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# Histamine restores biliary mass following carbon tetrachloride-induced damage in a cholestatic rat model

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ABSTRACT

*Background:* Bile duct ligation coupled with carbon tetrachloride induces apoptosis of large but not small cholangiocytes. Histidine decarboxylase regulates histamine synthesis. We have shown that: (i) cholangiocytes express histidine decarboxylase and secrete histamine and (ii) histamine stimulates biliary growth.
*Aims:* To demonstrate that histidine decarboxylase/histamine regulates cholangiocyte homeostasis after carbon tetrachloride treatment.
*Methods: In vivo*, normal and bile duct ligated rats were treated with saline or histamine (0.5 mg/kg body weight) and given carbon tetrachloride by gavage 2 days before sacrifice. Serum, liver blocks and large cholangiocytes were obtained. Histidine decarboxylase, bile duct mass and proliferation were measured in liver sections and in cholangiocytes. Apoptosis was measured by immunohistochemistry and gene expression. Histamine levels were evaluated in serum. *In vitro*, large cholangiocytes were treated with carbon tetrachloride in the absence/presence of histamine before evaluating proliferation.
*Results:* After bile duct ligation there was enhanced ductal mass, histidine decarboxylase expression and serum histamine levels. Carbon tetrachloride treatment enhanced biliary apoptosis, and decreased histidine decarboxylase and serum histamine levels and biliary proliferation, changes that were restored by histamine. *In vitro*, cholangiocytes treated with carbon tetrachloride had a lower proliferative capacity that was reversed when cells were pre-treated with histamine.
*Conclusion:* Histidine decarboxylase may be a key regulator of cholangiocyte homeostasis during biliary injury.

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## 1. Introduction

In addition to modify bile of canalicular origin [1,2], cholangiocytes are the target cells of a number of liver diseases, collectively known as "cholangiopathies" [3,4]. Primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) are devastating cholangiopathies that target the biliary epithelium [3,4]. Hallmark features of cholangiopathies are characterized by dysregulation of the balance between cholangiocyte proliferation/damage [3,4]. The cholestatic bile duct ligated (BDL) rat model is characterized by increased ductal secretory activity [5,6], and increased cAMP-dependent mitotic activity of large but not small cholangiocytes [6,7]. In contrast to BDL [6,7], treatment with the hepatotoxin, carbon tetrachloride ($CCl_4$) induces necrosis in hepatocytes coupled with functional damage (apoptosis) of large, cAMP-dependent cholangiocytes, damage that is associated with *de novo* proliferation and acquisition of large biliary phenotypes by small cholangiocytes to compensate for the loss of large cholangiocyte function [8].

Histamine is a biogenic amine that regulates numerous functions of the body. Histamine is formed after decarboxylation of the amino acid, histidine, by the enzyme L-histidine decarboxylase

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(HDC) [9–11]. After formation, histamine is released and either used or stored (mainly in mast cells) and can interact with specific G-protein coupled histamine receptor subtypes (H1HR–H4HR) [7,11,12]. Classically, the H1HR and H2HR have been found to have stimulatory cellular properties, whereas the H3HR and H4HR are negative regulators of cellular function [7,13,14]. While H1HR exert their functions by coupling to $G_q\alpha$ mobilizing intracellular $Ca^{2+}$ levels [15], H2HR effects are modulated by $G_s\alpha$ proteins coupled to adenylyl cyclase [16]. On the other hand, H3/H4HRs couple to $G_{i/o}\alpha$ resulting in negative regulation of cAMP [17]. In support of this background, we have shown that the H1HR agonists stimulate small cholangiocyte proliferation *via* a $Ca^{2+}$/CAM kinase-dependent pathway [14], whereas stimulation of H3HR downregulate the PKC/$Ca^{2+}$ pathway and inhibits cAMP synthesis and large biliary proliferation [7]. We have also shown that histamine stimulates the proliferation of small and large cholangiocytes by activation of inositol 1,4,5-trisphosphate ($IP_3$)/$Ca^{2+}$ and cAMP-dependent signalling mechanisms, respectively [13]. Moreover, studies have shown [9,10] that: (i) cholangiocytes regulate biliary mass by secretin histamine *via* HDC; and (ii) inhibition of HDC decreases both hyperplastic and neoplastic cholangiocyte proliferation *via* downregulation of vascular endothelial growth factor (VEGF), an important autocrine trophic factor for biliary growth [18]. Thus, based on this background we aim to demonstrate that histamine has a protective effect against $CCl_4$-induced damage of large cholangiocytes.

## 2. Materials and methods

All chemicals and reagents were purchased from Sigma Aldrich Co (St. Louis, MO) unless indicated otherwise. All primers, PCR reaction material and transfection reagents were purchased from SABiosciences (Frederick, MD). The substrate for γ-glutamyltranspeptidase (γ-GT), N (γ-L-glutamyl)-4-methoxy-2-naphthylamide, was purchased from Polysciences (Warrington, PA). The mouse anti-cytokeratin-19 (CK-19) antibody was purchased from Caltag Laboratories Inc. (Burlingame, CA, USA).

### 2.1. Animal models

Male Fisher rats (150–175 g) were purchased from Charles River (Wilmington, MA) and maintained in a temperature-controlled environment (20–22 °C) with 12:12-h light–dark cycles. Animals were fed *ad libitum* standard chow and had free access to drinking water. Before each experimental procedure, animals were injected with euthasol (50 mg/kg BW, IP). Study protocols were performed in compliance with the institutional guidelines set forth by the BaylorScott & White Healthcare Institutional Animal Care and Use Committee (IACUC). Our studies were performed in normal (sham) rats or rats that immediately after bile duct ligation (BDL) [5] for 7 days were treated with saline or histamine (0.5 mg/kg BW per day) [13,19] for 2 days in the presence of an acute administration of $CCl_4$ by gavage (0.5 ml/100 g BW) [8]; 2 days later (9 days after BDL) the animals were used for collection of serum, liver tissue and purified cholangiocytes [13].

### 2.2. Purified cholangiocytes and biliary cell lines

Since only large cholangiocytes proliferate after BDL and are damaged by $CCl_4$ treatment [6,8], most of the *in vivo* experiments were performed in these biliary subpopulations that were isolated by centrifugal elutriation followed by immunoaffinity separation [7,20,21] with a monoclonal antibody (a gift of Dr. R. Faris) against an unidentified antigen expressed by all intrahepatic rat cholangiocytes [20]. To validate our model of $CCl_4$-induced apoptosis of large cholangiocytes [8], some of the experiments were also performed in small cholangiocytes that are resistant to $CCl_4$ and *de novo* proliferate following functional damage of large cholangiocytes [8]. Cell count and viability (~97%) were measured by trypan blue exclusion. Purity (approximately 99%) was assessed by histochemistry for γ-GT [22]. The *in vitro* experiments were performed in our large murine cholangiocyte lines (LMCC) (see below) that display morphological, phenotypical and functional features similar to that of freshly isolated large cholangiocytes [13,23,24].

### 2.3. Expression of HDC and histamine receptor subtypes

We studied the expression of HDC and H1–H4HRs by: (i) immunoblots (20 μg protein) from freshly large cholangiocytes; and (ii) quantitative real-time PCR (1 μg total RNA) [13]. Immunoblots were normalized by β-actin. Band intensity was determined on the Odyssey system from LI-COR Biosciences (Lincoln, NE), Model #9120.

For all the real-time PCR reactions, rat primers (SABiosciences) were used; a ΔΔCT (delta delta threshold cycle) analysis was performed for real-time PCR. In total liver RNA, cytokeratin-19 (CK-19, a cholangiocyte-specific marker) [7] was utilized to determine the ratio of expression per cholangiocytes and all reactions were compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure proper RNA loading. We used RNA from rat brain and water as positive and negative controls, respectively.

### 2.4. Measurement of histamine levels in serum

To demonstrate a relationship between histamine levels (originating from basophil granulocytes, mast cells as well as cholangiocytes) [9,11,13,25] and biliary proliferation, we measured histamine levels in serum by EIA (Cayman Chemical, Ann Arbor, MI) as described [9,13].

### 2.5. Evaluation of biliary proliferation and apoptosis

Intrahepatic bile duct mass (IBDM) of small and large cholangiocytes was measured in paraffin-embedded liver sections (4 μm thick) by evaluating the percentage of intrahepatic bile ducts stained for cytokeratin-19 (CK-19, a specific marker of the biliary epithelium) [7] over the total liver mass. Briefly, in each liver section, IBDM was measured as area occupied by CK-19 positive bile duct/total area × 100 as described [24]. Sections were analyzed using a BX-51 light microscope (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI) and processed with an Image Analysis System (IAS: Delta Sistemi, Rome, Italy).

The number of proliferating large cholangiocytes were evaluated by immunohistochemistry for PCNA in liver sections as described [7]. Biliary proliferation was also evaluated by immunoblots [7] for PCNA in protein (10 μg) from lysate from total liver (positive control) and isolated large cholangiocytes from the selected experimental groups. Immunoblots were normalized by β-actin.

Since large (but not small) cholangiocytes are damaged by $CCl_4$ [8] we evaluated the percentage of large apoptotic cholangiocytes in paraffin-embedded liver sections (4–5 μm) by immunohistochemistry for BAX. Sections were examined in a coded fashion by BX-51 light microscopy (Olympus, Tokyo, Japan) equipped with a camera. Gene expression of Bax and pro-caspase 3 was also evaluated in total RNA extracted from large cholangiocytes by real-time PCR as described above [13].