Molecular mechanisms of pruritus in cholestasis
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Lysophosphatidic acid is a potential mediator of cholestatic pruritus


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LPA is a potential mediator of cholestatic pruritus
ABSTRACT

Background & Aims: Pruritus is a common and disabling symptom in cholestatic disorders. Its causes remain however unknown. We hypothesized that potential pruritogens accumulate in the circulation of cholestatic patients and activate sensory neurons.

Methods: Cytosolic free calcium ([Ca$^{2+}$]) was measured in neuronal cell lines by ratiometric fluorimetry upon exposure to serum samples of pruritic patients with intrahepatic cholestasis of pregnancy (ICP), primary biliary cirrhosis (PBC), other cholestatic disorders, and pregnant (PC), healthy and nonpruritic disease controls. Putative [Ca$^{2+}$]$_i$ inducing factors in pruritic serum were explored by analytical techniques including quantification by HPLC-MS. In mice, scratch activity after intradermal pruritogen injection was quantified using a magnetic device.

Results: Transients in neuronal [Ca$^{2+}$]$_i$, induced by pruritic PBC and ICP sera were higher than corresponding controls. Lysophosphatidic acid (LPA) could be identified as major [Ca$^{2+}$]$_i$-agonist in pruritic sera and LPA concentrations were increased in cholestatic patients with pruritus. LPA injected intradermally into mice induced scratch responses. Autotaxin (ATX), the serum enzyme converting lysophosphatidylcholine into LPA, was markedly increased in ICP vs. PC (P<0.0001) and cholestatic patients with vs. without pruritus (P<0.0001). ATX activity correlated with intensity of pruritus (P<0.0001), which was not the case for serum bile salts, histamine, tryptase, substance P or μ-opioids. In PBC patients who underwent temporary nasobiliary drainage both itch intensity and autotaxin activity markedly decreased during drainage and both returned to pre-existent levels after drain removal.

Conclusions: We suggest that LPA and ATX play a critical role in cholestatic pruritus and may serve as potential targets for future therapeutic interventions.
INTRODUCTION

Chronic pruritus is a disabling symptom accompanying a broad range of systemic disorders such as chronic liver diseases, chronic renal failure, malignancies, infections, endocrine and hematological diseases.\(^1,2\) Despite the recent discovery of itch-specific neuronal pathways, including novel itch mediators and their receptors,\(^1,3,4\) the pathogenesis of pruritus remains enigmatic. Regardless of the underlying cause, various cholestatic disorders, such as intrahepatic cholestasis of pregnancy (ICP), benign recurrent intrahepatic cholestasis (BRIC), progressive familiar intrahepatic cholestasis (PFIC), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) often induce pruritus. These cholestatic liver disorders are all characterized by an impairment of hepatocellular and/or cholangiocellular secretory function and bile flow.\(^5\) In these patients pruritus may become refractory to all medical treatments and can in severe cases be an indication for liver transplantation, even in the absence of liver failure.\(^6\) In the past, both enhanced serum levels of bile salts and \(\mu\)-opioids have been implicated in the etiology of cholestatic pruritus.\(^7\) However, neither correlations between itch intensity and bile salt or opioid levels nor a causative link have ever been established.

Autotaxin (ATX) was originally identified in the conditioned medium of human A2058 melanoma cells and described as an autocrine cell motility factor.\(^8\) ATX is overexpressed in several other tumor entities and has been linked to tumor cell proliferation, motility and formation of metastasis.\(^9\) Physiologically, ATX is required for angiogenesis and neuronal development, as indicated by ATX-deficient mice which are embryonic lethal due to vascular malformation and neuronal abnormalities.\(^10,11\) Recently, ATX could be identified as an extracellular, secreted enzyme with lysophospholipase D activity, which generates lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC).\(^12,13\) LPA is a small but potent bioactive phospholipid with a wide variety of effects in many cell types ranging from cytoskeletal (re-)organization and cell migration to cytokine production and platelet activation.\(^9,14\) Effects of ATX are believed to be mainly mediated by the enzymatic formation of LPA which activates at least six different G-protein-coupled receptors (GPCRs).\(^9,15\) Most interest in ATX has so far been directed toward its functions in cancer and early development. However, it was recently established that LPA plays a crucial role...
in the induction of neuropathic pain,\textsuperscript{16} and causes reprogramming of gene expression in different types of afferent nerve fibres.\textsuperscript{17}

Here, we report that levels LPA and ATX are markedly increased in serum of patients with cholestatic pruritus. Moreover, serum levels closely correlate with itch intensity and intradermal injections of LPA induce scratching behaviour in mice. We therefore suggest that LPA and ATX play a critical role in cholestatic itch and may serve as potential targets for future therapeutic interventions.

**MATERIALS AND METHODS**

**Human subjects.** Peripheral venous blood was obtained from healthy donors, pregnant women, and patients with cholestatic disorders after informed consent according to the Declaration of Helsinki. The study was approved by the local Medical Ethical Committees. Blood samples were immediately centrifuged at 4°C and serum was frozen in aliquots at -80°C. ICP was diagnosed, as previously described\textsuperscript{18}, in pregnant women with pruritus who had no rash in conjunction with raised serum liver transaminases and/or bile salts. Women were excluded if they had abnormal hepatitis serology (hepatitis A, B and C), or extrahepatic biliary obstruction following ultrasound examination. Pregnant controls had no history of liver dysfunction or any complication in the current or previous pregnancies.

**Animals.** A teflon-coated magnet was implanted in each hind paw of female C57BL/6J mice (6-8 weeks of age) one week prior to experiments. Mice were given 120 minutes to acclimate to the chamber surrounded by a magnetic coil before they were briefly removed from the chamber and intradermally injected with saline (50 μL) or lysophosphatidic acid (8-200 nmol in 50 μL) in the neck. Movements of the magnets induced an electric current in the magnetic field, which was registered by an oscillograph attached to a computer. The number of scratch bouts was analyzed as previously described.\textsuperscript{19} Software was used to count scratch movements with a low cut-off frequency of 10 Hz, a high cut-off frequency
of 20 Hz, a threshold level of 300 mV, a minimum of 4 beats per bout and a maximal coefficient of variation of 40% between the beats of a bout. The analytical procedure was validated with intradermal compound 48/80 showing a positive predictive value of 95% at a sensitivity of 50%. All mouse experiments were approved by the Institutional Animal Care and Use Committee.

**Materials.** Cell culture media were from Lonza (Basel, Switzerland); stearoyl-lysophosphatidic acid (LPA 18:1) and myristoyl-lysophosphatidyl choline (LPC 14:0) from Avanti Lipids (Alabaster, AL); choline oxidase, horseradish peroxidase, homovanillenic acid, pertussis toxin and ionomycine from Sigma-Aldrich (St. Louis, MO); Ki16245 and ATX-antibody for Western Blotting from Cayman (Ann Arbor, MI). Indo-1 AM was from Invitrogen (Carlsbad, CA), Microcon filters from Millipore (Billerica, MA).

**Cell culture.** SH-SY5Y cells were cultured in Ham’s F12K medium containing 10% (v/v) fetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (0.2 mmol/L) at 37°C in a humidified atmosphere of 5% CO2/95% air.

**Fluorimetric measurement of cytosolic free calcium.** SH-SY5Y cells were detached, washed twice and suspended in HEPES-buffered Hank’s balanced salt solution (HBSS). Cells were incubated with 10 μmol/L Indo-1 AM for 30 min at 37°C, washed, re-suspended in HEPES-buffered HBSS and incubated for another 30 min at 25°C to allow dye hydrolysis. After another wash step, cells were resuspended in HEPES-buffered HBSS. Analyses were performed in a Novostar analyzer (BMG Labtech GmbH, Offenburg, Germany; excitation 320 nm, emission 405 nm and 520 nm). Cell suspensions were allowed to adapt to 37°C for 10 min before serum or extracts were added. Receptor blockers were added 10 min prior to addition of serum. Cytosolic free calcium [Ca2+]i was calculated after calibration with ionomycine (10 μmol/L) and EGTA (5 mmol/L) according to Gryniewicz et al.20
Autotaxin activity assay. ATX activity was analyzed as recently described. In short, serum and bile samples were incubated with a buffer containing 1 mmol/L of LPC 14:0, 500 mmol/L NaCl, 5 mmol/L MgCl₂, 100 mmol/L Tris (pH = 9.0) and 0.05% Triton X-100 for 60 min at 37°C. The phosphodiesterase activity of ATX was determined by the amount of liberated choline, as detected by an enzymatic fluorimetric method using choline oxidase (2 U/mL), horseradish peroxidase (1.6 U/mL), and homovanillinic acid as substrate for peroxidase. After addition of both enzymes in a buffer (consisting of 20 mmol/L CaCl₂, 2 mmol/L homovanillinic acid, 50 mmol/L MOPS (pH = 8.0) and 0.1% Triton X-100) the increase in fluorescence was monitored at 37°C on a Novostar analyzer (excitation 320 nm, emission 405 nm).

Quantitative LPA determination. A detailed description of the procedure is given as a supplementary section. Briefly, serum lipids were extracted after addition of myristoyl-LPA as internal standard and analyzed by HPLC-MS.

Bile salt determination. Total serum bile salt levels were determined using Diazyme total bile salts kit (Diazyme Laboratories, Poway, CA) according to the manufacturer’s instructions.

Histamine determination. Serum histamine concentrations were measured by a competitive enzyme immunoassay (Immunotech, Marseille, France) based on the competition between free acylated histamine and alkaline phosphatase acylated histamine conjugate.
**Tryptase determination.** Serum tryptase concentrations were determined by a fluoroenzyme immunoassay (UNICAP Tryptase; Pharmacia Diagnostics, Freiburg, Germany) detecting both α- and β-tryptase.

**Determination of μ-opioid activity.** Total serum μ-opioid activity was determined as described by Swain et al.\(^{22}\)

**Determination of substance P.** Substance P concentrations were analyzed by a competitive enzyme immunoassay according to the manufacturer’s instructions (Bachem, Torrance, CA).

**SDS-PAGE and Western Blotting.** To concentrate ATX, 50 μL of serum and bile samples were first incubated for 4 hours at 4°C with immunoprecipitating ATX-antibody 5E5 (kindly provided by J. Aoki)\(^{10}\) bound to sepharose. After washing, sepharose beads were incubated for 10 min at 37°C with SDS-loading buffer containing β-mercapto-ethanol and spun down. Equal amounts of supernatant were separated by SDS-PAGE, blotted on PVDF membranes, blocked with 5% skim milk in PBS/0.05% Tween-20, and incubated with anti-ATX (1:1500, Cayman) overnight. Proteins were visualized with horseradish peroxidase-conjugated immunoglobulins and detected by enhanced chemoluminescence (Amersham, Buckinghamshire, UK).

**Statistical analysis.** Statistical differences were evaluated for two groups by Student’s \(t\)-test and for three or more groups by one-way ANOVA with Bonferroni correction using SPSS (version 16.0). Spearman’s correlation coefficient and corresponding \(p\)-values were calculated to assess the relationship between tested parameters. All data are expressed as means ± standard deviations (SD).
RESULTS

Neuron-activating serum factor identified as LPA

To identify potential pruritogens in cholestasis, we screened sera of pruritic patients for activation in different neuronal cell lines. We chose cytosolic free calcium ([Ca\(^{2+}\)]\(_i\)) as an indicator of neuronal activation, since [Ca\(^{2+}\)]\(_i\) is a key mediator of the neuronal secretory response towards diverse receptor-dependent and -independent stimuli. In the human neuroblastoma cell line SH-SY5Y we observed a dose dependent rise in intracellular calcium concentrations upon addition of serum (Figure 1a).

Figure 1: Cytosolic free calcium ([Ca\(^{2+}\)]\(_i\)) is increased in human neuroblastoma cell line SH-SY5Y by sera of patients with cholestatic pruritus more than by sera of healthy controls. PANEL A shows that serum induced a transient increase in [Ca\(^{2+}\)]\(_i\), which was dose-dependent. Note that even high serum dilutions of up to 1:320 induced an increase of [Ca\(^{2+}\)]\(_i\). PANEL B: Sera of women with ICP induced higher increases of [Ca\(^{2+}\)]\(_i\), compared to gestation-matched normal pregnancies (PC) and age-matched female controls (HC). PANEL C: Sera of PBC patients with and without pruritus induced a higher increase in [Ca\(^{2+}\)]\(_i\), compared to age-matched healthy female controls. *P<0.015, **P<0.005. \(\Delta[Ca^{2+}]_i\) represents the peak of calcium transient minus basal calcium concentration as shown in Figure 1A: maximal [Ca\(^{2+}\)]\(_i\) – basal [Ca\(^{2+}\)]\(_i\).
Interestingly, sera of women with intrahepatic cholestasis of pregnancy showed a markedly stronger neuronal activation compared to pregnant controls and healthy female controls (Figure 1b). Similarly, sera from patients with primary biliary cirrhosis (PBC) suffering from pruritus tended to induce higher transient rises in [Ca^{2+}]_i levels than sera from PBC patients without pruritus and healthy controls (Figure 1c). We further analyzed the serum samples to identify the neuron-activating serum factor. Pre-treatment of serum with 90% ethanol or proteinase K hardly diminished the [Ca^{2+}]_i response, indicating that the serum factor was not a peptide or protein (Figure 2a). Serum samples were centrifuged through filters to estimate the molecular size. The serum factor could pass a 100 kD filter, but not a 10 kD filter. Interestingly, pre-treatment of serum with 90% ethanol enabled the factor to partially pass through the 10 kD and even a 3 kD filter (Figure 2b). This observation could be explained by strong binding of the factor to albumin (~60kD). Thus, like unconjugated bilirubin, the substance appeared to be partially forced into solution upon ethanol-induced precipitation of albumin. Total recovery of the serum factor through a 10 kD filter was achieved by addition of cholate above its critical micellar concentration enabling a hydrophobic substance to be completely solved in an aqueous solution (Figure 2b). As those micelles have a size of approximately 4.4 kD, they barely pass a 3 kD filter. Hence, we were dealing with a small, hydrophobic substance. Its chemical properties were further analyzed by a two-phase Bligh and Dyer lipid extraction. At neutral pH the compound presented an amphiphilic character but dissolved better in the lower aqueous phase. Lowering the pH to 1.0 and thus potentially protonating the serum factor led to a recovery of the substance mainly in the upper lipid phase (Figure 2c). The increased hydrophobicity upon acidification could be explained by protonation of phosphate- or sulfate-groups in the molecule.

These observations suggested that a small phospholipid could be responsible for the activation of neuronal cells. As pertussis toxin diminished the rise in [Ca^{2+}]_i, signalling of the unknown substance appeared to occur via a G-protein-coupled receptor (Figure 2d). These observations rendered lysophosphatidic acid (LPA) a likely candidate because LPA has been reported to increase calcium in neuronal cells.
Figure 2: Identification of \([\text{Ca}^{2+}]_i\)-enhancing serum factor as lysophosphatidic acid (LPA). PANEL A shows the effect of pre-treatments of serum with 90% ethanol and proteinase K on \([\text{Ca}^{2+}]_i\), in neuronal cells. U: untreated; E: supernatant of 90% ethanol precipitation; Prot K: incubation for 24 hours with proteinase K; (n = 3). PANEL B gives the effect of filter experiments on serum. 100 kD, 10 kD: flow through of 100 kD and 10 kD-filter, respectively; E: supernatant of 90% ethanol precipitation (before filtering); CA: resuspension in 2 mM cholate (before filtering). (n = 3). PANEL C: Effect of Bligh & Dyer lipid extraction at pH values of 7.4 and 1.0. WP pH 7.4, LP pH 7.4, WP pH 1.0, LP pH 1.0: Water phase (WP) and lipid phase (LP) of serum after lipid extraction at pH values of 7.4 and 1.0. (n = 3). PANEL D: Effect of pertussis toxin (PTX) and LPA receptor blocker Ki16245 on cell activation by serum. (n = 3). \(\Delta 405/520\) represents the change in fluorescence at 405nm (\([\text{Ca}^{2+}]_i\)-sensitive signal) divided by that at 520nm (\([\text{Ca}^{2+}]_i\)-insensitive signal). PANEL E: LPA 18:1 is enhanced in ICP cases compared to gestation-matched non-cholestatic pregnant controls. LPA 18:1 is shown as the ratio to LPA 14:0 (added as an internal standard before extraction). *P<0.05, **P<0.01.
LPA is a potential mediator of cholestatic pruritus

Figure 3: Serum ATX is elevated in cholestatic patients with pruritus irrespective of the cause of cholestasis. PANEL A: ATX activity (measured as choline release upon incubation with LPC) is highly enhanced in ICP compared to pregnant controls and healthy female controls. ***P<0.0001. PANEL B: Western blot for ATX protein in patient sera. Recombinant ATX (rATX) was used as a positive control. PANEL C and D: ATX activity was highly enhanced in cholestatic women and men suffering from pruritus compared to non-pruritic cholestatic patients and healthy controls. **P<0.01, ***P<0.0001. PANEL E: Western blot for ATX protein in serum of PBC patients. Recombinant ATX (rATX) was used as positive control. PANEL F: ATX activity in patients with chronic hepatitis C was enhanced compared to controls, but significantly lower compared to cholestatic patients suffering from pruritus. ***P<0.0001.
Pre-treatment of the neuronal cells with Ki16425, a specific LPA-receptor blocker, significantly reduced the \([\text{Ca}^{2+}]_i\) rise, indicating that LPA was the major serum factor in our cholestatic serum samples (Figure 2d).

Analyzing the LPA content in serum samples by mass spectrometry indeed showed markedly higher concentrations of LPA 18:1 in sera of women with intrahepatic cholestasis of pregnancy compared to gestation matched pregnant controls (Figure 2e). Similar differences were observed for other LPA species such as LPA 16:0, 18:0, 18:2, 20:3 and 20:4 (data not shown).

**ATX is enhanced in pruritus of cholestasis**

LPA is formed in the blood through cleavage of choline from lysophosphatidylcholine (LPC) by autotaxin (ATX), which has recently been identified as a lysophospholipase D\(^{12,13}\). As LPC is present in plasma at relatively high concentrations (above 100 μM), the amount of LPA (in low μM range) in blood primarily depends on ATX activity\(^{11}\). Therefore, we analyzed whether ATX activity in blood also correlated with the occurrence of itch. We observed higher ATX activity in sera from women with pruritus due to ICP as compared to pregnant and non-pregnant controls (Figure 3a).
LPA is a potential mediator of cholestatic pruritus

Figure 4: Only ATX activity, but not histamine, nor tryptase, substance P, serum bile salts or μ-opioid activity in serum correlated with itch intensity of patients with cholestatic itch. PANEL A: ATX activity showed a significant linear correlation with the itch intensity represented as visual analogue scale ranging from 0 (no pruritus) to 10 (most severe form of pruritus). Spearman’s correlation coefficient: \( r = 0.7764 \), \( P < 0.0001 \). PANEL B-E: No correlation between histamine levels, tryptase concentrations, substance P levels or total serum bile salts and itch intensity. PANEL F: Total μ-opioid activity in female and pregnant controls compared to women with either ICP, PBC without pruritus or PBC with pruritus. n.s. = not significant.

The enhanced ATX activity correlated with increased ATX protein content in sera from these patients (Figure 3b). We studied whether this observation could be extended to other forms of cholestasis. Therefore, sera of patients with different cholestatic disorders with and without pruritus were analyzed. Quite strikingly, we found that, irrespective of the cause of cholestasis, ATX levels were markedly enhanced in patients suffering from pruritus, compared to patients without pruritus (Figure 3c-e).
Figure 5: Serum levels of ATX respond to therapeutic interventions. **PANEL A:** In PBC patients undergoing nasobiliary drainage (start on day 0), ATX activity dropped with pruritus scores and rose upon reappearance of pruritus several weeks later (day 15-144). Data are shown as percent changes of baseline values in four patients. *P<0.05. **PANEL B:** ATX activity in bile of patients undergoing nasobiliary drainage and in control bile. **PANEL C:** ATX protein could not be detected in bile by western blot. Recombinant ATX (rATX) was used as positive control.

Recently, in patients with chronic hepatitis C, liver ATX mRNA expression\(^{24}\) and serum ATX levels\(^{25}\) have been reported to be enhanced. ATX serum levels were also increased in our group of HCV patients when compared to healthy controls, but were significantly lower than in pruritic patients (Figure 3f).
ATX activity correlates with intensity of pruritus

Pruritus is a subjective perception which differs between individuals. Quantification of this symptom is difficult, but can be achieved using visual analogue scales (VAS). Patients quantified their itch intensity at the time point of blood drawing on a scale ranging from 0 (no pruritus) to 10 (most severe form of pruritus). Next we analyzed the correlation between itch intensities and the ATX activity in serum of these patients by linear regression analysis. We found a highly significant correlation between enzyme activity and intensity of itch perception (Figure 4a). In contrast, other agents proposed as potential pruritogens in cholestasis and other diseases in the past, did not show any correlation with itch intensity in our patient cohort. This was tested for histamine, tryptase, substance P and μ-opioid activity (Figure 4b-f). Even though cholestatic patients with pruritus as a group showed higher serum bile salt concentrations (Table 1), there was no correlation with itch intensity (Figure 4d). A direct role of histamine and bile salts as pruritogens has already been questioned in the past. An anti-pruritic effect of μ-opioid antagonists has been reported in some patients. However, μ-opioid activity was not enhanced in ICP patients compared to regular pregnancies and only very few PBC patients suffering of pruritus had increased μ-opioid levels, questioning a major causative role of opioids for the pathogenesis of pruritus in cholestasis (Figure 4f). In some patients with PBC, extensive long-lasting pruritus was intractable and did not adequately respond to any recommended medication. These patients underwent nasobiliary drainage for 2-7 days as an experimental treatment of most severe pruritus.

In all four procedures, this led to dramatic reduction or complete relief of pruritus within 24 hours which lasted for several days to weeks. Interestingly, concomitant with relief of pruritus, ATX activity dropped and rose back to pre-treatment levels when pruritus returned (Figure 5a). This effect was not due to direct biliary clearance of ATX as neither ATX activity (Figure 5b) nor protein (Figure 5c) could be found in bile of these and other patients.
### Table 1: Characteristics and serum chemistry of cholestatic patients with and without pruritus. All values are expressed as mean ± SD. P-values are for comparison between the subgroups of cholestatic patients with and without pruritus. Abbreviations: AP = alkaline phosphatase, γGT = γ-glutamyltransferase, BS = serum bile salts, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRP = C-reactive protein. The majority of samples were obtained from the outpatient clinic of the Academic Medical Center, Amsterdam. Additional samples were collected at the Universities of Utrecht, Rotterdam, Munich and Navarra.

<table>
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<th>Patients without pruritus (n=25)</th>
<th>Patients with pruritus (n=52)</th>
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<td>5/20</td>
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<td>Age (yrs)</td>
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<td>52.9 ± 13.0</td>
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<td>AP (IU/L)</td>
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<td>γGT (IU/L)</td>
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<td>Bilirubin (mg/dL)</td>
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<td>BS (μM)</td>
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<td>Albumin (g/dL)</td>
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<td>CRP (mg/dL)</td>
<td>0.4 ± 0.5</td>
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**Induction of pruritus by LPA**

To investigate a potential role of LPA in the induction of pruritus *in vivo* we used female C57BL/J6 mice. Scratch movements were registered as described previously. Intradermal injections of LPA, but not the carrier, induced significant scratching behaviour shown as number of scratch bouts per 5-minute intervals (Figure 6a), which was in line with a previous report. Furthermore, we could show that the induction of pruritus by LPA in...
mice was dose-dependent, showing an increased scratching behaviour from 20 nmol upwards (Figure 6b).

**Figure 6:** Dose-dependent induction of scratch responses by LPA in vivo. **PANEL A:** Intradermal injections of LPA (100 nmol) led to increased scratching behaviour compared to vehicle injections being significant during the first 15 minutes. Injections were performed in 7 mice. *P<0.05. **PANEL B:** Dose-dependent scratching behaviour after intradermal injections of LPA. Injections were performed in the indicated number of mice. *P<0.05, **P<0.01, ***P<0.001.

**DISCUSSION**

Pruritus is a common and disabling symptom in cholestatic liver diseases and many other systemic disorders including renal insufficiency, endocrine, hematologic and metabolic diseases, various infections as well as certain malignancies. The causal factors of pruritus are unknown in most of these diseases. Here, we provide clinical and experimental evidence that lysophosphatidic acid (LPA) is a potential mediator of cholestatic itch.
The discovery of itch-specific sensory neurons in the skin by Schmelz et al. revolutionized the research field of pruritus. Thus, primary sensory neurons could be localized that only responded to the pruritogen histamine, but were insensitive to mechanically-induced pain stimuli. Recently, another class of itch-specific sensory neurons has been described that mediates pruritic stimuli independent of histamine. Hence, pruritoceptive nerve fibres seem to consist of different subsets of neurons as was already known for nociceptive nerve fibres. This could explain the varying characters of itch sensations in different diseases ranging from “tickling” over “burning and painful” to agonizing pruritus. We are, however, still far away from an explanation for the itch sensation that is experienced by many patients with systemic disorders.

Cholestatic disorders are frequently accompanied by itch sensation and bile salts and endogenous opioid peptides have, among others, been hypothesized as pruritogens. However, in line with previous reports, we have found no correlation between the severity of itch and these two parameters. Furthermore, although histamine is a well established mediator of pruritus during allergic reactions, its serum levels showed no correlation with itch intensity in our patient cohort in line with the clinical observation that antihistamines are in general ineffective in the treatment of cholestatic itch. Serum tryptase is a marker of mast cell activation and tryptase has been reported to induce pruritus via protease-activated receptor 2 (PAR-2). However, in our study, tryptase concentrations were not enhanced in cholestatic patients and did not correlate with itch intensity. We observed similar negative results for substance P which represents a pruritogen in diseases such as the Sézary syndrome.

In contrast, we have found strong evidence that the occurrence of itch is associated with increased systemic levels of the signalling lipid mediator, lysophosphatidic acid (LPA). Both serum LPA and autotaxin levels were significantly elevated in cholestatic patients with itch as compared to non-pruritic patients. Autotaxin is a lysophospholipase D that hydrolyzes lysophosphatidylcholine into LPA and choline. Serum autotaxin activity in fact closely correlated with the extent of itch perception as objectified by the patient's scoring on a visual analogue scale. Moreover, we found that relief of itch in PBC patients by interruption of the enterohepatic cycle also significantly decreased the serum autotaxin levels, although the molecular mechanism remains yet unclear. It is attractive to speculate
that a yet undefined factor which undergoes enterohepatic circulation may increase autotaxin expression in cholestatic patients suffering from pruritus. We subsequently confirmed the finding reported by others\textsuperscript{31} that intradermal injection of LPA in mice caused short-lasting but significant scratching behaviour which was dose-dependent. Taken together these findings strongly suggest that autotaxin and its formed product, lysophosphatidic acid, play a causative role in the induction of itch during cholestasis.

Strikingly, we observed a much more clear difference between pruritic and non-pruritic patients in serum autotaxin levels than in serum LPA levels and concurrent serum-induced $\Delta[\text{Ca}^{2+}]_i$ in neuroblastoma cells (compare Figs. 1 and 2 vs. Fig. 3). One might argue that serum LPA levels (and the concurrent Ca$^{2+}$ transient) should closely follow the serum autotaxin level, as the latter is responsible for increased LPA levels. Although we did find a clear correlation between serum autotaxin and LPA levels as well as Ca$^{2+}$ transients (data not shown) the range of autotaxin levels was much more dynamic and levels were more consistently increased in pruritic patients. The most likely explanation for this discrepancy is that LPA is a highly unstable lipid derivative that undergoes rapid metabolism in the circulation. In addition, LPA can be formed during and after blood collection and therefore levels depend on the procedure of processing and storage. In contrast, the enzyme autotaxin turns out to be highly stable in vitro and therefore represents a much more direct and reliable parameter.

The source of increased serum autotaxin levels remains to be determined. Enhanced levels could either be caused by increased ATX expression or by reduced clearance of the enzyme. Recently, liver sinusoidal endothelial cells were shown to play an important role in uptake and degradation of ATX.\textsuperscript{38} This is in agreement with our observation that ATX could not be detected in bile. Although ATX has a half life of only several minutes in circulation, its activity can easily be detected in serum, suggesting a continuous synthesis and release by peripheral cells and tissues. Among these may be endothelial cells and adipocytes,\textsuperscript{39,40} but ATX was also reported to be expressed in the liver.\textsuperscript{41} Interestingly, in patients with chronic hepatitis C liver ATX mRNA expression was enhanced,\textsuperscript{24} which might lead to enhanced serum ATX concentrations in these patients.\textsuperscript{25} In our group of HCV
patients ATX levels were higher than in healthy controls but lower compared to cholestatic patients with pruritus.

ATX has originally been described as a motility-stimulating protein secreted from melanoma cells.\textsuperscript{8} Nowadays, the effects of ATX are thought to be mainly mediated by its enzymatic product LPA.\textsuperscript{14} The bioactive lipid LPA is an agonist of a family of at least six G-protein-coupled receptors that promote a great variety of biological processes, ranging from cell motility, proliferation, survival and tumor progression to vascular development and cytokine production.\textsuperscript{14} Furthermore, LPA has been implicated in neuronal cell functions such as brain development and neurite remodelling but also demyelination and after neurotrauma.\textsuperscript{42} In mice, LPA initiates neuropathic pain after a single intrathecal injection.\textsuperscript{16} Mice lacking LPA\textsubscript{1} receptor do not develop any signs of demyelination or neuropathic pain.\textsuperscript{16} It was demonstrated that LPA causes reprogramming of signal transmission through different nerve fibres. While transmission through type 3 A\textalpha;-fibres is increased, the transmission through type 1 C-fibres (involving substance P) is dramatically reduced.\textsuperscript{17} Thus, LPA may induce neuropathic pain via LPA\textsubscript{1} receptors on nociceptive nerve fibres and at the same time contribute to pruritus via LPA receptors on pruriceptive neurons. Alternatively, LPA might indirectly cause pruritus by stimulating the release of a pruritogenic cytokine or lipid mediator from cells located in the skin.\textsuperscript{43}

It is intriguing to speculate on the role of ATX and LPA in the pathogenesis of pruritus in other systemic diseases. Wound healing after injury or surgery is typically accompanied by local itch perception. LPA promotes re-epithelialization and healing of cutaneous wounds.\textsuperscript{44} High local concentrations of LPA might thus elicit itch-specific neurons leading to the well-known desire to scratch a wound during its healing process. Pruritus is also a common symptom in lymphoma patients, especially in those with Hodgkin’s disease.\textsuperscript{45} Recently, EBV-infected Hodgkin lymphoma cells have been shown to highly express ATX.\textsuperscript{46} Thus, these cells might release high amounts of ATX forming high local concentrations of LPA which not only promote tumor growth\textsuperscript{9} but may also activate the neuronal itch pathway. Intriguingly, patients with Hodgkin’s disease suffering of intense pruritus had a shorter survival than those without itch.\textsuperscript{47}
Unravelling the molecular mechanisms leading to pruritus in systemic diseases will have major impact on development of novel treatment strategies for this agonizing symptom. At least in cholestatic disorders, ATX inhibitors and LPA receptor blockers which are currently developed for the treatment of patients with malignancies to reduce disease progression and formation of metastasis\textsuperscript{48} might also represent a novel class of anti-pruritic drugs.

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SUPPLEMENTARY DATA

Quantitative LPA determination. As internal standard myristoyl-LPA (LPA 14:0) was added to serum samples reaching a final concentration of 1 μM. Lipids were subsequently extracted from 100 μL of serum by one-phase lipid extraction using 1 mL of methanol/chloroform (1:1, vol/vol). The extraction fluid was evaporated to dryness (45 °C, vacuum). The residue was dissolved in 100 μL of chloroform/methanol/water (50:45:5, v/v/v) containing 0.01% NH₄OH, and 10 μL of this solution was injected into the HPLC–MS system. The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller, and an autosampler (Thermo Electron, Waltham, MA, USA). The column temperature was maintained at 25°C. The lipid extract was injected onto a LiChrospher 2 × 250 mm silica-60 column, 5 μm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform/methanol, 97:3, v/v) and solution A (methanol/water, 85:15, v/v). Solutions A and B contained 1 and 0.1 mL of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 mL/min) was as follows: 0–10 min, 20% A–100% A; 10–12 min, 100% A; 12–12.1 min, 100% A–0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 μL/min eluent was introduced into the mass spectrometer. A TSQ Quantum AM (Thermo Electron) was used in the negative electrospray ionization mode. Nitrogen was used as the nebulizing gas. Argon was used as the collision gas. The skimmer offset was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300°C. Selected reaction monitoring (SRM) was used to monitor precursor to product ion transition of m/z 381.2 → 227.2 for LPA(14:0) and m/z 435.25 → 281.25 for LPA(18:1). Quadrupole 1 and quadrupole 3 were maintained at 0.3 and 0.7 unit resolution (FWHM) respectively. The collision gas pressure was 0.5 mTorr and the collision energy was set at 50 V. Dwell time was 0.150 s for both the analytes and IS. All the parameters of LC and MS were controlled by Xcalibur software version 2.0.7.
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