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Dose-Dependent Effects of Alpha-Naphthylisothiocyanate Disconnect Biliary Fibrosis from Hepatocellular Necrosis

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Abstract

Exposure of rodents to the xenobiotic α -naphthylisothiocyanate (ANIT) is an established model of experimental intrahepatic bile duct injury. Administration of ANIT to mice causes neutrophil-mediated hepatocellular necrosis. Prolonged exposure of mice to ANIT also produces bile duct hyperplasia and liver fibrosis. However, the mechanistic connection between ANIT-induced hepatocellular necrosis and bile duct hyperplasia and fibrosis is not well-characterized. We examined impact of two different doses of ANIT, by feeding chow containing ANIT (0.05%, 0.1%), on the severity of various liver pathologies in a model of chronic ANIT exposure. ANIT-elicited increases in liver inflammation and hepatocellular necrosis increased with dose. Remarkably, there was no connection between increased hepatocellular necrosis and bile duct hyperplasia and peribiliary fibrosis, as these pathologies increased similarly in mice exposed to either dose of ANIT. The results indicate that the severity of hepatocellular necrosis does not dictate the extent of bile duct hyperplasia/fibrosis in ANIT-exposed mice.

Keywords

liver; fibrosis; hepatocellular necrosis; bile duct; biliary hyperplasia; ANIT; alpha-naphthylisothiocyanate

INTRODUCTION

α -naphthylisothiocyanate (ANIT) is a xenobiotic utilized as an experimental tool to identify mechanisms of liver injury caused by bile duct damage (1–4). ANIT undergoes glutathione conjugation within hepatocytes and is transported into the bile by the transporter, MRP2 (5). The ANIT-glutathione conjugate is unstable in bile, which produces a scenario where ANIT undergoes repetitive rounds of absorption and metabolism via enterohepatic recirculation

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(4,6,7). This produces cytotoxic concentrations of ANIT in the bile, causing initial selective damage to bile duct epithelial cells (BDECs) (5,7,8).

Administration of a single, large dose of ANIT to rodents produces acute cholestatic liver injury characterized by focal hepatocellular necrosis (3,9–11). Preventing ANIT transport into bile reduces ANIT-induced hepatocellular necrosis, implying ANIT biliary injury is a prerequisite for hepatocellular necrosis (5). Focal hepatocellular necrosis in rodents given an acutely toxic dose of ANIT resembles early liver pathology in mice subjected to bile duct ligation (BDL), an experimental setting of acute, obstructive cholestatic liver injury (2,3,9,10). In both acute ANIT- and BDL-induced liver injury, hepatic neutrophil accumulation is observed and exaggerated neutrophil activation is critical for hepatocellular necrosis (3,12,13). Collectively, these studies support the hypothesis that acute hepatocellular necrosis in acute cholestasis is mediated by inflammatory cells, activated in part by the release of proinflammatory bile acids into the liver parenchyma (14).

Persistent exposure of rodents to ANIT through the diet represents an experimental setting of bile duct hyperplasia and peribiliary fibrosis (15–17). Mechanisms of peribiliary fibrosis in chronic ANIT exposure resemble other models of chronic cholestatic liver injury (long-term BDL; *Mdr2*^{−/−} mice) (16,18–24). Expression of the heterodimeric $\alpha_v\beta_6$ integrin by BDECs leads to local activation of latent-transforming growth factor β (TGF β) and activation of neighboring fibroblasts (16,25–28). Exaggerated peribiliary fibrosis resembles the onion skin-like fibrosis observed in patients with primary sclerosing cholangitis (29). Interestingly, in mice exposed chronically to ANIT, serum markers of hepatocellular injury are minimally elevated, and focal hepatocellular necrosis is minimal (16,20,30). Although both peribiliary fibrosis and hepatocellular necrosis are observed in mice chronically exposed to ANIT (15,18,20,31), it is currently unknown whether these two pathologies are intrinsically connected. To begin to address this question, we examined whether the ANIT dose-response relationship could reveal disconnects between hepatocellular necrosis, inflammation, bile duct hyperplasia and peribiliary fibrosis in an experimental setting of chronic ANIT exposure.

MATERIALS AND METHODS

Mice

Age-matched 9-week-old male mice on a C57BL/6J background (Jackson Laboratory, Bar Harbor, ME) were used for these studies. Mice were housed at an ambient temperature of approximately 22°C with alternating 12 hour light/dark cycles and provided water and rodent chow *ad libitum* prior to 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 Michigan State University Institutional Animal Care and Use Committee.

ANIT diet model

Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was a standard rodent chow diet containing either 0.05% or 0.1% ANIT (Sigma-Aldrich, St. Louis, MO).

Based on average chow consumption, the daily dose of ANIT is approximately 60 mg/kg and 120 mg/kg, respectively. Groups of mice were fed ANIT diet for a total of 4 weeks, *ad libitum*. Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into an empty syringe, allowed to clot, and spun at $10,000 \times g$ for 2 minutes for collection of serum. The liver was excised and briefly immersed 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 left lateral lobe was fixed in 10% neutral buffered formalin for 96 hours prior to routine processing. The remaining liver was cut into approximately 100 mg sections and flash-frozen in liquid nitrogen.

Histopathology, clinical chemistry and immunohistochemistry

For analysis of liver histopathology by light microscopy, formalin-fixed paraffin-embedded liver sections were cut at 5 μm and stained with hematoxylin and eosin (H&E), sirius red, or immunohistochemically for cytokeratin-19 (CK-19) antigen by the Michigan State University Investigative Histopathology Laboratory as described previously (18,31). Hepatocellular necrosis was quantified in a masked fashion by examining 5 representative 40X H&E-stained liver sections from each animal and expressed as percent of total tissue area. For quantification of sirius red (collagen deposits) and CK-19 staining (BDECs), images of stained liver sections were captured using a Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20X objective. The area of positive sirius red and CK-19 staining in at least 200 images per tissue was determined in an automated and unbiased fashion using a batch macro and the color de-convolution tool in ImageJ. Serum activity of alanine aminotransferase (ALT) was determined using commercial reagents (Infinity ALT/GPT Thermo Fisher, Waltham, MA).

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated from approximately 20 mg of snap-frozen liver using TRI Reagent according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). 1 μg of total RNA was utilized for the synthesis of cDNA, accomplished using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). SYBR Green quantitative real-time PCR (qPCR) amplification was performed using a CFX Connect thermal cycler (Bio-Rad) with primers purchased from IDT (Coralville, IA). Primers were purchased from IDT (Coralville, IA). The expression of each gene was normalized to the housekeeper gene GAPDH and the relative levels of each gene were evaluated using the $\Delta\Delta\text{Ct}$ method. Primer sequences are listed in Table 1.

Statistics

Comparison of 3 or more groups was performed using one-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

Dose-dependent increase in hepatocellular necrosis and inflammatory gene induction in ANIT-exposed mice

Liver histology was unremarkable in mice fed control chow (Figure 1A). In agreement with previous studies, ANIT-exposed mice developed marked bile duct hyperplasia (Figure 1A, asterisks). Exposure of mice to 0.05% ANIT diet for 4 weeks was associated with very mild hepatocellular injury, indicated by increased serum ALT activity (~1.5 fold increase) and very minimal hepatocellular necrosis (Figure 1B–C). Mice fed 0.1% ANIT diet developed significantly more hepatocellular injury, as indicated by a more marked increase in serum ALT activity (~5 fold increase) and area of hepatocellular necrosis (Figure 1A–C, arrows). Compared to mice fed a control diet, hepatic expression of pro-inflammatory genes CCL2, IL6 and CXCL2 increased in mice exposed to 0.05% ANIT. Notably, hepatic expression of each of these mRNAs increased further in mice exposed to 0.1% ANIT (Figure 2A–C). In addition, induction of IL1 β and CXCL1 mRNAs increased only in mice exposed to 0.1% ANIT (Figure 2D–E). Consistent with this observation, evaluation of H&E-stained liver sections suggested increased neutrophil accumulation in livers of mice exposed to 0.1% ANIT compared to 0.05% ANIT (Figure 1A). Collectively, the results indicate that hepatocellular necrosis and inflammation are dose-dependent in mice exposed to ANIT chronically via the diet.

ANIT-induced biliary hyperplasia is disconnected from hepatocellular necrosis

To quantify ANIT-induced bile duct hyperplasia, we stained liver sections for cytokeratin-19 (CK-19), a marker of bile ducts in mouse liver. Bile duct area increased significantly in mice exposed to 0.05% ANIT, compared to control diet, indicating development of classic biliary hyperplasia (Figure 3A–B). Surprisingly, ANIT-induced bile duct hyperplasia was similar in mice exposed to 0.1% ANIT diet (Fig. 3A–B). Expression of ITG β 6 mRNA, which encodes the β subunit of the $\alpha_v\beta_6$ integrin, is largely restricted to BDECs in settings of cholestatic liver injury (16,25,26,28). Consistent with this, expression of hepatic ITG β 6 mRNA, one component of the BDEC-selective profibrogenic $\alpha_v\beta_6$ integrin, increased similarly in mice exposed to 0.05% and 0.1% ANIT diet (Figure 3C). The results indicate that increased hepatocellular necrosis is not paralleled by increased bile duct hyperplasia in ANIT-exposed mice.

ANIT-induced peribiliary fibrosis is connected to bile duct hyperplasia, not hepatocellular necrosis

Exposure to 0.05% ANIT in chow for 4 weeks increased peribiliary collagen protein deposition and COL1A1 mRNA levels in liver compared to control-diet fed mice (Figure 4A–C). Interestingly, COL1A1 mRNA induction and increased collagen protein deposition were similar in mice exposed to 0.1% ANIT (Figure 4A–C), suggesting that increased hepatocellular necrosis in mice exposed to 0.1% ANIT (Figure 1) was not a mechanistic determinant of fibrosis severity. Indeed, in mice exposed to 0.1% ANIT, collagen deposits were not observed within areas of hepatocellular necrosis (Figure 4C).

DISCUSSION

Experimental ANIT exposure has been utilized to discover mechanisms of liver disease stemming from biliary injury (1,2). Here we found that hepatocellular necrosis and hepatic inflammatory gene induction increased in a dose-dependent manner in ANIT-exposed mice. However, whereas bile duct hyperplasia and fibrosis were increased by ANIT, a connection to ANIT dose was not observed. The results suggest that hepatocellular necrosis is not required for ANIT-induced bile duct hyperplasia. Moreover, the observation that hepatocellular necrosis and biliary fibrosis do not uniformly increase with dose suggests a distinct mechanistic basis for each lesion.

Upregulation of the heterodimeric $\alpha_v\beta_6$ integrin is evident on BDECs in multiple models of cholestatic liver injury/fibrosis, including in mice exposed chronically to ANIT (16,25–27). The $\alpha_v\beta_6$ integrin converts latent TGF β 1 into its active form, and multiple studies have shown this pathway contributes to biliary fibrosis (16,18,25,26). Notably, inhibition of $\alpha_v\beta_6$ reduces liver fibrosis in a modified BDL where hepatocellular injury is not a primary feature (26). Moreover, inhibition of $\alpha_v\beta_6$ had no effect on serum ALT activity in ANIT-exposed mice (16). These studies identify $\alpha_v\beta_6$ integrin-mediated TGF β 1 activation as a profibrogenic pathway distinct from the mechanisms controlling hepatocellular necrosis in cholestatic liver injury. Indeed, whereas hepatic ITG β 6 mRNA induction tracked with bile duct hyperplasia but not hepatocellular necrosis at different doses of ANIT.

Previous studies have implicated neutrophils in the progression of hepatocellular injury caused by cholestasis. Neutrophil accumulation is a hallmark of focal hepatocellular necrotic lesions observed in acute cholestasis (3,12,32–34). Neutrophil depletion reduces hepatocellular necrosis caused by ANIT and BDL (13,33,35) and inhibition of CXC chemokine-mediated neutrophil recruitment or deficiency in critical adhesion molecules reduces necrosis (32,33). In agreement, we observed a marked increase in hepatic proinflammatory gene induction and histological evidence of neutrophil accumulation coincident with increased hepatocellular necrosis in 0.1% ANIT-exposed mice. Of importance, this increase in inflammation and hepatocellular necrosis occurred without a reflective increase in peribiliary fibrosis, further suggesting fibrosis and hepatocellular necrosis are mechanistically distinct lesions. Moreover, areas of focal hepatocellular necrosis in ANIT-exposed mice were devoid of collagen, suggesting these lesions are adequately repaired. Overall, this disconnect is supported by other studies noting that neutrophils are not major contributors to liver fibrosis in chronic cholestatic liver injury (32).

Understanding the unique driving forces of hepatocellular necrosis and peribiliary fibrosis during cholestasis has several important implications, including but not limited to the importance of characterizing dose-related effects of ANIT in experimental settings. The observation that hepatocellular necrosis and biliary fibrosis are disconnected is important for interpretation of studies seeking novel antifibrotic drugs. For example, in models where hepatocellular injury is intrinsically connected to fibrosis, such as chronic administration of the hepatotoxicant carbon tetrachloride (36,37), drugs could be inferred as antifibrotic even if their mode of action is to inhibit carbon tetrachloride hepatotoxicity. In contrast, drugs that prevent hepatocyte necrosis may not have direct effects on biliary hyperplasia and fibrosis in

ANIT-exposed mice. This emphasizes the need to consider etiology of liver fibrosis when comparing effects of interventions in different models of chronic liver injury. In summary, our studies reveal an important disconnect between hepatocellular necrosis and bile duct hyperplasia/fibrosis in mice exposed chronically to ANIT, providing potential explanation for prior studies identifying dichotomy in the mechanisms driving fibrosis in different experimental settings.

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Figure 1. Dose-dependent increase in liver injury in ANIT-exposed mice

Male, wild-type C57Bl/6J mice were fed standard control rodent chow or identical chow containing 0.05% or 0.1% ANIT for 4 weeks. (A) Representative photomicrographs show liver sections stained with hematoxylin and eosin. Asterisks indicate biliary hyperplasia. Arrows indicate hepatocellular necrosis. (B) Serum ALT activity and (C) area of hepatocellular necrosis were determined as described in Materials and Methods. Data are expressed as mean + SEM; $n=5-10$ mice per group. * $p<0.05$ vs. control diet. ** $p<0.05$ vs. 0.05% ANIT-exposed mice.

Figure 2. Dose-dependent increase in inflammatory gene induction in ANIT-exposed mice
Male, wild-type C57Bl/6J mice were fed standard control rodent chow or identical chow containing 0.05% or 0.1% ANIT for 4 weeks. Hepatic expression of mRNAs encoding the proinflammatory genes (A) CCL2 (B) IL6 (C) CXCL2 (D) IL1 β and (E) CXCL1 was determined by real-time qPCR. Data are expressed as mean + SEM; n=5–10 mice per group. *p<0.05 vs. control diet. #p<0.05 vs. 0.05% ANIT-exposed mice.

Figure 3. Effect of ANIT exposure on bile duct hyperplasia

Male, wild-type C57Bl/6J mice were fed standard control rodent chow or identical chow containing 0.05% or 0.1% ANIT for 4 weeks. (A) Representative photomicrographs show liver sections stained for cytokeratin-19 (CK-19, brown). (B) CK-19 staining was quantified as described in Materials and Methods. (C) Hepatic expression of ITGβ6 mRNA was determined by real-time qPCR. Data are expressed as mean + SEM; n=5–10 mice per group.

*p<0.05 vs. control diet.

Figure 4. Effect of ANIT exposure on biliary fibrosis in mice

Male, wild-type C57Bl/6J mice were fed standard control rodent chow or identical chow containing 0.05% or 0.1% ANIT for 4 weeks. (A) Quantification of sirius red staining was performed as described in Material and Methods. (B) Hepatic expression of profibrogenic COL1A1 mRNA was determined by real-time qPCR. (C) Representative photomicrographs show liver sections stained for collagen deposits with sirius red. The dashed outline

demarcates an area of hepatocellular necrosis. Data are expressed as mean + SEM; n = 5–10 mice per group. *p<0.05 vs. control diet-exposed mice.

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Table 1

Gene names and primer sequences (5' → 3') for transcripts verified by qPCR.

Gene Name	Gene ID	Gene Symbol	Species	Forward primer	Reverse primer
Glyceraldehyde-3-phosphate dehydrogenase	14433	<i>Gapdh</i>	mouse	GTGGACCTCATGGCCCTACAT	TGTGAGGGAGATGCTCAGTG
Collagen, type I, alpha 1	12842	<i>Col1a1</i>	mouse	GAGCGAGAGTACTGGATCG	GCTTCTTTTCCCTTGGGGTTTC
Integrin beta 6	16420	<i>Iigb6</i>	mouse	CTCACGGGTACAGTAAACGCA	AAATGAGCTCTCAGGCAGGC
Interleukin 1 beta	16176	<i>Il1b</i>	mouse	AGCTTCCTTGTGCAAGTGTCT	GACAGCCAGGTCAAAGGTT
Interleukin 6	16193	<i>Il6</i>	mouse	TCCTCTCTGCAAGAGACTTCC	TTGTGAAGTAGGGAAGGCCG
Chemokine (C-C motif) ligand 2	20296	<i>Ccl2</i>	mouse	GTCCCTGTGATGCTTCTGG	GCTCTCCAGCCCTACTCATTTG
Chemokine (C-X-C motif) ligand 1	14825	<i>Cxcl1</i>	mouse	GCTGGGATTACACCTCAAGAA	TGGGGACACCTTTTAGCATC
Chemokine (C-X-C motif) ligand 2	20310	<i>Cxcl2</i>	mouse	AAGTTTGCCTTGACCCCTGAA	AGGCACATCAGGTACGATCC