocellular injury, which increases after 3 hours.[3] Remarkably, after a 30-minute bile duct ligation there was an increase in a biomarker of coagulation (i.e., thrombin-antithrombin; TAT) independent of an elevation of a biomarker of hepatocellular injury (i.e., alanine aminotransferase; ALT). This suggests that hepatocellular injury is not a requirement for the activation of coagulation and opens the possibility for another mechanism whereby coagulation is activated.

Hepatocyte TF has been shown to drive coagulation in models of liver injury.[4, 5] Using a strain of mouse characterized by TF deficient hepatocytes, researchers documented that hepatocyte TF drives coagulation after a 14 day bile duct ligation.[4] However, the potential for coagulation to be activated after a shorter time point (i.e., 30 minutes) was not known. Moreover, by identifying a hepatocyte TF-dependent mechanism at a shorter time point, it is possible to explore potential triggers of hepatocyte TF before the onset of injury. Therefore, I considered the possibility hepatocyte TF contributed to coagulation after a 30-minute bile duct ligation. I utilized mice expressing a conditional TF allele crossed with mice expressing *Cre* recombinase under control of the albumin promoter ($TF^{flox/flox}$/AlbCre; $HPC^{\Delta TF}$), which results in TF deficient hepatocytes. In agreement with the prior study documenting the role of hepatocyte TF in a 14-day bile duct ligation, it was found that after 30-minute bile duct ligation, there was a significant decrease in the levels

of TAT in the plasma of mice with TF deficient hepatocytes, which suggests that hepatocyte TF contributes to the activation of coagulation. However, one limitation to using $HPC^{\Delta TF}$ mice is that although this approach is liver specific, it is not hepatocyte specific.[6, 7] Indeed, it is possible that another TF expressing cell with the liver is injured, resulting in coagulation. Moreover, the marker injury used (i.e., alanine aminotransferase release) is specific for hepatocellular injury. Thus, it is difficult to determine from these results if another TF expressing cell within the liver is injured, which ultimately results in the activation of coagulation. Future studies could strengthen my initial finding by using an adeno-associated viral vector (AAV8) in which *Cre* recombinase is expressed under control of the thyroxine binding globulin promoter. Prior studies have demonstrated that this approach is highly hepatocyte specific.[8, 9] Preliminary studies indicate effective deletion of TF (>90%) from hepatocytes isolated from $TF^{flox/flox}$ mice inoculated with AAV8-Cre compared to mice inoculated with AAV8-GFP control vector. These studies would ensure that hepatocyte TF drives coagulation after a 30-minute bile duct ligation.

Although, hepatocyte TF contributed to coagulation after a 14-day bile duct ligation, there was little evidence for the trigger of TF activity. My bile duct ligation studies documented an increase in TAT and a concurrent increase in bile acids after a 30-minute bile duct ligation, which suggests a temporal connection between bile acids and the activation of coagulation. The next logical step to these studies is to determine the role of bile acids in activating coagulation after bile duct ligation. Bile duct ligation allows me to rapidly increase plasma concentrations of bile acids. If I were able to increase the available pool of bile acids within the gallbladder and associate this increase with increased TAT, then I would further support my findings that bile acids activate coagulation. Therefore, future studies would focus on altering the levels of bile acids to determine their effects on coagulation after bile duct ligation. Prior studies have shown that feeding cholic

acid (1%) increases the pool of available bile acids. [10, 11] By increasing the pool of available bile acids, I could test the hypothesis that increasing the total pool of bile acids further activates coagulation. I would anticipate higher levels of TAT in mice that were fed cholic acid versus mice fed normal diet after bile duct ligation. Furthermore, from this study, levels of individual bile acids can be measured and associated with changes in coagulation. This could allow me to differentiate bile acids based on their ability to activate coagulation, which could allow for the identification of key bile acids that are responsible for driving coagulation.

Additionally, I could use a pharmacologic approach to decrease the amount of bile acids by feeding the bile acid sequestrant, cholestyramine, to mice. Cholestyramine limits reabsorption of bile acids from the intestines, increasing excretion into the feces.[12, 13] A previous study documented that feeding cholestyramine to mice reduces bile acid concentrations in the bile by approximately 50%.[10] Future studies looking at the effect of depleting bile acids in the context of bile duct ligation would further support my hypothesis that bile acids activate coagulation. Future experiments would involve feeding a diet containing cholestyramine (0.2%) to mice before performing bile duct ligation surgery. After bile duct ligation, I would anticipate a lower concentration of plasma bile acids and subsequently lower plasma TAT levels versus mice fed a normal diet.

In addition to cholestyramine, I can use genetically modified mice that are deficient in critical enzymes for bile acid synthesis (*Cyp7a1* and *Cyp27a1*). Of interest, these mice have a significant reduction in liver and gallbladder concentrations of bile acids (~60% and 76% respectively).[14] Mice deficient in *Cyp7a1* and *Cyp27a1* would allow me to test the hypothesis that a reduction in the available pool of bile acids reduces the activation of coagulation after bile duct ligation. I would anticipate a reduction in plasma TAT levels in *Cyp7a1*$^{-/-}$, *Cyp27a1*$^{-/-}$, and double

knockouts (i.e., *Cyp7a1*$^{-/-}$/*Cyp27a1*$^{-/-}$) compared to wild-type comparators after bile duct ligation. Indeed, in preliminary studies performed at Rutgers by Dr. Bo Kong under the supervision of Dr. Grace Guo) *Cyp7a1*$^{-/-}$/*Cyp27a1*$^{-/-}$ mice and control mice underwent sham or bile duct ligation, and 30 minutes later plasma ALT activity, bile acids, and TAT levels were determined. Plasma TAT levels were significantly increased after BDL in wild-type mice alongside an increase in ALT activity. Interestingly, increases in both TAT and ALT were diminished in *Cyp7a1*$^{-/-}$/*Cyp27a1*$^{-/-}$ mice (not shown). This supports the hypothesis that bile acids increase coagulation. One challenge with interpreting this initial data was the observation that ALT levels increased 30 minutes after BDL, unlike our initial studies. This makes it challenging to determine whether the reduction in plasma TAT levels occurs due to a reduction in bile acids or secondary to a reduction in hepatocyte injury. Furthermore, one limitation to using these mice is that they still have bile acids, albeit at far lower levels compared to wild-type mice. However, further studies using mice deficiency in single bile acid synthesis enzymes (*Cyp7a1* and *Cyp27a1*) compared to the double knockouts would be beneficial to associate a decrease in chenodeoxycholic acid derived versus cholic acid derived bile acids to a decrease in the activation of coagulation after bile duct ligation.

Based on the *in vivo* data suggesting bile acids activate coagulation, I sought to determine the effect of bile acids on hepatocyte TF:FVIIa. Pathologically relevant concentrations of glycochenodeoxycholic acid increased hepatocyte TF:FVIIa activity without inducing cellular injury. Next, I sought to determine the mechanism whereby bile acids activate TF:FVIIa, considering the possibility that bile acids directly activate the TF:FVIIa complex. From these studies, it was documented that pathologically relevant concentrations of both glycochenodeoxycholic acid and taurochenodeoxycholic acid—a predominantly murine bile acid—increased the activity of relipidated and soluble TF:FVIIa. It is entirely possible that bile

acids increase TF:FVIIa activity as a byproduct of their detergent like structure. To address this possibility, I used a detergent structurally similar to taurochenodeoxycholic acid, CHAPS,[15] and determined the effect of CHAPS on relipidated and soluble TF:FVIIa activity. Surprisingly, there was no increase in TF:FVIIa activity after treatment with CHAPS, which suggests that the bile acid-mediated effect is not driven by the detergent-like characteristics of bile acids.

My initial findings that glycochenodeoxycholic and taurochenodeoxycholic acid increased TF:FVIIa activity led me to pursue structurally different bile acids. Notably, there are two primary bile acids, chenodeoxycholic acid and cholic acid, which differ by the presence of a hydroxyl group on cholic acid. Glycochenodeoxycholic and taurochenodeoxycholic acid are derived from chenodeoxycholic acid whereas taurocholic acid is the taurine conjugate of cholic acid. Interestingly, I found that taurocholic acid increased hepatocyte TF:FVIIa activity but did not directly activate relipidated TF:FVIIa. Indeed, this was in contrast to taurochenodeoxycholic acid, which increased relipidated TF:FVIIa activity. This suggests that subtle structural changes to the bile acid (i.e., presence of hydroxyl group on taurocholic acid) can affect the mechanism whereby they increase TF:FVIIa activity. It was also found that taurocholic acid had a lasting effect on TF:FVIIa activity as its removal did not abolish the increase in TF:FVIIa activity. Bile acids have been shown to activate several intracellular pathways,[16] many of which have been shown to modify TF expression and activity.[17] However, it was found that pretreatment with various pharmacological inhibitors for intracellular pathways had no effect on the taurocholic acid-mediated increase in hepatocyte TF:FVIIa activity. Next, we explored the possibility that taurocholic acid was affecting the composition of the cellular membrane, given the connection between changes in this cell compartment and TF:FVIIa activity.. To explore this possibility, I determined the effect of taurocholic acid on the externalization of phosphatidylserine. Surprisingly,

it was discovered that taurocholic acid caused non-apoptotic externalization of phosphatidylserine, which led to an increase in TF:FVIIa activity. This suggested that the structure of the bile acids could affect the mechanisms whereby they increase TF:FVIIa activity. In a proof-of-principle study, the phosphatidylserine binding protein, lactadherin, reduced the taurocholic acid-mediated increase in hepatocyte TF activity. This finding was significant, because this suggests that subtle structural differences (i.e., the presence or absence of a hydroxyl group) affects the ability for bile acids to increase TF:FVIIa procoagulant activity. This could have tremendous implications on disease, and future studies looking into the association of individual bile acids to disease development would be beneficial.

One limitation to the studies involving bile acids was that I used a predominantly human bile acid (i.e., glycochenodeoxycholic acid) on primary mouse hepatocytes. Prior studies show that primary rat hepatocytes undergo apoptosis after treatment with human bile acids (e.g., glycochenodeoxycholic acid), whereas human hepatocytes do not undergo apoptosis.[18] From my studies, glycochenodeoxycholic acid treatment did not induce cytotoxicity, but it is possible that the direct effect of glycochenodeoxycholic acid on mouse hepatocyte TF:FVIIa activity does not translate to human hepatocytes. It is possible that glycochenodeoxycholic functions the same as taurocholic (i.e., externalize phosphatidylserine), but this similarity was not observed because the wrong cell type was used (i.e., mouse hepatocytes). Future studies could strengthen my findings by using predominantly human bile acids (i.e., glycine conjugated) on primary human hepatocytes. One challenge to using primary human hepatocytes is that they are often cryopreserved, which could complicate interpretations as freeze-thawing increases TF:FVIIa activity. In addition to using human primary hepatocytes, it would be beneficial to determine the role of bile acids in activating TF present on cell lines derived from humans, such as HepG2 cells that have inducible

TF activity *in vitro*.[19] Overall, using cell lines derived from humans could validate my finding that glycochenodeoxycholic acid directly activates TF:FVIIa.

Although I discovered that taurocholic acid externalized phosphatidylserine, the precise mechanism whereby this externalization occurs is not known. Normally phosphatidylserine levels are low in the outer leaflet of the membrane where TF:FVIIa is present. Phosphatidylserine levels are kept low by active flippase and inactive scramblase enzymes. Of importance, increased levels of intracellular calcium lead to the inactivation of flippase and the activation of scramblase, which results in a net increase in externalized phosphatidylserine.[20-22] Therefore, I could utilize intracellular calcium chelators (e.g., BAPTA-AM) to determine the role of calcium in the bile acid-mediated increase in phosphatidylserine externalization The outcome of this experiment would document the role of intracellular calcium in the bile acid-mediated increase in TF:FVIIa activity.

Currently, it is unclear if the bile acids need to enter the cell to increase hepatocyte TF:FVIIa activity. Bile acids are taken up by hepatocytes through the Na-taurocholate cotransporting polypeptide (NTCP). In a preliminary study, I found that treating primary mouse hepatocytes with a sodium free media containing taurocholic acid did not affect the taurocholic acid-mediated increase in TF:FVIIa activity. This suggests that hepatocellular uptake of taurocholic acid is not required for the increase in hepatocyte TF:FVIIa activity. I can expand on my initial findings by further blocking hepatocellular uptake of bile acids. A prior study found that biotinylated bile acids (e.g., cholic acid) inhibited the uptake of taurocholic acid by blocking bile acid transporters.[23] Utilizing these bile acids, I would be able to determine whether hepatocellular uptake of taurocholic acid is required to increase TF:FVIIa activity.

Although my *in vitro* results with glycochenodeoxycholic acid suggests directly interacts with the TF:FVIIa complex, these studies do not reveal interaction sites on the complex. My data

suggest that modest amounts of phosphatidylserine (1%) limit the effect of bile acids on relipidated TF. From my studies, I found that adding 1% phosphatidylserine into vesicles significantly increased relipidated TF:FVIIa activity. Moreover, adding bile acids (i.e., glycochenodeoxycholic and taurochenodeoxycholic acid) had no effect on the activity of TF:FVIIa relipidated in 1% PS containing vesicles, which suggests the possibility that bile acids interact at sites where phosphatidylserine interacts. Interestingly, there are TF mutants that lack the binding site for phosphatidylserine.[24] Notably, when a serine-163 on TF is mutated to an alanine (S163A), there is a significant reduction of TF:FVIIa activity. Future studies would explore the hypothesis that bile acids can substitute for phosphatidylserine. In these studies, TF mutants lacking phosphatidylserine binding sites would be relipidated and I would determine the ability for bile acids to increase TF:FVIIa activity. Moreover, prior studies suggest that phosphatidylethanolamine can synergize with phosphatidylserine, increasing its ability to activate TF:FVIIa.[25, 26] Future studies looking at the capacity for phosphatidylethanolamine to increase glycochenodeoxycholic acid-mediated effect would be mechanistically informative. The anticipated results from these studies would reveal if bile acids could substitute for phosphatidylserine.

In parallel with experiments looking at the potential for bile acids to substitute for phosphatidylserine, I can use surface plasmon resonance (SPR) to further determine the role of bile acids in activating TF:FVIIa. SPR involves the amine-coupling of a protein (the ligand) to a microchip and flowing a compound (the analyte) over to determine the binding kinetics. Prior studies have used SPR to identify the binding kinetics of FVIIa to TF.[27, 28] Future studies could utilize SPR to determine whether glycochenodeoxycholic acid can interact with relipidated TF. Furthermore, SPR can be used to determine whether the PS-binding domains present on TF are

required for GCDCA binding. These experiments would utilize TF lacking the phosphatidylserine domain (S163A). Furthermore, it is possible that glycochenodeoxycholic acid will increase the binding of FX to the TF:FVIIa complex. These studies can be performed by amine-coupling FX to the chip and flowing various concentrations of bile acids and relipidated TF:FVIIa over the chip. For these studies, it will be necessary to include a direct FXa inhibitor (i.e., rivaroxaban) to prevent conversion of FX to FXa. This study would allow me to determine the role of bile acids in increasing the binding of FX to the TF:FVIIa complex.

The use of a single bile acid was essential for defining the different mechanisms whereby bile acids increase TF:FVIIa activity. However, using a single bile acid limits the *in vivo* relevance as numerous bile acids increase in plasma and liver parenchyma during liver disease. There are several approaches to address this, the first being treating hepatocytes with bile. In these experiments, I could harvest bile from mice and treat primary mouse hepatocytes with various concentrations of bile. Another approach is using a solution containing relevant concentrations of bile acids present in the plasma of patients with liver disease. Moreover, this would allow the control of bile acid levels and can be used to find an association between individual bile acids and their ability to increase TF:FVIIa activity. If an association was found between the occurrence of activation of coagulation and certain bile acid species, this would open the door to the possibility of modulating specific species of bile acids to prevent pathologic coagulation.

In this dissertation, I focused on hepatocyte TF, as prior studies found that hepatocyte TF played a critical role in activating coagulation.[4, 5] However, the role of hepatocyte TF has not been broadly investigated in the context of CCl_4-induced hepatotoxicity. Therefore, I determined the role of hepatocyte TF in the activation of coagulation after challenge with carbon tetrachloride (CCl_4) (chapter 4). My data suggest that 6 hours after challenge with CCl_4, there is a profound

increase in liver damage without an increase in TAT. This result was surprising, as injury is often coupled with activation of coagulation. One challenge to the interpretation of these results was that this study involved a small number of animals (n = 3). It is possible that injury to another cell type has occurred, which may not be detectable by measuring alanine aminotransferase activity.[29] Furthermore, , 24 hours after challenge with CCl_4 there was no effect of hepatocyte TF deficiency on injury nor coagulation. This suggests that hepatocyte TF does not contribute to coagulation in the CCl_4-induced liver injury model. A plausible explanation is that another TF-expressing cell type is driving coagulation. I could determine the role of myeloid-derived TF after CCl_4 by using a mouse expressing Cre under control of the M Lysozyme promoter crossed with $TF^{flox/flox}$ mice. There is a LysMCre strain that would allow for a myeloid cell lineage specific knockout of TF. These mice will allow me to determine if myeloid cell lineage TF is driving coagulation rather than hepatocyte TF. In addition to determining the role of monocyte TF, I can test whether stellate cell TF is responsible for activating coagulation. I could cross $TF^{flox/flox}$ mice with mice expressing *Cre* recombinase controlled by the lecithin retinol acyltransferase, which would generate stellate cells deficient in TF. This would allow me to determine the role of other sources of TF in activating coagulation after CCl_4.

Next, I wanted to determine the effect of TF deficiency on coagulation after chronic challenge with CCl_4. This is important to study, because activation of coagulation has been shown to affect the progression of liver injury. Although the acute studies did not reveal a link between hepatocyte TF and the activation of coagulation, I sought to determine the contribution of hepatocyte TF to the development of fibrosis. Notably, chronic challenge with CCl_4 induced collagen gene expression and collagen deposition within the liver; however, there was no difference between wild-type and mice with TF deficient hepatocytes. Moreover, there was no

increase in TAT after chronic challenge. One limitation to interpreting the effect of TF deficiency on the activation of coagulation was a lack of an increase in TAT, which was used as a marker of coagulation. There are several possible explanations, the first being the time after the last injection. It is entirely possible that TAT did increase, but I collected samples 3 days after the final injection and the increase was missed. In future studies, samples could be collected at various times after the final injection to determine the precise time course of TAT elevation after CCl_4 challenge.

Future studies should also utilize a more organ-specific marker of activated coagulation, as TAT cannot always reflect an individual tissue's contribution to the activation of coagulation. In order to determine the role of hepatocyte TF, tissue specific markers of coagulation should be used. A prior study utilized a non-invasive procedure to detect protease activity within rodents using the IVIS imaging system.[32] In this study, mice that underwent $FeCl_3$-induced thrombosis were given a fluorescein-conjugated probe, which allowed researchers to detect thrombin activity. Moreover, this method would allow for the detection of coagulation activity within individual organs (e.g., liver and lung). For example, another study using intravital imaging to detect thrombin activity within the liver. [33] For acute studies involving CCl_4, 24 hours after CCl_4 challenge may work, as TAT complexes were elevated. Using a thrombin probe would beneficial for detecting thrombin activity in the chronic samples, because we may have missed the change in plasma TAT. Future studies would focus on utilizing a thrombin probe to detect changes in intravascular coagulation. Mice would be injected in CCl_4 for 6 weeks, and then a thrombin probe would be introduced into the circulation. This thrombin probe would then be detected via intravital imaging of the liver, which would allow for a more sensitive detection of thrombin activity. In summary, using methods that allow for more sensitive detection of thrombin activity would allow me to determine the role of hepatocyte TF in activating coagulation after CCl_4.

In the context of the studies presented in chapter 4, there was no difference in TAT levels between mice deficient in hepatocyte TF and wild-type mice, which suggests that hepatocyte TF does not drive coagulation after challenge with CCl_4. This result was surprising as hepatocyte TF played a critical role in activating coagulation in other models of liver injury (e.g., acetaminophen, bile duct ligation). This suggests that another cellular source may be driving coagulation after challenge with CCl_4, and future studies would address this by exploring the possibility that non-parenchymal cells activate coagulation after challenge with CCl_4. Of interest, there was a difficulty in detecting an increase in plasma TAT after chronic challenge. A potential solution to this would be to detect thrombin activity within the liver using intravital imaging, which would allow for more sensitive detection of thrombin activity.

Overall, the studies in this dissertation address the potential mechanisms underlying the activation of TF:FVIIa in the context of liver disease. This is critical information as there is little known about how coagulation becomes activated during liver disease, which limits potential therapeutics. The studies from chapter 2 suggest the possibility for bile acids to drive TF:FVIIa activity. Furthermore, the studies in chapter 3 expand on the initial finding that bile acids drive TF:FVIIa activity by identifying different mechanisms whereby this activation occurs. Together, these studies have advanced our understanding of how pathological mediators that increase (e.g., bile acids) during liver disease can ultimately drive coagulation. Interestingly, the studies in chapter 2 suggest this activation is driven by hepatocyte TF. However, the studies in chapter 4 suggest the possibility that hepatocyte TF is not solely responsible for activating coagulation during liver injury. Indeed, my findings suggest that another cell type can activate coagulation during injury, and this finding challenged the previous notion that hepatocyte TF is solely

responsible for driving coagulation. In summary, this work has expanded our knowledge on the fundamentals of how the coagulation cascade becomes activated during liver disease.

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