Research Article

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Autotaxin activity has a high accuracy to diagnose intrahepatic cholestasis of pregnancy

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Background & Aims: Intrahepatic cholestasis of pregnancy (ICP) is defined by pruritus, elevated total fasting serum bile salts (TBS) and transaminases, and an increased risk of adverse fetal outcome. An accurate diagnostic marker is needed. Increased serum autotaxin correlates with cholestasis-associated pruritus. We aimed at unraveling the diagnostic accuracy of autotaxin in ICP.
Methods: Serum samples and placental tissue were collected from 44 women with uncomplicated pregnancies and 105 with pruritus and/or elevated serum transaminases. Autotaxin serum levels were quantified enzymatically and by Western blotting, autotaxin gene expression by quantitative PCR.
Results: Serum autotaxin was increased in ICP (mean ± SD: 43.5 ± 18.2 nmol ml^{-1} min^{-1}, n = 55, p <0.0001) compared to other pruritic disorders of pregnancy (16.8 ± 6.7 nmol ml^{-1} min^{-1}, n = 33), pre-eclampsia complicated by HELLP-syndrome (16.8 ± 8.9 nmol ml^{-1} min^{-1}, n = 17), and pregnant controls (19.6 ± 5.7 nmol ml^{-1} min^{-1}, n = 44). Longitudinal analysis during pregnancy revealed a marked rise in serum autotaxin with onset of ICP-related pruritus. Serum autotaxin was increased in women taking oral contraceptives. Increased serum autotaxin during ICP was not associated with increased autotaxin mRNA in placenta. With a cut-off value of 27.0 nmol ml^{-1} min^{-1}, autotaxin had an excellent sensitivity and specificity in distinguishing ICP from other pruritic disorders or pre-eclampsia/HELLP-syndrome. Serum autotaxin displayed no circadian rhythm and was not influenced by food intake.
Conclusions: Increased serum autotaxin activity represents a highly sensitive, specific and robust diagnostic marker of ICP, distinguishing ICP from other pruritic disorders of pregnancy and pregnancy-related liver diseases. Pregnancy and oral contraception increase serum autotaxin to a much lesser extent than ICP.

Keywords: Autotaxin; Bile salts; Cholestasis; Pregnancy; Pruritus.
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Introduction

Intrahepatic cholestasis of pregnancy (ICP), also known as obstetric cholestasis, is a pregnancy-specific liver disorder with onset mainly in the third trimester of pregnancy. ICP is characterized by pruritus, elevated serum fasting bile salts and transaminases and an increased risk of adverse fetal outcomes [1–3]. This disorder typically affects 0.2–2% of all pregnant women. The incidence of ICP, however, varies considerably with ethnicity and geographical location, with the highest rates observed in Northern Europe and Southern America [2,4]. Pruritus is the defining symptom of ICP, which progressively worsens as pregnancy advances. Pruritus may considerably reduce quality of life, lead to sleep deprivation, depressed mood, and even suicidal ideation in more severe cases. In contrast to other more commonly observed pruritic dermatoses of pregnancy [5], a concern in ICP is the increased risk of adverse fetal outcomes [1,2]. ICP increases the risk of fetal distress, cardiotocography abnormalities, preterm labour and sudden intrauterine death, particularly in those women with total serum fasting bile salt (TBS) levels exceeding 40 μmol/L [3,4,6,7]. Therefore, a proper diagnosis is essential to enable pharmacological treatment with ursodeoxycholic acid (UDCA), close antenatal monitoring and potentially the induction of labour after 37 weeks, with the aim of reducing fetal distress and intrauterine death [8,9].

The diagnosis of ICP is currently based on the presence of pruritus, raised fasting serum TBS levels above 10 μmol/L, and/or elevated serum transaminases (in the absence of diseases that cause cholestasis or pruritus) as well as spontaneous relief of signs and symptoms within four to six weeks after delivery [1,10]. However,

diagnosis of ICP may be difficult when considering other pregnancy-associated dermatoses, liver diseases and their possible co-existence. The most sensitive marker for ICP is a raised fasting level of TBS, while serum transaminases may be normal in up to 30% of cases [6,11]. However, an asymptomatic elevation of TBS levels, hypercholanaemia, is observed in approximately 10% of pregnant women [12], and has been reported to affect up to 40% of Argentinean pregnancies [13]. In addition, serum TBS increase upon food intake, thereby increasing variation, unless serum is collected upon fasting. Elevated serum transaminases during the 3rd trimester of pregnancy are seen in women with HELLP-syndrome (hemolysis, elevated liver enzymes and low platelet count), pre-eclampsia, acute fatty liver of pregnancy and other non-pregnancy-related liver disorders, including obesity [1,2,6]. These women also hold an increased risk for fetal adverse outcomes, but have an etiology that differs from women with ICP. Furthermore, the management of these conditions is different from that of ICP.

Autotaxin (ATX) is a lysophospholipase D essential for angiogenesis and neuronal development during embryogenesis [14]. Other physiological functions attributed to ATX include cellular motility, proliferation, and lymphocyte homing [15]. The effects of ATX are largely mediated by the enzymatic formation of lysophosphatidic acid (LPA), which may act via one of at least six different LPA receptors [14,16]. ATX levels have been reported to be increased during pregnancy and correlate positively with gestational age [17]. To identify the pruritogens of cholestasis, we recently screened sera from ICP women for activation of neuronal cells and identified LPA as a potent neuronal activator [18]. LPA and ATX levels were significantly increased in ICP women compared to gestation-matched pregnant controls. LPA could be related to pruritus during ICP as intradermal injection of LPA in mice caused a dose-dependent scratch response [18].

In this study, we analyzed serum ATX levels in women with ICP, other pruritic dermatoses of pregnancy and pregnancy-related liver disorders, to determine whether ATX may represent a diagnostic marker for ICP. Furthermore, ATX expression in placental tissue was analyzed to determine the source of increased circulating ATX levels during ICP, as placenta was suggested to be the source of enhanced serum ATX during uncomplicated pregnancy. Finally, the influence of oral female steroid hormones and food intake on serum ATX was determined.

Materials and methods

Human subjects

Peripheral venous whole blood samples were collected prospectively from pregnant women with pruritus and/or elevated serum transaminases, as well as newborn babies and placental tissue from pregnant women after delivery, who were seen at the Women's and Children's Clinic, Academic Medical Center, University of Amsterdam, Amsterdam, from December 2005 until March 2010, and via the research team at Queen Charlotte's and Chelsea Hospital, London, from January 2006 to June 2010. Samples were also taken from non-pregnant women with a history of ICP and controls with a previous uncomplicated pregnancy. ICP cases and healthy volunteers were only enrolled after giving informed consent. The study was conducted according to the Declaration of Helsinki and approved by the local Medical Ethical Committees (Reference numbers: 21233.018.07, 05.17.0936, and 08/H0707/21). Blood samples were allowed to clot for an hour before they were centrifuged for 10 min at 1000*g* and 4 °C. The serum supernatant was aliquoted and cryopreserved at −80 °C until measurements were performed. Placental tissue was snap frozen using liquid nitrogen and cryopreserved at −80 °C for later RNA isolation.

ICP was diagnosed in pregnant women with pruritus, but without rash, in conjunction with raised serum transaminases and/or fasting serum TBS (>10 μmol/L), as described previously [1,10]. If ICP was suspected but TBS and transaminases were normal at first presentation, measurements were repeated weekly and patients only classified as ICP if these parameters became abnormal. Most of the women diagnosed with ICP received UDCA treatment according to guidelines [8]. Women were excluded if they had signs of acute or chronic hepatitis infection (hepatitis A, B or C), other non-viral hepatitis etiologies or extrahepatic biliary obstruction, following ultrasound examination. Pregnant and non-pregnant controls had no history of liver dysfunction or any complication in the current or previous pregnancies.

Pruritic dermatoses of pregnancy consisted of atopic eruption of pregnancy and polymorphic eruption of pregnancy without raised serum transaminases or serum TBS levels, as defined recently [19].

The diagnosis of HELLP-syndrome was defined according to the National Heart, Lung and Blood Institute Working Group criteria [20] and was based on hemolysis (haptoglobin <0.20 g/L and/or lactate dehydrogenase (LDH) >600 IU/L), elevated aspartate aminotransaminase (AST) and/or alanine aminotransaminase (ALT) >70 U/L and low platelet count (platelets $<100 \times 10^9$/L). Pre-eclampsia was defined as arterial hypertension with two blood pressure measurements $\geqslant$140/90 mmHg more than 4 h apart or a diastolic blood pressure of $\geqslant$110 mmHg combined with proteinuria (>300 mg/24 h) that developed after 20 weeks of gestation in a formerly normotensive woman.

Healthy controls consisted of women and men without a significant past medical history. Women on oral contraceptives took either a combined pill containing both estrogen and progestin or a progestin-only pill.

All autotaxin and total serum bile salt measurements were performed by observers blind to patient status, and results were interpreted without knowledge of diagnosis.

Materials

Choline oxidase, horseradish peroxidase, and homovanillinic acid were purchased from Sigma-Aldrich (St. Louis, MO); Myristoyl-lysophosphatidylcholine (LPC 14:0) was from Avanti Polar Lipids (Alabaster, AL).

Autotaxin activity assay

ATX activity was quantified as recently described [21]. Briefly, serum samples were diluted and incubated with a buffer containing 1 mmol/L of LPC 14:0 for 60 min at 37 °C. The lysophospholipase D activity of ATX was determined by the amount of liberated choline, using an enzymatic fluorimetric method. Samples were added to a buffer containing choline oxidase (2 U/ml), horseradish peroxidase (1.6 U/ml), and homovanillinic acid as substrate for peroxidase. The increase in fluorescence was monitored at 37 °C on a Novostar analyzer. Both the interassay and the intra-assay variance of the assay was <10%.

RNA isolation and quantification of transcript levels

Total RNA was extracted from placental tissue using Trizol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized from total RNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Real-time PCR measurements were performed at 60 °C in a Lightcycler apparatus (Roche, Mannheim, Germany) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to the housekeeping gene, 36b4 (acidic ribosomal phosphoprotein P0). For qPCR experiments, the following primer sequences were used: *ATX* forward: TGCAATAGCTCAGAGGACGA; *ATX* reverse: AGAAGTCCAGGCTGGTGAGA; *36B4* forward: TCATCAACGGTACAAACA; and *36B4* reverse: GCCTTGACCTTTTCAGCAAG; *HPRT* forward: AGTTCTGTGGCCATCTGCTT; *HPRT* reverse: GTTAAACAACAATCCGCCCA; *GAPDH* forward: GTCAGTGGTGGACCTGACCT; *GAPDH* reverse: TGAGCTTGACAAAGTGGTCG.

Total serum bile salt determination

Serum TBS levels were quantified using Diazyme total bile salts kit (Diazyme Laboratories, Poway, CA), according to manufacturer's instructions.

SDS-PAGE and Western blotting

ATX was extracted from 20 μl of serum samples by incubation with immunoprecipitating ATX-antibody 5E5 (kindly provided by J. Aoki) [22] bound to sepharose for 4 h at 4 °C. After washing, sepharose beads were incubated for 10 min at 37 °C