The role of autotaxin in cholestatic pruritus

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CHAPTER 3

ENTEROENDOCRINE CELLS ARE A POTENTIAL SOURCE OF SERUM AUTOTAXIN IN MEN

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CHAPTER 3

ABSTRACT

Objective: Serum autotaxin (ATX) activity is significantly increased in cholestatic patients. Our study aimed to unravel the source(s) of ATX in cholestasis.

Materials and methods: ATX activity and protein were measured in sera of healthy (n=33) and cholestatic patients (n=152), including women with intrahepatic cholestasis of pregnancy. ATX mRNA and protein expression were analyzed in various tissues from mice and men. Induction of ATX activity was assessed in mouse models of extrahepatic (bile duct ligation) and intrahepatic cholestasis (Atp8b1<sup>G308V/G308V</sup>, 0.1% cholate-supplemented diet). ATX clearance in cholestatic and control mice was assessed after intravenous injection of recombinant ATX. Human hepatic clearance was estimated by comparing ATX activity in portal and hepatic vein serum.

Results: Serum ATX activity and ATX protein concentration tightly correlated under all conditions in patients and controls (p<0.0001). In humans ATX mRNA was highly expressed in small intestine, whereas in mice Atx was expressed mainly in brain and placenta but not in small intestine. Extensive ATX protein expression was identified in human, but not murine intestinal enteroendocrine cells. In murine models of cholestasis and cholestatic pregnancy plasma ATX activity was only mildly elevated (up to 2.1-fold). Atx tissue expression and rATX clearance after parenteral administration did not differ between cholestatic and control mice.

Conclusions: Serum ATX activity during cholestasis and itch is enhanced by increased protein concentration rather than enzymatic induction. In mice, clearance of ATX is not affected by cholestasis. Small intestinal ATX expression by enteroendocrine cells might represent an important source of cholestasis-induced serum ATX activity in men.

INTRODUCTION

Serum autotaxin (ATX) is a lysophospholipase D (lysoPLD) and the major source of extracellular lysophosphatidic acid (LPA).<sup>1,2</sup> ATX and LPA serum levels tightly correlate in humans and mice.<sup>3-5</sup> LPA mediates diverse (patho)physiological processes through 6 different G-protein coupled LPA-receptors (LPAR<sub>1</sub>-<sub>6</sub>).

Numerous cell types express ATX.<sup>7-10</sup> Increased ATX and/or LPAR expression levels are described in a yet increasing variety of tissues during embryonic development as well as disease, reviewed in.<sup>11</sup>

Moreover, increased activity of ATX secreted in the systemic circulation was recently proposed to contribute to the pathophysiology of pruritus in various cholestatic liver diseases<sup>12,13</sup> and in atopic dermatitis (AD).<sup>14,15</sup> Increased serum ATX activity is a feature of uncomplicated pregnancy, but a marked elevation is diagnostic for intrahepatic cholestasis of pregnancy (ICP),<sup>16</sup> a cholestatic condition during pregnancy by definition accompanied by pruritus.

The source of increased serum ATX activity in pruritus of cholestasis remains to be unravelled. A possible mechanism might be an increase of the actual serum ATX protein concentration, which is only supported by scarce data from cholestatic patients and pregnant women (western blot)<sup>12</sup> as well as patients with atopic dermatitis (ELISA).<sup>13</sup> Whereas adipose tissue is known to be a significant contributor to serum ATX levels in mice,<sup>18</sup> the only (easily accessible) human tissue in which ATX expression was studied as a potential source of elevated serum levels is placenta. No difference in placental ATX mRNA levels between patients with ICP and pregnant controls was observed although serum ATX activity was markedly higher in ICP patients,<sup>17</sup> virtually excluding the placenta as a major source of elevated serum ATX activity in ICP. As an alternative explanation for elevated ATX activity in cholestasis-associated pruritus such as in ICP, serum factors inhibiting or inducing the enzymatic activity of ATX might be altered during disease, as was proposed for atopic dermatitis.<sup>16</sup> A third optional explanation might be decreased clearance of serum ATX under cholestatic conditions, which was proposed to be mediated by sinusoidal cells in the liver.<sup>19</sup>

In the current study, we aimed to identify the source(s) of increased ATX lysoPLD activity during cholestasis, cholestasis-associated pruritus and (cholestatic) pregnancy.

METHODS

Human subjects: Healthy control tissue samples were obtained anonymously from surgical resection specimens. Serum samples from (pregnant) controls and cholestatic patients were obtained in the Academic Medical Center in Amsterdam, Hospital Clinic in Barcelona as well as the Friedrich-Alexander-University Hospital of Erlangen. All sample collection was performed after informed consent in accordance with the Declaration of Helsinki.

Experimental animals: Experiments were performed with female C57Bl/6 wild type (Harlan Laboratories) and Atp8b1<sup>G308V/G308V</sup> mice (C57Bl/6 background, bred in the Academic Medical....
Center, Amsterdam, hereafter referred to as Atp8B1 mutant). For experiments with pregnant mice, the day of conception was determined by inspection of vaginal plugs. Mice were between 8 weeks and 8 months of age and housed conventionally with ad libitum drinking water and, unless stated otherwise, semi-synthetic diet (20% casein, ref. no. 4068.02, Arie Blok BV, The Netherlands) either or not supplemented with 0.1% cholic acid (0.1%CA). Bile duct ligation was performed in wild type animals as a model of extrahepatic cholestasis by ligature and resection of the common bile duct and the gallbladder. Blood withdrawal by cheek vein puncture was performed weekly to determine plasma ATX activity levels and concentrations of total bile salts and bilirubin.

In order to investigate if cholestasis affects clearance of ATX, we injected rATX intravenously via a vena jugularis canula in a dose of 1 mg which led to a serum ATX activity of about 10 times of normal right after injection. Repetitive withdrawal of a few drops of blood for plasma ATX activity measurements was done from a tail wound at different time points within an hour after rATX injection. Experiments were conducted according to the institutional Animal Care and Use Committee regulations.

**Generation and purification of recombinant ATX:** HEK293T cells overexpressing his-tagged autotaxin (ATX^HIS) were cultured at ~70-100% confluency in FCS-free medium for ~72 hours. The medium was collected and concentrated about 10 times using Pierce® Concentrators (20K MWCO/20mL, Thermo Scientific). Medium was incubated overnight at 4°C with nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). A column of packed beads was made and washed with a 20mM imidazole buffer before eluation of the rATX with 500mM imidazole. Fractions with highest ATX activity were pooled and dialysed using a Slide-A-Lyzer^® Dialysis Cassette (Thermo Scientific) against 0.9% NaCl pH7.4 at 4°C. Purity of the preparation was tested by SDS-PAGE and Coomassie staining.

**Autotaxin lysoPLD activity assay:** As described before, serum or plasma samples were incubated in a 1:40 dilution for 60 minutes at 37°C with 1mM LPC 14:0 in a buffer of 500mM NaCl, 5mM MgCl_2, 100mM Tris (pH 9.0), and 0.05% Triton X-100. The lysophospholipase D (lysoPLD) activity of ATX was measured and calculated by the amount of liberated choline in the incubated sample after subtraction of the endogenous choline of the same sample incubated in buffer without LPC. Choline was detected by increase in fluorescence after addition of 2U/mL choline-oxidase and 2mM homovanillic acid in a 2500U/mL peroxidase, 50mM 3-[(N-morpholino)propanesulfonic acid (pH 8.0), 20mM CaCl_2, 0.1% Triton X-100, at 37°C in a NOVostar analyzer (excitation 320nm and emission 405nm; BMG Labtech GmbH, Offenburg, Germany). Inter-assay variance of the assay was <15%, intra-assay variance <10%.

**Autotaxin immunoassay:** The Quantikine sandwich ELISA kit (R&D systems DENP20) was used to determine ATX protein concentration in human sera. Reagents and samples were prepared according to the manual, using dilutions from 20-fold for healthy controls up to 160-fold for ICP sera.

**HPLC for determination of total serum bile salt concentration:** Bile salt species were separated and quantified by reverse-phase HPLC, as published before. For this purpose, plasma samples (20µL) were deproteinized by addition of 5 volumes of acetonitrile. Following centrifugation (10min, 20000g), solven was evaporated from supernatants and bile salts were solubilized in 200µL 25% methanol. 100µL sample was applied to a Hppers C18 HPLC column (internal diameter: 3µm, column length: 15cm; Thermo Scientific, Breda, The Netherlands) operated at 20°C. The starting eluent consisted of 6.8mM ammoniumformate (pH 3.9), followed by linear gradient or isocratic elution with acetonitrile at the indicated concentration: 27% (1min), 42% (13min), 42% (19min), 60% (20min), 66% (26min), 80% (27min), 80% (29min) and 0% (30min). The flow rate was 0.8mL perminute. Detection was performed using a Nano Quantity Analyte Detector QT-500 (Quan technologies, Blaine, USA). Quantification of the different bile salt species was performed using a calibration curve for all different bile salt species.

**Immunohistochemistry:** After deparaffinization, tissue slides were heated to 37°C (for pepsin buffer) or 98°C (for sodium citrate and Tris-EDTA buffers) as indicated in table 1. Slides were incubated with Ultra-V block (ThermoScientific) for 30 minutes to diminish background. Primary antibodies and dilutions used for incubation are described in table 1. For ATX, an unlabeled rabbit-anti-rabbit antibody (Southern Biotech 6130-01), incubated 1:2000 for 15 minutes at room temperature, was used as a bridge to the secondary poly-AP labeled anti-rabbit antibody (Bight Vision, Immunologic, diluted 1:1, 30 minutes incubation at room temperature). Goat-anti-rabbit and -mouse AP labeled secondary antibodies (Dako D0487 and Biorad 170-6520), incubated 1hr at room temperature in a 1:1000 dilution, were used for all other stainings. Vector blue and red AP substrate kits (Vector lab) were used to develop (co-)staining. In all procedures, a section devoid of primary antibody was taken along as a negative control.

**Real Time Quantitative polymerase chain reaction:** Extraction of mRNA from tissues was performed by Iysis in TriPure Isolation Reagent (Roche, 11667165001), subsequent incubation with chloroform followed by centrifugation and precipitation by iso-propanol. Samples were stored at -80°C until further processing. cDNA was synthesized from 1.5ug total mRNA after DNase treatment (Promega, M6101) by an oligoDT primer and Superscript III reverse transcriptase (Invitrogen). A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. Real-time qPCR analyses were performed in a Lightcycler apparatus (Roche, Mannheim, Germany) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche, 4070516001). Transcript levels were normalized to the geometric mean of at least 2 reference genes. Primer sequences for each gene are depicted in table 2.

**Statistics:** Pearson’s correlation was calculated between serum ATX activity and ATX protein concentration. Statistical differences between two groups were evaluated by unpaired Student’s t-test, differences in animals within the same group at two different time points by paired Student’s t-test. The difference between three or more groups was estimated by one-way analysis of variance, either or not for repeated measurements. For the in vivo rATX clearance experiment, the area under the curve for each animal separately was calculated and subsequently the two
groups were compared using an unpaired Student's t-test. Calculations were performed with GraphPad Prism version 6.0.

Table 1. Procedure and antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Target</th>
<th>Antigen retrieval</th>
<th>Primary antibody</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotaxin (ATX)</td>
<td>100mM Tris-EDTA, pH 9.0</td>
<td>Rat monoclonal 4F1, provided by J. Aoki</td>
<td>1:20,000, 60hrs at 4°C</td>
</tr>
<tr>
<td>Chromogranin A (CgA)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, GeneTex GTX 15160</td>
<td>1:2000, 1hr at RT</td>
</tr>
<tr>
<td>Glucagon</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit monoclonal, Thermo Scientific RB1422A</td>
<td>1:200, 1hr at RT</td>
</tr>
<tr>
<td>Glucagon like peptide 1 (GLP1)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit monoclonal, LifeSpan Bioscience LS-C138187</td>
<td>1:250, 1hr at RT</td>
</tr>
<tr>
<td>Insulin</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Mouse monoclonal, Thermo Scientific MS1379</td>
<td>1:1000, 1hr at RT</td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td>0.5% pepsin 5mM HCl, pH 2.0</td>
<td>Mouse monoclonal, Thermo Scientific MS1431S</td>
<td>1:100, 1hr at RT</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, Dako A0566</td>
<td>1:3200, 1hr at RT</td>
</tr>
<tr>
<td>Substance P (SP)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, Amersham RPN1572</td>
<td>1:2000, 1hr at RT</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit monoclonal, Thermo Scientific MA1-39558</td>
<td>1:150, O/N at 4°C</td>
</tr>
<tr>
<td>Transmembrane G-protein coupled Receptor 5 (TGR5)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, Thermo Scientific PA5-27076</td>
<td>1:1000, O/N at 4°C</td>
</tr>
<tr>
<td>Tuft cells (Dclk1/DCAMKL1)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, Abcam AB31704</td>
<td>1:200, O/N at 4°C</td>
</tr>
<tr>
<td>Vasointestinal peptide (VIP)</td>
<td>10mM sodium citrate, pH 6.0</td>
<td>Rabbit polyclonal, Monosan PSX1016</td>
<td>1:1000, 1hr at RT</td>
</tr>
</tbody>
</table>

Table 2. Primer sequences used for RT-qPCR experiments.

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>hATX</td>
<td>TGCATAGCTCGAGAGGACGA</td>
</tr>
<tr>
<td>h36B4</td>
<td>TACATGCCGCTCAAAACA</td>
</tr>
<tr>
<td>hHPRT</td>
<td>AGTCCGCGTGACCGCTGGAT</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>CTGAGCTGCGTCGAGATCTG</td>
</tr>
<tr>
<td>mATX</td>
<td>AGAAAGAATGGGCTACAGG</td>
</tr>
<tr>
<td>m36B4</td>
<td>GGACCGGAGAGACCTCCCTT</td>
</tr>
<tr>
<td>mUbiquitin</td>
<td>AGCCCGATTCAGCGACCAAG</td>
</tr>
</tbody>
</table>

RESULTS

Serum autotaxin activity and protein levels tightly correlate in men

The difference in serum ATX activity between control and cholestatic patients could be caused by a possible induction of ATX's enzymatic activity, e.g. by circulating cholephiles. To distinguish this from an actual increase in ATX protein concentration, we measured both ATX protein and activity in sera from non-pregnant and pregnant controls, cholestatic patients with and without itch and women with ICP. Patient characteristics by means of serum liver tests are provided in table 3, per subgroup of patients included. As can be derived from the table, most cholestatic patients were PBC patients. Non-PBC patients represent a rather heterogeneous group, as indicated in the legend. Figure 1 shows a strong correlation (p<0.0001, Pearson's linear correlation) between ATX activity and ATX protein concentration that endures within all groups indicated. This indicates that increased ATX activity associated with pruritus is not caused by activation of the enzyme but must be attributable to increased ATX protein synthesis and/or reduced clearance.

Clearance of autotaxin from the circulation in mice

We subsequently addressed the question whether the clearance of ATX is affected during cholestasis. It has been reported that in mice clearance of ATX is mainly mediated by endothelial cells of the liver. To further investigate clearance of ATX under normal and cholestatic conditions, we intravenously injected recombinant ATX (rATX) in mice and analyzed its disappearance from the peripheral circulation. As a model for cholestasis we used Atp8b1

Figure 1. Correlation between ATX activity and ATX protein concentration in serum samples from healthy controls (p=0.01, r²=0.33, n=18, open circles), cholestatic patients without pruritus (p=0.0001, r²=0.60, n=41, black dots), cholestatic patients with pruritus (p<0.0001, r²=0.86, n=99, black squares), pregnant controls (p<0.0001, r²=0.87, n=15, open triangles) and women with ICP (p<0.0001, r²=0.91, n=12, black triangles).
Table 3. Serum liver tests of patient groups included in figure 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Bilirubin (μmol/L)</th>
<th>ALP (U/L)</th>
<th>γGT (U/L)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>Gestation (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>12</td>
<td>&lt;5.0</td>
<td>143.6 ± 49.6</td>
<td>38.1 ± 13.1</td>
<td>18.1 ± 8.4</td>
<td>36.0 ± 2.4</td>
<td>n/a</td>
</tr>
<tr>
<td>Cholestasis without pruritus - PBC</td>
<td>35 (85%)</td>
<td>20.8 ± 40.8</td>
<td>260.5 ± 190.4</td>
<td>99.6 ± 138.6</td>
<td>54.3 ± 66.6</td>
<td>40.4 ± 57.7</td>
<td>n/a</td>
</tr>
<tr>
<td>Cholestasis without pruritus - non-PBC*</td>
<td>6 (15%)</td>
<td>78.8 ± 87.8</td>
<td>403.2 ± 305.8</td>
<td>279.7 ± 275.9</td>
<td>88.7 ± 51.9</td>
<td>54.3 ± 66.6</td>
<td>n/a</td>
</tr>
<tr>
<td>Cholestasis with pruritus - PBC</td>
<td>77 (78%)</td>
<td>27.1 ± 49.1</td>
<td>435.9 ± 486.0</td>
<td>156.1 ± 172.7</td>
<td>59.2 ± 48.7</td>
<td>59.2 ± 48.7</td>
<td>n/a</td>
</tr>
<tr>
<td>Cholestasis with pruritus - non-PBC</td>
<td>22 (22%)</td>
<td>96.7 ± 121.0</td>
<td>395.8 ± 226.7</td>
<td>245.0 ± 338.7</td>
<td>99.3 ± 82.1</td>
<td>118.0 ± 73.1</td>
<td>n/a</td>
</tr>
<tr>
<td>Pregnant controls</td>
<td>15</td>
<td>&lt;5.0</td>
<td>143.6 ± 49.6</td>
<td>38.1 ± 13.1</td>
<td>18.1 ± 8.4</td>
<td>36.0 ± 2.4</td>
<td>n/a</td>
</tr>
</tbody>
</table>

The clearance of intravenously injected recombinant autotaxin (rATX, 1μg) in cholestatic mice (n=7, black squares) was similar to non-cholestatic controls (n=7, open circles). Data represent mean ± S.E.M. (B+C) Plasma ATX activity was similar in samples from portal and hepatic veins in control (B) and cholestatic (C) mice, one hour after rATX injection. Horizontal bars represent the median. (D) ATX immunostaining of mouse liver sacrificed 60 minutes after iv injection shows accumulation of rATX in the cells lining the sinusoidal spaces.
in the sinusoidal spaces, which most likely represents uptake by sinusoidal endothelial cells (figure 2D). As will become apparent in figure 5H, no such pattern could be seen in livers of control or cholestatic mice without injection of ATX.

**Hepatic clearance of autotaxin from the circulation in men**

In attempt to estimate the role of the liver in the clearance of ATX from the circulation in humans, we sampled blood from vena portae and vena hepatica from patients during transjugular intrahepatic portosystemic shunt (TIPS) placement (n=14). As depicted in figure 3, serum ATX activity was significantly lower in vena hepatica compared to vena portae (p=0.03, paired t-test).

**Autotaxin mRNA expression pattern in mice and men**

In an attempt to identify the source of increased serum autotaxin during cholestasis, Atx expression was quantified by qPCR in a wide range of organs from cholestatic and control mice (non-pregnant, except for placenta). Atx expression in mice was high in brain and placenta with lower levels in liver, lung, kidney, spleen and adipose tissue. None of the organs from cholestatic mice showed a significant upregulation of Atx expression compared to non-cholestatic controls (figure 4A). Instead, Atx mRNA expression in placenta was decreased during cholestatic pregnancy.

In contrast to the murine expression pattern, a screening of human healthy control organs revealed high ATX mRNA expression in human intestine – comparable to adipose tissue levels – whereas expression in liver was low (figure 4B).

**Autotaxin is expressed in intestinal enteroendocrine cells of men, but not mice**

To further characterize the ATX expression in small intestine, we performed immunohistochemistry on a wide range of tissues of both humans and mice. Figure 5 shows that ATX (in blue) was prominently present in a subset of human healthy control small intestine epithelial cells (figure 5A+C, pictures representative for staining pattern in duodenum, jejunum and ileum slides) as well as in pancreatic islets (figure 5E+G). Co-staining with chromogranin A (CgA, in red, figure 5C+G) identified these cells as enteroendocrine cells (EECs).

As can be seen in figure 6, ATX-immunoreactivity was also detected in mouse pancreatic islet EECs (figure 6D+F), but mouse small intestine was completely devoid of ATX (figure 6A+C), despite the presence of CgA positive EECs (figure 6B+C). Slides of human and
mouse choroid plexus, known to highly express ATX, were stained simultaneously as a positive control for the staining procedure (figure 5H and 6G).

In an attempt to further specify these ATX positive cells, co-staining for different markers for subsets of EECs and/or tuft cells was performed. The EEC markers glucagon, glucagon like peptide 1 (GLP1), insulin, serotonin (5-HT), somatostatin, substance P (SP), synaptophysin or vasoactive intestinal polypeptide (VIP) showed only marginal or no overlap with ATX (data not shown). In addition, there was no co-localization with the bile acid receptor TGR5 (figure 5D) that is thought to be expressed by EECs as immunoreactivity was merely seen in crypt paneth cells. ATX positive cells in small intestine and pancreas did also not show overlap with the tuft cell marker double cortin-like kinase 1 (DCLK1). Of note, even during cholestasis no ATX staining was seen in human liver (figure 5I), and only weak positivity in mouse liver (figure 6I).

Plasma autotaxin activity is only moderately induced in mice upon acute and chronic cholestasis as compared to humans

In wild type mice (n=5), complete ligation of the common bile duct (BDL) was conducted as a model of acute extrahepatic cholestasis. Chronic intrahepatic cholestasis was induced, as published before, by feeding a 0.1% cholate-supplemented diet to Atp8b1 mutant mice for 2 to 3 weeks (n=17-22). Cholestasis was confirmed in both models by increase of total plasma bile salts (figure 7A+B) and bilirubin (data not shown), as compared to sham operated (n=5) and wild type controls fed the same diet (n=7-19). Plasma ATX was transiently but moderately induced from 4.3 ± 0.1 to 6.6 ± 0.4 nmol·min⁻¹·mL⁻¹ (mean ± S.E.M; p=0.0028, paired t-test) on the first day after BDL (compared to sham-operated animals) (figure 7C). In Atp8b1 mutant mice ATX activity rose from 6.2 ± 0.4 to 11.5 ± 1.0 nmol·min⁻¹·mL⁻¹ (p<0.0001, paired t-test) after 1 week of 0.1% cholate diet. The induction remained stable during 3 weeks of cholestasis. After that period the mice had to be sacrificed because of excessive weight loss.

Plasma autotaxin activity is moderately induced during pregnancy in mice

We measured induction of ATX during pregnancy in wild type C57Bl6 (n=7) and Atp8b1 mutant mice (n=7). As a potential mouse model for ICP, another group of Atp8b1 mutant mice was fed with a 0.1%CA diet (n=7) starting at day 12 of pregnancy. Cholestasis was confirmed in the latter group by means of total plasma bile salts (figure 8A, p=0.13, paired t-test day 8 vs. day 15) and serum bilirubin (data not shown). Plasma ATX on average induced 2.1-fold to 12.6 ± 1.3 nmol·min⁻¹·mL⁻¹ at day 18 (mean ± S.E.M.; p=0.0003, paired t-test) with no significant difference between the three groups (figure 8B, p=0.78, one-way ANOVA on three groups at day 18). Hence, the combination of cholestasis with pregnancy did not lead to an additional rise in serum ATX activity, as is the case in women with ICP, who on average have a 16-fold induced serum ATX activity.12
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DISCUSSION

The lysophospholipase D, autotaxin (ATX), provides targeted LPA release and signaling in a broad range of (patho)physiological processes. A physiological role of the abundant ATX in the systemic circulation has not yet been identified. In cholestasis and in atopic dermatitis serum ATX and LPA levels correlate with the intensity of pruritus (itch).

We therefore hypothesized that ATX-derived LPA plays a key role in systemic pruritus of patients with cholestatic liver disease. Till date, the origin of increased serum ATX activity during cholestasis and pregnancy was unknown. We hypothesized that increased activity could be the result of an increase in protein concentration (due to either increased production or reduced clearance) or increased enzymatic activity.

In the current study, we provide evidence that enhanced ATX activity correlates well with increased ATX protein concentration during cholestasis and pregnancy (Figure 1). Hereby we have ruled out the possibility of ATX enzyme activation in these conditions, e.g. by circulating cholephiles. ATX clearance in mice was, as reported before, very rapid but it was unaffected by cholestasis (Figure 2A). Serum ATX seems to be cleared by endothelial cells (Figure 2B), but it is likely that the liver is not only the primary site of clearance, given that serum ATX activity was only slightly lower in vena hepatic compared to vena portae, only in humans reaching an acceptable level of significance (Figure 2B+C and 3). However, the human samples were obtained during TIPS procedure in cirrhotic patients possibly accompanied by endothelial dysfunction that may underestimate the extent of clearance of ATX by the liver. Similar, yet invasive sampling would have to be collected from cholestatic patients to definitely reject the hypothesis that the increased serum ATX protein level during cholestasis is caused by a decreased clearance in the liver.

Organ-specific induction of ATX expression during cholestasis is difficult to investigate in humans. We therefore analyzed organ-specific Atx mRNA expression in mice with experimental cholestasis. We did not observe relevant organ-specific upregulation of Atx mRNA expression in cholestatic compared to control animals (Figure 4A). However, the increase in serum ATX activity levels in mice was small compared to that in patients (Figure 7, 8). A striking difference between mice and men was that Atx mRNA expression was low in small intestine of mice, but impressively high in small intestine of humans (Figure 4B). Considering the mass of small intestinal tissue relative to total body weight, the small intestine must be regarded as a relevant source of systemic ATX activity in humans.

Immunochemistry revealed ATX expression in a subset of epithelial cells in the human small intestine (Figure 5A), showing considerable overlap with Chromogranin A (CgA) staining (Figure 5C). This indicated that intestinal ATX expression is localized in enteroendocrine cells (EECs).

EECs have been described as a heterogeneous group of cells with variable and partly overlapping expression patterns for diverse secretory peptides. In our study, most but not all human CgA-positive EECs showed co-staining for ATX (Figure 5F). Co-staining for a range of endocrine markers (Table 1) did not show sufficient expression overlap to attribute the ATX-

Figure 7. Total plasma bile salts increased upon (A) bile duct ligation (BDL, black squares) and (B) 0.1% cholic acid diet (0.1%CA) in Atp8b1 mutant mice (black squares). Plasma ATX activity increased (C) transiently after BDL (black squares) and (D) stably after introduction of 0.1%CA diet in Atp8b1 mutant (black squares) but not wt (open circles) mice. Data represent mean ± S.E.M.

Figure 8. (A) Total plasma bile salts increased during pregnancy only in the Atp8b1 mutant mice after start of the 0.1%CA diet at day 12 of the pregnancy (black squares), mimicking intrahepatic cholestasis of pregnancy (ICP) in humans. (B) Plasma ATX activity increases during the course of the pregnancy to a similar extent in all three groups. Data represent mean ± S.E.M.
positive cells to any of the currently described EEC subpopulations (data not shown). Moreover, no co-expression of ATX with the bile salt receptor TGR5 (figure 5D) or the tuft cell marker Dclk1 (data not shown) was found.

Thus, ATX expressing cells in the small intestine represent a hitherto unreported subset of EECs (figure 5A+C). In small intestine of mice, EECs were present to a similar extent as in humans, as shown by chromogranin A (CgA) expression, but these cells did not show ATX-immunopositivity (figure 6A+C). This striking species difference coincides with our observation that in mice serum ATX activity was increased much less during intrahepatic cholestasis (1.9-fold, figure 7D) and (cholestatic) pregnancy (2.1-fold, figure 8B) than in cholestatic patients (5.5-fold in non-pregnant cholestatic patients with pruritus and 16-fold during ICP,12,13 as can be derived from figure 1. Taken together, we believe small intestinal EECs can be regarded as a major source of serum ATX in humans.

In contrast to humans, cholestatic mice do not develop pruritus, as measured by chronic scratch activity (unpublished observations). The markedly elevated serum ATX activity during cholestasis in humans, but not mice, together with the strong small intestinal expression of ATX by specific EECs in humans but not mice may contribute to development of pruritus in humans. The mechanism of putatively enhanced small intestinal ATX expression during cholestasis requires further research. Unfortunately, collection of intestinal biopsies from cholestatic patients to test this hypothesis cannot be performed due to ethical concerns. Of note, the trigger(s) for ATX production in EECs may come from the luminal as well as the basolateral site, due to changed composition of bile or accumulation of cholephiles in the systemic circulation, respectively.

The fact that cholestatic mice did show some increase in systemic ATX activity levels suggests that there is an accessory systemic, EEC-independent mechanism of ATX upregulation. The sustained increase in plasma ATX activity levels in our mouse models of intrahepatic (figure 7D) but not extrahepatic cholestasis (figure 7C), suggests that enterohepatic cycling is crucial here. The observation that pruritus intensity and serum ATX levels rapidly decrease shortly after disruption of the enterohepatic cycle by nasobiliary drainage in patients12,13 fits with this hypothesis. Still, therapeutic targeting of small intestinal ATX expression might become a promising anti-pruritic strategy in the future.

In conclusion, our study unravels small intestinal EECs as a potential source of serum ATX in humans, but not mice, and stimulated ATX release as a likely mechanism responsible for elevated serum ATX activity during cholestasis and cholestasis-associated pruritus. The species-specific ATX expression pattern could help to understand why humans, but not mice develop itch in cholestasis.

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