Thrombin inhibition with dabigatran protects against high fat diet-induced fatty liver disease in mice

Anna K. Kopec, Nikita Joshi, Keara L. Towery, Karen M. Kassel, Bradley P. Sullivan, Matthew J. Flick, and James P. Luyendyk

Department of Pathobiology & Diagnostic Investigation (AKK, KLT, JPL), Department of Pharmacology & Toxicology (NJ), Michigan State University, East Lansing, MI 48824; Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160 (KMK, BPS); Cancer and Blood Diseases Institute, Division of Experimental Hematology and Cancer Biology, Cincinnati Children’s Hospital, Cincinnati, OH 45229 (MJF)

Current affiliation: Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence Kansas 66047 (BPS)
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Contact Information for Corresponding Author:

James P. Luyendyk, Ph.D.
Pathobiology & Diagnostic Investigation
Michigan State University
253 Food Safety and Toxicology Building
1129 Farm Lane
East Lansing, MI 48824
517-884-2057
517-432-2310 (fax)
E-mail: luyendyk@cvm.msu.edu

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Abbreviations: Non-alcoholic fatty liver disease (NAFLD); Non-alcoholic steatohepatitis (NASH); Dabigatran etexilate (DE); High fat diet (HFD); Control diet (CD); Protease activated receptor 1 (PAR-1); Alanine aminotransferase (ALT); Thrombin-antithrombin (TAT); Farnesoid
X receptor (FXR); G protein-coupled bile acid receptor 1 (TGR5); Cluster of differentiation 36 (CD36); Tumor necrosis factor (TNF); Chemokine (C-C motif) ligand 2/Monocyte chemoattractant protein 1 (CCL2/MCP-1), Stearoyl-CoA desaturase 1 (SCD1); Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (CIDEA); Peroxisome proliferator-activated receptor gamma (PPARγ); Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1); Cytochrome P450, family 8, subfamily B, polypeptide 1 (CYP8B1); Cytochrome P450, family 7, subfamily B, polypeptide 1 (CYP7B1); Cytochrome P450, family 39, subfamily A, polypeptide 1 (CYP39A1); Low-density lipoprotein (LDL); Low-density lipoprotein receptor (LDLR); Atrial fibrillation (A-FIB); Apolipoprotein B (APOB); Fatty acid synthase (FASN); Acyl coenzyme A oxidase 1 (ACOX1); Acetyl coenzyme A carboxylase alpha (ACACA).
ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of obesity and metabolic syndrome. Robust coagulation cascade activation is common in obese patients with NAFLD. We identified a critical temporal relationship between thrombin generation and the manifestation of hepatic steatosis, inflammation and injury in C57BL/6J mice fed a high fat diet (HFD) for 1, 2 and 3 months. Mice fed a HFD exhibited dramatic increases in hepatocellular injury and inflammation over time. Hepatic fibrin deposition preceded an increase in serum alanine aminotransferase, and the most dramatic changes in liver histopathology occurred in conjunction with a detectable increase in plasma thrombin-antithrombin levels at 3 months. To directly determine whether thrombin activity promotes NAFLD pathogenesis, mice were fed a HFD and simultaneously treated with the direct thrombin inhibitor dabigatran etexilate for 3 months. Notably, dabigatran treatment significantly reduced hepatic fibrin deposition, hepatic inflammation, hepatocellular injury and steatosis in mice fed a HFD. Of interest, dabigatran treatment also significantly attenuated HFD-induced body weight gain. Gene expression analysis suggested that thrombin potentially drives NAFLD pathogenesis by altering the expression of genes associated with lipid metabolism and bile acid synthesis. Collectively, the results suggest that thrombin activity is central to HFD-induced body weight gain, liver injury and inflammation, and provide the proof-of-principle evidence that pharmacological thrombin inhibition could be effective in limiting NAFLD and associated pathologies.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common feature of obesity that increases the risk of developing cardiovascular disease and Type II diabetes (Targher et al., 2010a). Atypical accumulation of triglycerides within hepatocytes occurs as a consequence of unbalanced lipid metabolism, which manifests histologically as micro- and macrovesicular hepatic steatosis. Hepatic inflammation accompanying the NAFLD spectrum varies in severity, and can progress to severe steatohepatitis (NASH) and fibrosis, a leading cause of liver-related morbidity. Hepatic inflammation and injury dramatically increase the risk of cardiovascular disease, the leading cause of morbidity and mortality in patients with NAFLD/NASH (Targher et al., 2005; Hotamisligil, 2006; Targher et al., 2010a; Ndumele et al., 2011). Despite the identified relationship between liver inflammation and poor patient outcomes, the precise triggers of disease-amplifying processes, such as local inflammatory cell activation, are not completely understood.

Profound activation of the blood coagulation cascade is evident in patients with obesity and NAFLD, as indicated by increased plasma levels of thrombin, a serine protease (Ay et al., 2010; Beijers et al., 2010; Fritsch et al., 2010; Semeraro et al., 2012). Similarly, coagulation is evident in mouse models of NAFLD/NASH (Kassel et al., 2010; Luyendyk et al., 2010; Kassel et al., 2011; Owens et al., 2012). Recent studies indicate that the coagulation cascade activation contributes to obesity-associated sequelae, including fatty liver disease (Kassel et al., 2011; Kassel et al., 2012; Owens et al., 2012). Studies in mice suggest that procoagulant and signaling functions of tissue factor contribute to high fat diet (HFD)-induced obesity and fatty liver disease (Kassel et al., 2011; Owens et al., 2012). Mice lacking the thrombin receptor, protease activated receptor (PAR-1) are protected from HFD-induced NAFLD, despite similar body weight gain...
(Kassel et al., 2011). However, the broader role of thrombin activity including the relationship between clotting function and HFD-induced NAFLD has not been completely addressed.

In this study, we determined the temporal connection between coagulation cascade activation relative to the pathogenesis of systemic and hepatic features of HFD-induced NAFLD in mice. Moreover, we tested the hypothesis that prophylactic administration of the FDA-approved orally bioavailable thrombin inhibitor, dabigatran etexilate, would inhibit HFD-induced obesity and NAFLD.
MATERIALS AND METHODS

Mice and diets: Wild-type male C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were between 6 and 7 weeks of age at the start of each study. For the time course study mice were fed a control diet [AIN-93M (10% kcal from fat); Dyets Inc., Bethlehem, PA] or a HFD [Diet #100244 (40% kcal from milk fat); Dyets Inc.] for 1, 2 or 3 months. For the thrombin inhibitor study, mice were fed the same HFD formulated to contain the pulverized content of dabigatran etexilate capsules such that drug content was 10 g/kg for 3 months. An identical diet without drug material was fed for comparison. To avoid the challenge associated with bitter taste of the drug, both diets contained non-nutritive peanut flavoring and peanut butter (2 g/kg and 10 g/kg, respectively). After one week of acclimation, food intake for each cage and body weights of the mice were measured weekly. Animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care approved facilities at either the University of Kansas Medical Center or Michigan State University. Mice were housed at an ambient temperature of ~22±2°C with alternating 12 hour light/dark cycles and provided free access to purified drinking water. All animal procedures were approved by the University of Kansas Medical Center and/or Michigan State University Institutional Animal Care and Use Committees.

Sample collection: Food was removed overnight prior to sample collection. Mice were anesthetized using isoflurane and blood was collected from the caudal vena cava into a syringe containing sodium citrate (0.38% final) for collection of plasma and into an empty syringe for collection of serum. The liver was excised and washed in phosphate buffered saline. A small section (~20 mg) of the right medial lobe was collected for RNA isolation and snap frozen in
liquid nitrogen. The remainder of the right medial lobe was affixed to a cork using optimal cutting temperature compound (Fisher Scientific, Pittsburgh, PA) and immersed for approximately 3 minutes in liquid nitrogen-chilled 2-methylbutane (Fisher Scientific). Multiple sections from the left lateral lobe were fixed in 10% neutral-buffered formalin for approximately 48 hours and then embedded in paraffin. The remaining liver was snap frozen in liquid nitrogen.

**Serum alanine aminotransferase and plasma thrombin-antithrombin determination:** Blood samples were spun at 4000 x g for 10 minutes or at 10000 x g for 2 minutes for collection of plasma or serum, respectively. Serum alanine aminotransferase (ALT) activity was measured using a commercially available reagent (Infinity ALT/GPT, Thermo Fisher, Waltham, MA). Plasma thrombin-antithrombin (TAT) levels were determined using a commercial enzyme-linked immunosorbent assay kit (Enzygnost TAT micro, Siemens Healthcare Diagnostics, Deerfield, IL). For each assay, data were collected using an Infinite M200 plate reader (Tecan, Durham, NC).

**Histopathology and immunohistochemistry:** Paraffin-embedded livers were sectioned at 5 µm and stained with hematoxylin and eosin by the Michigan State University (MSU) Investigative HistoPathology Laboratory, a division of Human Pathology. At least 2 to 3 sections of liver from the left lateral lobe from each animal were evaluated by light microscopy to qualitatively determine the severity of micro- and macrovesicular steatosis. The average number of inflammatory foci (>5 clustered mixed inflammatory cells) in each 100X field was determined for all sections in each liver sample.
Paraffin-embedded sections were stained for insoluble fibrin using a polyclonal rabbit anti-mouse fibrin antibody, as described previously (Flick et al., 2007), with alkaline phosphatase conjugated anti-rabbit IgG (H+L) (Sigma, St. Louis, MO) used with Fast Red detection system (Sigma). Macrophage staining was performed by the MSU Histopathology Laboratory. Briefly, paraffin-embedded livers were sectioned at 4 µm, deparaffinized and subjected to enzymatic epitope retrieval using 0.03% Pronase E (Sigma). Following blocking with rabbit serum, slides were incubated with rat anti-mouse F4/80 antibody (clone CI:A3-1, AbD Serotec, Raleigh, NC) at 1:100 dilution in normal antibody diluent (NAD, Scytek Laboratories, Logan, UT) for 60 minutes. Tissues were then incubated with biotinylated rabbit anti-rat IgG (H+L), mouse absorbed (Vector Laboratories, Burlingame, CA), antibody at 10 µg/ml in NAD and incubated for 30 minutes, followed by R.T.U. Vector Elite Peroxidase Reagent (Vector Laboratories) for 30 minutes. Slides were developed utilizing Vector NovaRED kit (Vector Laboratories) peroxidase chromogen incubation for 15 minutes followed by counterstain in Gill Hematoxylin (Thermo Fisher) for 30 seconds, differentiation, and dehydration, clearing and mounting with Permount mounting media.

**Determination of triglycerides and cholesterol in serum and liver and plasma glucose and insulin levels:** Serum triglyceride and cholesterol levels were determined using Triglycerides GPO Liquid and Cholesterol Liquid reagent sets (Pointe Scientific Inc., Canton, MI), respectively according to the manufacturer’s protocol. For hepatic sample analyses, lipids were extracted from 100 mg frozen livers as described previously (Luyendyk et al., 2010). Plasma insulin was determined using a commercial ultrasensitive insulin ELISA (Alpco, Salem, NH) and
plasma glucose was measured using commercial reagent (Pointe Scientific Inc.) according to the manufacturer protocols.

**RNA isolation, cDNA synthesis and quantitative real-time PCR:** RNA isolation, cDNA synthesis and quantitative real-time PCR were performed as described previously (Kassel et al., 2011; Kassel et al., 2012). Gene expression was normalized to *Hprt* levels. A complete list of all gene names and primer sequences are provided in *Supplementary Table 1*.

**Statistical analyses:** Comparison of 2 groups was performed using Student’s *t*-test. Comparison of 3 or more groups was performed using two-way analysis of variance and Student-Newman-Keuls *post-hoc* test. Data were considered significant when the p-value was less than 0.05.
RESULTS

HFD-induced systemic and hepatic coagulation activity track with the severity of hepatic steatosis and inflammation as well as body weight gain, liver weight and lipid profiles. To determine the temporal relationship between the development of fatty liver disease and evidence of local coagulation system activity, mice were fed either a CD or HFD for 3 months with subsets of animals taken at monthly intervals for analysis. Liver histology in mice fed a CD at each time was similar and unremarkable. Minimal microvesicular steatosis was evident in livers of mice fed a HFD for 1 month (Figure 1A). Widespread microvesicular steatosis and infrequent macrovesicular steatosis was evident in livers of mice fed a HFD for 2 months (Figure 1A); whereas marked pericentral mixed macro- and microvesicular steatosis was observed in livers of mice fed a HFD for 3 months (Figure 1A). At the same time points and with samples from the same animals in which liver histology was evaluated, plasma thrombin-antithrombin (TAT) levels, a stable marker of thrombin generation and robust indicator of coagulation activity, as well as hepatic fibrin deposition, were measured. Little to no hepatic fibrin deposition (red staining) was observed in mice fed a CD (Figure 1B). In contrast, fibrin deposition was readily evident in mice fed a HFD for 2 and 3 months, and was generally localized in the liver sinusoids (Figure 1B). Compared to mice fed a CD, plasma TAT levels increased significantly in mice fed a HFD only at 3 months (Figure 1C). Together, these results suggest that local hepatic coagulation, as indicated by fibrin deposition, precedes a detectable increase in plasma TAT. Moreover, there is a temporal connection between HFD-induced fatty liver progression and activation of coagulation.

Hepatic inflammation, marked by an increase in foci of mixed-inflammatory cell infiltration (Figure 2A) alongside marked elevations in serum ALT activity (Figure 2B) was
present in mice fed a HFD for 3 months. Immunohistochemical analysis confirmed distinct macrophage accumulation at 3 months (Figure 2C), corresponding with increased gene expression of proinflammatory cytokines, \( \text{Ccl2} \) and \( \text{Tnf} \) at this time point (Figure 2D). The increased hepatic inflammation and hepatic injury paralleled the robust increase in hepatic steatosis and coagulation activity at 3 months.

As expected, the development of hepatic steatosis, coagulation activity, and inflammation in mice fed a HFD coincided with significantly increased body weight, relative to mice fed a CD. The largest weight gain occurred between 2 and 3 months (Table 1). Whole liver weight and relative liver weight increased significantly at 2 and 3 months in mice fed a HFD (Table 1). Compared to mice fed a CD, serum triglyceride levels transiently decreased at 1 month in mice fed a HFD but were otherwise unaffected (Table 1). In contrast, liver triglyceride levels increased at both 2 and 3 months in HFD-fed mice (Table 1). A time-dependent increase in serum cholesterol occurred in HFD-fed mice (Table 1). In contrast, hepatic cholesterol levels were similarly elevated at each time point (Table 1). Plasma glucose and plasma insulin levels showed a time-dependent increase in animals fed a HFD, with significant increases at 2 and 3 months (Table 2).

**Pharmacologic thrombin inhibition protects against HFD-induced NAFLD and weight gain in mice.** Our time course study indicated that increased cellular inflammation, severe steatosis and elevated serum ALT levels were closely preceded by hepatic fibrin deposition and tightly linked to significantly increased plasma TAT levels. This suggested a direct connection between thrombin activity and fatty liver pathogenesis in HFD-fed mice. Accordingly, we hypothesized that administration of a direct thrombin inhibitor would reduce primary features of HFD-induced
fatty liver disease in mice. To test this hypothesis we utilized dabigatran etexilate, a novel, orally bioavailable direct thrombin inhibitor, utilized clinically to prevent thrombosis in non-valvular atrial fibrillation (A-fib) (Hankey and Eikelboom, 2011). Following treatment with dabigatran, liver histopathology revealed a near complete prevention of steatosis relative to mice fed only a HFD (Figure 3A). Thrombin inhibition with dabigatran significantly reduced serum ALT levels (Figure 3B), focal hepatic mixed inflammatory cell infiltration (Figure 3C), macrophage accumulation (Figure 3D) and hepatic mRNA expression of proinflammatory cytokines Ccl2 and Tnf (Figure 3E) in mice fed a HFD for 3 months. Moreover, compared to mice fed a HFD for 3 months, plasma thrombin time was markedly prolonged by dabigatran treatment (not shown). Finally, dabigatran also dramatically reduced hepatic fibrin deposition in mice fed a HFD (Figure 3F), consistent with previous studies demonstrating a dose of 10 g/kg chow *ad libitum* is anticoagulant in mice (Bogatkevich et al., 2011; Antoniak et al., 2013). Surprisingly, dabigatran significantly reduced body weight gain in mice fed a HFD (Figure 4A-B). This could not be attributed to a difference in food consumption (not shown). Dabigatran significantly decreased liver weight and relative liver weight, indicating a body weight-independent effect of dabigatran on liver size (Figure 4C-D). Moreover, thrombin inhibition significantly reduced serum and hepatic triglyceride levels (Figure 4E-F), as well as serum and hepatic cholesterol in mice fed a HFD (Figure 4G-H). Finally, dabigatran did not have any effect on plasma insulin or plasma glucose in mice fed a HFD (Figure 4I-J).

**Thrombin inhibition suppresses genes controlling lipid metabolism and induces genes implicated in bile acid synthesis in mice fed a HFD.** Thrombin inhibition with dabigatran etexilate significantly reduced body and liver weights and hepatic steatosis in mice fed a HFD.
for 3 months. To anchor these phenotypic differences to underlying gene expression changes, we used qPCR to assess hepatic expression of putative gene products known to control diet-induced obesity and fatty liver, including the fatty acid desaturase Scd1 (Figure 5A), fatty acid transporter Cd36 (Figure 5B), fatty acid “sensor” Cidea (Figure 5C), and nuclear receptor Pparg (Figure 5D) (Scaglia et al., 2005; Zhou et al., 2008; Moran-Salvador et al., 2011; Zhou et al., 2012). Analysis revealed a temporal connection between the onset of hepatic fibrin deposition and up-regulation of each gene (Figure 5A-D) in HFD-fed mice, with the largest magnitude of gene induction occurring at 3 months, a time coinciding with a significant increase in plasma TAT levels. Importantly, thrombin inhibition with dabigatran significantly suppressed induction of each gene in HFD-fed mice (Figure 5A-D). Notably, dabigatran treatment did not universally reverse changes in hepatic lipogenic gene expression, as expression of several genes coordinating lipid metabolism, including fatty acid synthesis and fatty acid beta oxidation (e.g., Fasn, Acox1 and Acaca) were not significantly impacted by dabigatran treatment (not shown).

Previous studies found that transgenic overexpression of Cyp7a1, the rate-limiting enzyme in hepatic bile acid metabolism, inhibited HFD-induced obesity coincident with a reduction in hepatic triglycerides and hepatic cholesterol (Li et al., 2010). In agreement, we identified that pharmacologic thrombin inhibition increased the expression of genes related to bile acid synthesis (Figure 5E-H). This included induction of the “classic pathway” genes with the rate-limiting enzyme Cyp7a1 (Figure 5E) and increase in Cyp8b1 (Figure 5F) expression, as well as induction of the “alternative pathway” genes, including Cyp7b1 and Cyp39a1 (Figure 5G-H). In contrast, expression of these genes was suppressed in mice fed a HFD at 3 months relative to CD-fed animals (Figure 5E-H). This finding suggests one important mechanism
whereby thrombin inhibition with dabigatran protects against diet-induced obesity and NAFLD by increasing bile acid synthesis.
DISCUSSION

Previous studies have linked a procoagulant state to metabolic syndrome and NAFLD development in patients (Ay et al., 2010; Beijers et al., 2010; Fritsch et al., 2010; Targher et al., 2010b; Semeraro et al., 2012). Additionally, perturbations in the expression and activity of coagulation factors has been observed in mice fed a HFD (Kassel et al., 2011; Owens et al., 2012). Here, we defined the temporal relationship between the onset of coagulation cascade activation and HFD-induced progression of fatty liver. Mild changes in serum lipids and hepatic steatosis were evident prior to activation of the coagulation cascade in mice fed a HFD. Marked increases in hepatic inflammation and injury coincided with coagulation cascade activation, suggesting a temporal link between coagulation cascade activation and exacerbation of HFD-induced NAFLD in mice.

Our studies with the thrombin inhibitor dabigatran, demonstrate that coagulation system activity is not simply a biomarker of fatty liver disease severity, but also that thrombin-mediated proteolysis is mechanistically-linked to the development and progression of NAFLD. This likely relates in part to the exciting observation that HFD-induced body weight gain is reduced in mice treated with dabigatran. The protection from HFD-induced body weight gain and hepatic steatosis mediated by dabigatran did not appear to be linked to altered glucose handling, at least given the statistical power of the current study. However, more definitive studies of putative effects of dabigatran on glucose metabolism are ongoing. Intriguingly, the reduction in liver weight in dabigatran-treated mice could not be fully explained by body weight changes, raising the possibility that thrombin-mediated induction of genes controlling de novo lipogenesis contributes directly to hepatic steatosis. Indeed, HFD-induced hepatic expression of several genes (e.g., Cidea, Pparg, Scd1, Cd36) is attenuated in PAR-1-null mice, which are protected
from fatty liver, despite equivocal weight gain when fed a HFD (Kassel et al., 2011). Collectively, the results support the hypothesis that coagulation is central to the pathogenesis of HFD-induced NAFLD, driven through both local changes in gene expression and systemic effects including body weight gain.

Previous studies found that reducing hematopoietic cell tissue factor expression decreased thrombin generation, body weight gain, and NAFLD in HFD-fed Ldlr⁻/⁻ mice (Kassel et al., 2011; Owens et al., 2012). Our results with prophylactic administration of a clinically-utilized thrombin inhibitor are consistent with a reduction in tissue factor-driven thrombin generation conferring protection against HFD-induced obesity and NAFLD. Thus, it is surprising that short-term (4 weeks) thrombin inhibition with argatroban did not significantly impact body weight gain in obese hypercholesterolemic Ldlr⁻/⁻ mice (Kassel et al., 2012). However, argatroban is a thrombin inhibitor of limited utility and potency due to poor pharmacokinetics (e.g., t₁/₂ of only 50 minutes) (Kondo et al., 2001), a requirement of parenteral delivery and a complex drug formulation. It is conceivable that in the previous study the degree or duration of thrombin inhibition with argatroban was insufficient to impact body weight gain, despite the observed reduction in hepatic inflammation and correction of serum cholesterol in argatroban-treated mice (Kassel et al., 2012). Nevertheless, the current and previous studies highlight the critical role of thrombin signaling in the regulation of HFD-induced fatty liver and associated pathologies.

Inflammatory cells and the mediators they produce have emerged as critical regulators of obesity and metabolic disease (Hotamisligil et al., 1993; De Taeye et al., 2007; Huang et al., 2010; Stienstra et al., 2010; Henao-Mejia et al., 2012). Obesity is associated with a pathologic state, termed metabolic inflammation, in which control of energy usage and hepatic and adipose
metabolite fate are directed by accumulated immune cells and mediators they produce (e.g., cytokines) (Hotamisligil, 2006; Gregor and Hotamisligil, 2011; Cao, 2014). In liver, accumulation and activation of inflammatory cells, particularly activation of Kupffer cells and recruitment of monocytes/macrophages, has been shown to promote hepatic lipid dysmetabolism and tissue injury in NAFLD/NASH (Baffy, 2009; Huang et al., 2010; Stienstra et al., 2010). Thus, one possible mechanism whereby thrombin activity could promote fatty liver disease and obesity is by exacerbating inflammatory cell activity and associated metabolic inflammation. Indeed, coagulation and inflammatory pathways are deeply integrated, and thrombin is well appreciated to increase macrophage proinflammatory function (Esmon, 2003; Opal, 2003; Esmon, 2004). Thrombin signaling through PAR-1 expressed by hepatic nonparenchymal cells, such as macrophages and stellate cells, has the potential to trigger additional inflammatory cell recruitment and activation via up-regulation of cytokine expression (Gaca et al., 2002; Copple et al., 2003; Fiorucci et al., 2004; Chen and Dorling, 2009). We found that thrombin inhibition with dabigatran markedly reduced hepatic inflammation, alongside decreased mRNA expression of macrophage chemotactic factors (e.g., MCP-1/CCL2) and macrophage-derived proinflammatory cytokines (e.g., TNF). PAR-1−/− mice were also protected from HFD-induced NAFLD and displayed a similar dramatic reduction in hepatic TNF and MCP-1/CCL2 expression and macrophage accumulation (Luyendyk et al., 2010). Collectively, these results suggest that potentiation of hepatic inflammation by thrombin-PAR-1 signaling is one mechanism whereby thrombin activity alters hepatic lipid metabolism and contributes to NAFLD/NASH pathology.

In addition to thrombin signaling, fibrin deposition is associated with obesity and multiple components of the metabolic syndrome (Rugman et al., 1994; Nienaber et al., 2008). Notably, a key functional link to metabolic disease sequelae is the concept that fibrin can exert
powerful proinflammatory activities. Specifically, cross-linked hepatic fibrin deposition may
direct macrophage localization and activation through direct engagement of the $\alpha_M\beta_2$ integrin
expressed by macrophages in the liver (Ugarova et al., 1998). Thrombin activation of factor XIII
and thrombin-activatable fibrinolysis inhibitor can support inflammation via stabilization of
fibrin (Myles et al., 2003). Moreover, outside of its traditional targets in hemostasis, thrombin
increases the proinflammatory potential of osteopontin, a molecule linked to obesity and NAFLD
(Sahai et al., 2004). Taken together, these studies suggest multiple pathways whereby thrombin
targets drive inflammation that promotes the pathogenesis of NAFLD.

The finding that dabigatran treatment markedly reduces hepatic inflammation sets the
stage for additional mechanistic studies determining the contribution of multiple thrombin targets
to NAFLD pathology. Specifically, our proof-of-principle findings with dabigatran reinforce the
possibility that pharmacologic inhibition of thrombin or factor Xa, or their targets, may form a
novel treatment strategy for obesity and NAFLD/NASH. Clinical investigation of this connection
is highly feasible, insofar as patients with NAFLD/NASH and/or obesity often have a concurrent
indication for anticoagulation, such as non-valvular A-Fib. For example, existing clinical
evidence suggests that compared to warfarin, patients taking dabigatran have significantly lower
plasma ApoB levels (Joseph et al., 2013). These findings demonstrate yet another metabolic
benefit of direct thrombin inhibition in managing stroke and potentially decreasing
atherosclerotic complications in patients with A-Fib.

Thrombin inhibition led to induction of bile acid synthesis genes which catalyze steps in
the classical and alternative pathways of bile acid synthesis from cholesterol. Others have shown
that bile acid activation of the nuclear receptor FXR inhibits progression of NAFLD/NASH and
activation of the G-protein coupled receptor TGR5 promotes thermogenesis in brown adipose
(Kong et al., 2009; Zhang et al., 2009; Pols et al., 2011). In agreement with these functions of bile acids, transgenic mice overexpressing hepatic Cyp7a1 are resistant to HFD-induced obesity (Li et al., 2010). Thus, the protective effects of dabigatran on body weight gain could relate to enhanced expression of bile acid synthesizing enzymes. Indeed, PAR-1 deficiency was not associated with up-regulation of Cyp7a1 (not shown), providing potential explanation as to why deficiency in this thrombin target failed to impact body weight gain (Kassel et al., 2011). Moreover, induction of bile acid synthesis could be the mechanism by which thrombin inhibition reduces serum cholesterol. We demonstrated herein, and previously in hypercholesterolemic mice (Kassel et al., 2012), that inhibition of thrombin reduces serum cholesterol levels. Additional studies are required to both fully characterize the impact of thrombin activity on the size and composition of the bile acid pool and evaluate whether the thrombin contribution to bile acid dynamics is direct or secondary to other thrombin-mediated changes in the microenvironment (e.g., enhancing local hepatic inflammation).

The coagulation system has emerged as an important contributor to multiple facets of metabolic disease (Ay et al., 2010; Beijers et al., 2010; Fritsch et al., 2010; Targher et al., 2010b; Semeraro et al., 2012), with mouse models suggesting a connection to HFD-induced obesity, insulin resistance, and fatty liver disease (Kassel et al., 2011; Antoniak et al., 2013). Identifying the mechanisms whereby thrombin and its targets promote NAFLD/NASH could identify novel strategies to inhibit this disease process without affecting hemostasis. Our data also suggest another potential therapeutic benefit of direct thrombin inhibitors in patients with an existing indication such A-Fib. Not surprisingly, NAFLD/NASH is tightly linked to obesity (Fabbrini et al., 2010), the latter being an important risk factor for A-Fib (Badheka et al., 2010). Indeed, elevated liver enzymes (e.g., ALT) were recently found to be associated with the risk of A-Fib in
the Framingham Heart Study (Targher et al., 2013). Viewed in the context of our findings, the use of direct thrombin inhibitors to reduce stroke in obese patients with A-Fib may also correct NAFLD/NASH and potentially have beneficial effects on obesity. Furthermore, a reduction in liver enzymes (and potentially hepatic inflammation) could in turn reduce the risk of A-Fib. However, the comparative benefit of anticoagulant therapy with either novel oral direct inhibitors of thrombin or factor Xa or with conventional agents (e.g., warfarin, heparin) in models of obesity or obese patients has not yet been evaluated. Additional mechanistic studies elucidating the effects of direct thrombin inhibitors on limiting obesity and associated sequelae including fatty liver, hepatic inflammation and injury in mouse models have the potential to guide future clinical investigation related to anticoagulant indications and drug selection.

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AUTHOR CONTRIBUTIONS

Participated in research design: Kopec, Joshi, Kassel, Sullivan, Luyendyk

Conducted experiments: Kopec, Joshi, Towery, Kassel, Sullivan, Flick, Luyendyk

Performed data analysis: Kopec, Joshi, Kassel, Sullivan, Flick, Luyendyk

Wrote or contributed to the writing of the manuscript: Kopec, Joshi, Flick, Luyendyk
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

FIGURE 1.
Temporal effects of high fat diet (HFD) on severity of hepatic steatosis, fibrin deposition and plasma thrombin-antithrombin (TAT) levels in C57BL/6J mice. (A) Representative photomicrographs showing hematoxylin and eosin-stained liver sections from mice fed either a control (CD, AIN-93M) or a HFD for 1, 2 or 3 months. (B) Hepatic fibrin deposition (red) was evaluated immunohistochemically at every time point. (C) Plasma TAT levels. Data are expressed as mean ± SEM, n=7-9 mice per group. Asterisk (*) indicates p<0.05 compared to time-matched CD measured by analysis of variance followed by Student-Newman-Keuls post-hoc test. Scale bars = 100 µm.

FIGURE 2.
Temporal effects of high fat diet (HFD) on hepatocyte injury and inflammation. C57BL/6J mice were fed either a control (CD, AIN-93M) or a HFD for 1, 2 or 3 months. (A) Hepatic inflammatory foci were quantified in hematoxylin and eosin-stained sections and expressed as the average number per 100X field. (B) Serum levels of alanine aminotransferase (ALT). (C) Macrophage accumulation was evaluated by F4/80 staining in HFD livers at 3 months. (D) Hepatic Ccl2 and Tnf gene expression. Data are expressed as mean ± SEM, n=7-9 mice per group. Asterisk (*) indicates p<0.05 compared to time-matched CD measured by analysis of variance followed by Student-Newman-Keuls post-hoc test or Student’s t-test. Scale bars = 100 µm.
FIGURE 3.
Effects of pharmacologic inhibition of thrombin with dabigatran on high fat diet (HFD)-induced NAFLD. C57BL/6J mice were fed either a HFD or a HFD with dabigatran etexilate (DE) for 3 months. (A) Liver sections were stained with hematoxylin and eosin and evaluated by light microscopy. (B) Activity levels of alanine aminotransferase (ALT) were quantified in serum and (C) number of hepatic inflammatory foci was determined using hematoxylin and eosin-stained sections. (D) Macrophage accumulation was evaluated by F4/80 immunohistochemical staining. (E) Hepatic Ccl2 and Tnf gene expression. (F) Hepatic fibrin deposition (red) was evaluated immunohistochemically. Data are expressed as mean + SEM, n=4 mice per group. Asterisk (*) indicates p<0.05 compared to HFD measured by Student’s t-test. Scale bars = 100 µm.

FIGURE 4.
Effects of pharmacologic inhibition of thrombin with dabigatran on high fat diet (HFD)-induced obesity and serum and hepatic triglyceride and cholesterol levels. C57BL/6J mice were fed either a HFD or a HFD with dabigatran etexilate (DE) for 3 months. (A) Terminal body weight was measured weekly after 1 week acclimation on the diet. (B) Body weight gain is expressed as a terminal body weight divided by initial body weight after 1 week of acclimation on the diet. (C) Liver weight was collected at the time of sacrifice and (D) relative liver weight is expressed as % of terminal body weight. (E) Serum triglycerides; (F) liver triglycerides; (G) serum cholesterol; (H) hepatic cholesterol; (I) plasma glucose and (J) plasma insulin were
quantified using commercially available reagents. Data are expressed as mean + SEM, n=4 mice per group. Asterisk (*) indicates p<0.05 compared to HFD measured by Student’s t-test.

FIGURE 5.

Thrombin inhibition with dabigatran etexilate (DE) alters genes associated with hepatic metabolism and bile acid synthesis in C57BL/6J mice fed a high fat diet (HFD). Gene expression analysis by qPCR was performed on the HFD time course and thrombin inhibition study hepatic samples and identified changes in (A) Scd1, (B) Cd36, (C) Cidea and (D) Pparg levels. Moreover, HFD suppressed bile acid metabolism genes, including (E) Cyp7a1, (F) Cyp8b1, (G) Cyp7b1 and (H) Cyp39a1. Data are expressed as mean + SEM, n=4 or n=7-9 mice per group. Time course: Fold change is expressed relative to CD fold change at 1 month. Asterisk (*) indicates p<0.05 compared to time-matched CD measured by one-way analysis of variance followed by Student-Newman-Keuls post hoc test. Thrombin inhibition study: Fold change in HFD+DE is expressed relative to the HFD fold change at 3 months, which is set to 1. Asterisk (*) indicates p<0.05 compared to HFD measured by Student’s t-test.
Table 1. Temporal effects of high fat diet (HFD) and control diet (CD) on terminal body weight\textsuperscript{a}, body weight gain\textsuperscript{b}, liver weight, relative liver weight and serum and hepatic triglyceride and cholesterol levels in C57BL/6J mice.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Diet</th>
<th>Body weight (g)</th>
<th>Body weight gain (%)</th>
<th>Liver weight (g)</th>
<th>Relative liver weight</th>
<th>Serum triglycerides (mg/dl)</th>
<th>Hepatic triglycerides (mg/g liver)</th>
<th>Serum cholesterol (mg/dl)</th>
<th>Hepatic cholesterol (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>CD</td>
<td>25.7 ± 1.6</td>
<td>22.3 ± 10.7</td>
<td>0.86 ± 0.08</td>
<td>3.3 ± 0.2</td>
<td>115.5 ± 13.4</td>
<td>20.3 ± 5.5</td>
<td>133.4 ± 8.9</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>27.3 ± 1.6</td>
<td>37.6 ± 11.0*</td>
<td>1.00 ± 0.14</td>
<td>3.7 ± 0.4</td>
<td>91.5 ± 7.4*</td>
<td>15.3 ± 5.5</td>
<td>126.3 ± 9.0</td>
<td>7.8 ± 1.2*</td>
</tr>
<tr>
<td>2 months</td>
<td>CD</td>
<td>28.5 ± 1.5</td>
<td>46.3 ± 11.2</td>
<td>0.90 ± 0.07</td>
<td>3.2 ± 0.1</td>
<td>110.8 ± 11.1</td>
<td>19.7 ± 4.1</td>
<td>132.1 ± 11.4</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>32.5 ± 3.4*</td>
<td>73.0 ± 18.8*</td>
<td>1.44 ± 0.45*</td>
<td>4.4 ± 0.9*</td>
<td>103.4 ± 12.8</td>
<td>26.3 ± 7.6*</td>
<td>156.1 ± 26.4*</td>
<td>7.3 ± 1.4*</td>
</tr>
<tr>
<td>3 months</td>
<td>CD</td>
<td>29.4 ± 2.5</td>
<td>57.6 ± 5.7</td>
<td>0.91 ± 0.11</td>
<td>3.1 ± 0.1</td>
<td>111.7 ± 17.5</td>
<td>23.1 ± 2.7</td>
<td>131.4 ± 8.9</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>43.1 ± 3.2*</td>
<td>120.6 ± 23.2*</td>
<td>2.58 ± 0.66*</td>
<td>5.9 ± 1.2*</td>
<td>108.0 ± 5.1</td>
<td>31.5 ± 4.8*</td>
<td>199.8 ± 22.2*</td>
<td>7.6 ± 0.7*</td>
</tr>
</tbody>
</table>

Data: mean ± SD, for 7-9 mice per group.

\*p<0.05 compared to time-matched CD measured by ANOVA followed by Student-Newman-Keuls post-hoc test.

\textsuperscript{a}Terminal body weight was measured prior to overnight food removal.

\textsuperscript{b}Body weight gain represents terminal body weight divided by body weight at the start of the study.
Table 2. Temporal effects of high fat diet (HFD) and control diet (CD) on plasma insulin and plasma glucose levels in C57BL/6J mice.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Diet</th>
<th>Plasma insulin (pmol/l)</th>
<th>Plasma glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>CD</td>
<td>17.8 ± 4.9</td>
<td>150.4 ± 17.8</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>19.2 ± 4.0</td>
<td>164.4 ± 34.3</td>
</tr>
<tr>
<td>2 months</td>
<td>CD</td>
<td>18.0 ± 7.0</td>
<td>133.8 ± 31.1</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>37.0 ± 21.2*</td>
<td>196.8 ± 60.2*</td>
</tr>
<tr>
<td>3 months</td>
<td>CD</td>
<td>20.7 ± 9.1</td>
<td>112.3 ± 17.2</td>
</tr>
<tr>
<td></td>
<td>HFD</td>
<td>51.9 ± 17.0*</td>
<td>187.6 ± 13.4*</td>
</tr>
</tbody>
</table>

Data: mean ± SD, for 7-9 mice per group.

*p<0.05 compared to time-matched CD measured by ANOVA followed by Student-Newman-Keuls post-hoc test.
**Figure 1**

Panel A: Confocal images demonstrating lipid accumulation.

Panel B: Histological sections showing differences in tissue morphology.

Panel C: Graph depicting plasma TAT levels across different conditions and time points.

*Plasma TAT ng/ml*
Figure 2

A) Hepatic inflammatory foci

<table>
<thead>
<tr>
<th></th>
<th>CD 1 month</th>
<th>CD 2 months</th>
<th>CD 3 months</th>
<th>HFD 1 month</th>
<th>HFD 2 months</th>
<th>HFD 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>number/100x field</td>
<td>10 ± 2</td>
<td>12 ± 3</td>
<td>15 ± 4</td>
<td>8 ± 1</td>
<td>10 ± 2</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>

B) Serum ALT

<table>
<thead>
<tr>
<th></th>
<th>CD 1 month</th>
<th>CD 2 months</th>
<th>CD 3 months</th>
<th>HFD 1 month</th>
<th>HFD 2 months</th>
<th>HFD 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/L</td>
<td>50 ± 5</td>
<td>60 ± 6</td>
<td>70 ± 7</td>
<td>100 ± 10</td>
<td>120 ± 12</td>
<td>150 ± 15</td>
</tr>
</tbody>
</table>

C) Histological images

CD 3 months

HFD 3 months

D) Hepatic Ccl2 and Tnf expression

<table>
<thead>
<tr>
<th></th>
<th>CD 3 months</th>
<th>HFD 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>fold change Ccl2</td>
<td>2 ± 0.5</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>fold change Tnf</td>
<td>1 ± 0.2</td>
<td>4 ± 0.8</td>
</tr>
</tbody>
</table>

*Significant difference compared to CD group.
Figure 3

A. HFD vs HFD+DE

B. Serum ALT

C. Hepatic inflammatory foci

D. HFD vs HFD+DE

E. Hepatic Ccl2 fold change

F. Hepatic Tnf fold change

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Figure 4

A) Body weight

B) Body weight gain

C) Liver weight

D) Relative liver weight

E) Serum triglycerides

F) Hepatic triglycerides

G) Serum cholesterol

H) Hepatic cholesterol

I) Plasma glucose

J) Plasma insulin
Figure 5

A. Hepatic Scd1 fold change

B. Hepatic Cd36 fold change

C. Hepatic Cidea fold change

D. Hepatic Pparg fold change

E. Hepatic Cyp7a1 fold change

F. Hepatic Cyp8b1 fold change

G. Hepatic Cyp7b1 fold change

H. Hepatic Cyp39a1 fold change

* denotes significance at p < 0.05.