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Original

Plasma lipoxin A4 levels in childhood chronic spontaneous urticaria

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Chronic spontaneous urticaria (CSU) is an idiopathic inflammatory disorder. Despite great research progress, the pathogenesis of the disease is still not fully understood. Lipoxins (LXs) are autacoid lipid metabolites that are the first discovered members of a new genus named called "specialized proresolving mediators". In this study, we aimed to investigate the possible role of LXA_4 in the pathogenesis of CSU.

Forty-two children with CSU and 25 healthy children were enrolled in the study. The demographic and clinical features of patients were evaluated, autologous serum skin tests (ASSTs), and routine laboratory assessments were performed. Disease activity was determined using the urticaria activity score. An enzyme-linked immunosorbent assay was used to evaluate LXA_4 plasma levels.

The median value of plasma LXA_4 was found to be 60.8 ng/ml (interquartile range, 48.1–71.8) in CSU patients and 137.4 ng/ml (121.4–150.8) in the control group. The difference between the groups was statistically significant ($p<0.001$). Additionally, the median plasma LXA_4 levels in the ASST-positive patients were significantly reduced compared to the ASST-negative ones (45.8 [36.7–67.6] versus 63.8 [58.3–78.9] ng/ml, respectively, $p<0.05$).

Our results showed that diminished LXA_4 biosynthesis may be a critical part of CSU pathogenesis in children, especially in patients with an autoimmune component.

Key words: chronic spontaneous urticaria, children, lipoxin A4, disease severity.

Chronic urticaria is an inflammatory disorder of the skin characterized by the presence of the daily or near daily appearance of urticaria (hives) with/or without angioedema for more than six weeks.[1] The disease has two subtypes—chronic inducible urticaria and chronic spontaneous urticaria (CSU).[2] If an urticarial event is triggered after a physical stimulus, such as cold, a vibration, pressure, or water contact, it constitutes chronic inducible urticaria. If the urticarial event is not associated with a physical stimulus, it is classified as CSU.[2]

Despite much research in last few decades, the etiopathogenesis of CSU is still not fully understood. Although some controversy exists, there is strong evidence of an underlying autoimmune mechanism in 30%–40% of CSU patients.[3] It is well known that persistent and uncontrolled mast cell activation is the hallmark of disease.[4,5] Although the cause of this phenomenon is unknown, it is thought that the proinflammatory cytokines released from the inflammatory cells that migrate to the dermis decrease the sensitivity threshold of the mast cells for degranulation.[5]

Acute inflammation is an innate immune response to pathogens, toxins, and injuries and is vital, protective, and useful.[6,7] The best end-point of this process is complete resolution. If this does not happen, uncontrolled inflammation can cause chronic inflammation, tissue injury, and scarring.[8] Recent studies have clearly shown that the resolution of inflammation is a tightly regulated, active course shaped by a family of lipid mediators called "specialized proresolving lipid mediators (SPMs)".[7] The defined members of this family are lipoxins (LXs), protectins, resolvins, and maresins.[8]

LXs are the first discovered proresolving eicosanoids that are synthesized from arachidonic acid via lipoxygenases with different pathways in various cells, including neutrophils, eosinophils, platelets, alveolar macrophages, and airway epithelial cells.[7, 9-11] LXA_4 exerts its bioactions mainly through the agonism of a G protein-coupled receptor called formyl peptide receptor-2 (FPR2/ALX), in addition to the agonism of the aryl hydrocarbon receptor and the partial antagonism of the cysteinyl leukotriene receptor 1.[9,12-14] FPR2/ALX is expressed on a number of cells, including polymorphonuclear cells, eosinophils, monocytes/macrophages, B-lymphocytes, fibroblasts, enterocytes, airway epithelia, and vascular smooth muscle cells.[9,13,15-18]

LXA_4 and its analogues have potent anti-inflammatory and immunoregulatory features. They inhibit eosinophil and neutrophil chemotaxis, vascular adhesion, transendothelial migration, reactive oxygen species (ROS) generation, and azurophilic degranulation of neutrophils.[7] LXA_4 reduces nuclear factor (NF)-κB activation and the release of inflammatory cytokines.[7] It also directly influences vascular endothelia, inhibits ROS production, decreases the expression of adhesion molecules and vascular permeability.[7,13,19]

Direct and indirect evidence obtained from recent studies has shown that LXs play prominent roles in the pathogenesis of various disorders, such as cystic fibrosis, acute lung injury, sepsis, peritonitis, rheumatoid arthritis, and Alzheimer's disease, and in some allergic diseases, such as asthma, allergic rhinitis, atopic dermatitis, and exercise-induced bronchoconstriction.[7, 20-23] Despite all its

Table I. Demographic and Clinical Features of the Study Group.

	CSU patients (n= 42)
Age in years (mean, ±SD)	11.0±4.2
Gender (M/F)	25/17
Angioedema (n[%])	16 (38%)
Total Ig E>100 Iμ/L (n[%])	12 (29%)
Eosinophils>5% (n[%])	3 (7%)
Aeroallergen sensitization (n[%])	5/32 (16%)
ASST positivity (n[%])	
ASST-positive	10/32 (31%)
ASST-negative	22/32 (69%)
UAS (median, [IQR])	3.0 (2.8–5)
Treatment (n[%])	
Second-generation antihistamines at licensed doses	28 (67%)
Second-generation antihistamines at high doses	4 (10%)
Combination of second-generation antihistamines + montelukast	10 (23%)

CSU, chronic spontaneous urticaria; SD, standard deviation; IQR, interquartile range; ASST, autologous serum skin test; UAS, urticaria activity score.

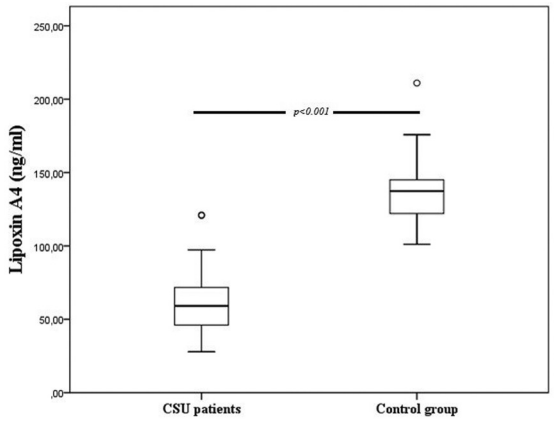

Fig. 1. Plasma LXA_4 levels in chronic spontaneous urticaria (CSU) patients and control group participants.

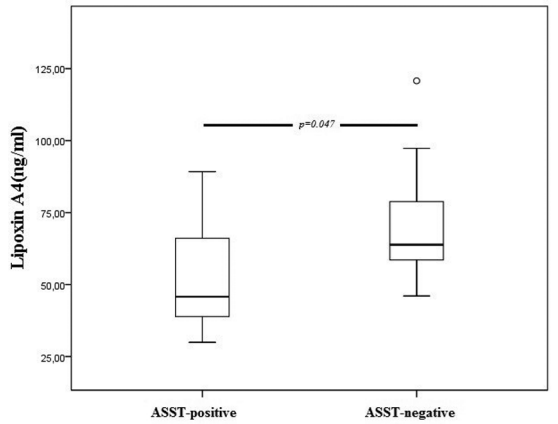

Fig. 2. Plasma LXA_4 levels in autologous serum skin test (ASST)-positive and -negative patients.

extraordinarily promising bioactivities, the possible role of LXA_4 in the pathogenesis of CSU has not been investigated until now. Therefore, we hypothesize that inadequate LX biosynthesis in patients with CSU may cause a defect in the resolution of dermal inflammation that leads to chronic, uncontrolled inflammation and that this phenomenon may be an unresolved aspect of the pathogenesis of CSU.

Material and Methods

Patients

This study was conducted between April 2016 and July 2016 in outpatient clinics at two university hospitals in the same city. A total of 58 pediatric CSU patients who were admitted during the previous six months were invited to join the study. Patients who were diagnosed with

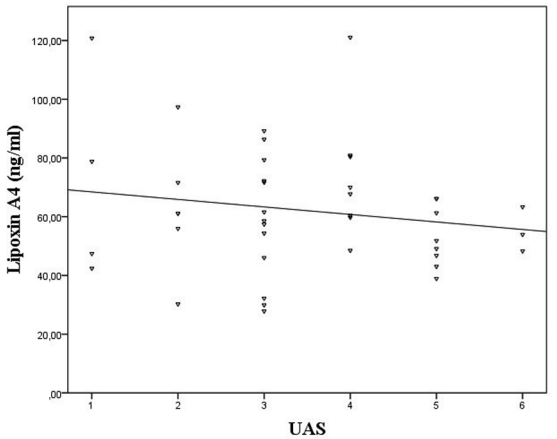

Fig.3. Scatter graph showing the LXA_4 and urticaria activity score (UAS) relationship in patients with chronic spontaneous urticaria (CSU).

chronic inducible urticaria and for whom the underlying causes of CSU had been identified (outside of autoimmunity) were excluded from the study. Diagnosis and classification were based on the European Academy of Allergy and Clinical Immunology guidelines.[2] Children with diseases or conditions that can potentially alter plasma LXA_4 levels, including concomitant diagnosed atopic dermatitis, allergic rhinitis, asthma, or any other chronic disease, or children who are taking any other drugs or nutritional supplements (e.g., fish oil) except antihistaminics or montelukast were excluded from the study. After all exclusions, 42 patients with CSU were finally enrolled in the study.

The control group consisted of 25 healthy children who were periodically attending pediatric welfare clinics in the same hospitals for regular checkups. These children had no history, signs, or symptoms of acute or chronic diseases and were not taking any medications or nutritional supplements. The study was performed in accordance with the tenets of the Declaration of Helsinki and good clinical practice and was approved by the institutional ethical committee (No.15728). All study participants and their parents were given information about the study, and signed consent was obtained from the parents.

Assessment of disease activity

Disease activity was calculated using the urticaria activity score (UAS).[24] UASs were determined based on interviews with patients and their families and on physical examination

findings. The UAS consisted of the sum of the wheal number score and the pruritus score, which ranged from 0–6.

Blood sample collection

All drugs used by patients were discontinued at least 24 hours before blood sampling. After overnight fasting, peripheral blood samples were collected from an antecubital vein into heparinized tubes; thereafter, blood was centrifuged at 3000×g for 10 min to obtain the plasma. The separated plasma was frozen immediately at −80°C until further analysis of plasma LXA_4 levels. Blood samples from the patient and control groups were taken at the same time each day to avoid any possible diurnal variation of LXA4 levels.

Laboratory investigations

The following tests were routinely performed in all cases: a complete blood count, a liver function test, a thyroid-stimulating hormone test, free thyroxine test, an anti-thyroid peroxidase test, an anti-thyroglobulin antibody test, a total immunoglobulin E test, and a microscopic investigation of stool for parasites.

Atopic status was defined as at least one positive reaction to 10 common aero-allergens using a skin prick test. Standard commercial allergen solutions and lancets (Stallergenes® and Stallerpoint®, Paris, France) were used for the skin prick tests. The inhalant panel consisted of *Dermatophagoides pteronyssinus, Dermatophagoides farinae*, a grass pollen mixture, a weed pollen mixture, an aspergillus mixture, alternaria, cypress, birch, dog epithelia, and cat epithelia. The skin prick test was accepted positive with the existence of a wheal of at least 3 mm maximum diameter after subtracting the negative value. An autologous serum skin test (ASST) was conducted following the procedure recommended by Sabroe et al.[25]. We were not able to perform skin prick tests and ASSTs on 10 patients because their antihistamine-containing treatments could not be discontinued due to the intensity of their symptoms.

Measurement of plasma LXA_4 levels

LXA_4 plasma levels were assessed using a human LXA_4 enzyme linked immunosorbent assay (ELISA) kit (Shanghai Sunred Biological Technology Co., Shanghai, China). Samples were thawed at room temperature and then centrifuged at 3000 rpm for 10 min. The samples were then diluted to obtain the appropriate concentrations, and an ELISA was performed according to the manufacturer's instructions. The minimal detection limit was 1 ng/ml, and assay sensitivity was 0.52 ng/ml.

Statistical analyses

A Shapiro–Wilk test was used to test distributions for normality. Parametric data were expressed as the mean±standard deviation (SD), and non-parametric data were expressed as the median, interquartile range (IQR). A Mann–Whitney U test was used to calculate the differences between two parameters in the groups. The correlation between two variables was assessed using the Spearman rank correlation coefficient. Categorical data were evaluated using the chi square test; $p<0.05$ was accepted as statistically significant. Statistical analyses were performed using IBM SPSS 19 (IBM, Chicago, IL, USA).

Results

The study group consisted of 25 boys and 17 girls, and the control group consisted of 13 boys and 12 girls. The mean ages of the CSU patients and the control group participants were 11.0±4.2 and 9.5±3.9 years, respectively. No significant differences in age or gender existed between the groups ($p>0.05$). Liver function tests and thyroid-stimulating hormone and free thyroxin levels were within normal limits, and anti-thyroid peroxidase and anti-thyroglobulin antibody tests and stool investigations for parasites were negative for all patients.

The median value of UASs was 3.0 (2.8–5), and there was no statistically significant difference between ASST-positive and ASST-negative patients ($p>0.05$). Some demographic and clinical features of the patients are shown in Table I.

The median value of plasma LXA_4 was significantly lower in the patient group compared to the control group (ng/ml, median [IQR], 60.8 [48.1–71.8] vs. 137.4 [121.4–150.8]). The difference between the groups was statistically significant ($p<0.001$; Fig. 1). Additionally, median [IQR] plasma LXA_4 levels were 45.8 ng/ml [36.7–67.6] in the ASST-positive CSU patients and 63.8 ng/ml [58.3–78.9] in the ASST-negative CSU patients. There was also

a statistically significant difference between these two groups ($p<0.05$; Fig. 2). Despite the negative relationship between the LXA_4 levels and UASs, a correlation analysis did not show any statistical significance ($p>0.05$; Fig.3). Plasma LXA_4 levels did not vary between the groups in terms of gender, presence or absence of angioedema and atopic status ($p>0.05$). Also, there was not a correlation between plasma Ig E and LXA_4 levels ($p>0.05$).

Discussion

Our study results showed that patients with CSU have obviously reduced plasma LXA_4 levels compared to healthy controls and that ASST-positive patients' LXA_4 levels were even lower than those of ASST-negative patients. These results highlight the possible role of LXA_4 in the pathogenesis of CSU for the first time in the literature. Although there was a negative relationship between plasma LXA_4 levels and UASs, a correlation analysis did not show a statistical significance ($p>0.05$). We chose to use UAS instead of UAS7 (sum of UAS scores for seven consecutive days) in this study because LXA_4 has a short half-life and we aimed to compare instant UAS and plasma LXA_4 levels as much as possible[12]. Although UAS is the only validated tool for assessing the disease activity of CSU,[2,24] it has some limitations. For example, it includes subjective interpretations of families or patients and reflects disease activity in the last 24 hours instead of momentary activity. These limitations and the small sample size may be the reasons the correlation analysis did not result in significance. However, it is challenging to be able to create a larger pediatric patient group when the disease being investigated is CSU because the prevalence in children is only one-tenth of that in adults.[3,26,27]

Asthma is the best studied disease that shows the role of lipoxinA4 in allergic inflammation. LXs can be demonstrated in sputum and bronchoalveolar lavage fluids of asthmatic patients.[6] LXs block airway responsiveness, reduce airway inflammation, reduce granulocyte infiltration to airways, lower the number of inflammatory cells and the levels of pro-inflammatory mediators, such as cysteinyl leukotrienes, and decrease oxidative stress.[28-30] Gagliardo et al.[31] showed defective LXA_4 generation and FPR2/ALX receptor expression in children with severe asthma. Karra and co-workers reported that LXB_4 significantly reduces nasal mucosal leukocytes, eosinophil chemotaxis, and degranulation of mast cells in a murine model of allergic rhinitis.[20] Wu at al.[23]demonstrated that topical application of 15(R/S)-methyl-LXA_4, a LXA_4 agonist, is well tolerated, has no side effects, and significantly improves all features of atopic dermatitis. The anti-inflammatory effect of 15(R/S)-methyl-LXA_4 cream was found to be similar to that of mometasone cream in this study.

Reduced LXA_4 biosynthesis in CSU patients, which we identified in our study, can explain the vast majority of what we know about the pathogenesis of CSU. Wheals, flares, and angioedema, which are characteristic of CSU, arise as a result of increased vascular permeability and extravascular leakage of intravascular fluid and proteins.[32] In their studies, Ereso at al.[33] and Pang et al.[34] determined that LXA_4 decreases vascular hyperpermeability and microvascular fluid leakage.

Neutrophils are the main inflammatory cells that compose the cellular infiltrates of urticarial plaque, and they exist within one hour after stimulus in the urticarial wheal.[4,35] LXA_4 inhibits chemotaxis, adhesion, transmigration, and cytokine production of neutrophils.[7] Eosinophils are also involved and are located mainly in the perivascular dermis.[35] According to researchers who state that the coagulation cascade is activated in CSU, eosinophil activation is an initial event and triggers urticarial episodes.[36] LXA_4 inhibits chemotaxis, interleukin (IL)-5, and eotaxin secretion of eosinophils.[7] Additionally, LXA_4 can block granulocyte-macrophage colony-stimulating factor-induced activation signaling in eosinophils, which is one of the crucial cytokines affecting eosinophil activation and survival.[37,38] Moreover, LXA_4 induces NK cell-induced apoptosis of both eosinophils and neutrophils.[39] Mast cell numbers remain unaltered in patients with CSU and are similar in uninvolved skin and healthy control subjects.[40] However, inappropriate activation and degranulation of mast cells are key pathophysiological events.[4] Two previous studies reported that LXA_4 is a potent inhibitor of immunoglobulin (Ig) E-mediated mast cell

degranulation in a dose-dependent manner.[20,41]

An autoimmune origin has become the most accepted hypothesis in recent years.[4] Functional autoantibodies in the sera of CSU patients have been demonstrated against IgE and FcεRIα.[42] In our study, ASST-positive patients had reduced plasma LXA_4 levels compared to ASST-negative patients. This may be partly explained in two ways; (1) FcεRI signaling is transmitted intracellularly by the NF-κB pathway in mast cells.[43] Additionally, activation and cytokine production is mainly dependent on this pathway.[43-45] (2) LXA_4 decreases immunoglobulin production on memory B-cells in a dose-dependent manner.[15] LXA_4 can reduce both autoantibody secretion and FcεRI signaling via NF-κB inhibition and in this way may play a more dominant role in CSU with autoimmune origins.

Although elevated levels by themselves cannot explain the entire disease pathogenesis, the increased production of some cytokines, chemokines, or metabolites—including IL-6, vascular endothelial growth factor (VEGF), matrix metallopeptidase 9 (MMP-9), and reactive oxygen species—was reported in previous clinical studies.[46-49] LXs can inhibit the generation of pro-inflammatory cytokines and chemokines via the inhibition of transcription factors, such as NF-κB and activator protein 1.[50-52] In this regard, several in vitro studies and animal models have proven that LXA_4 and its analogues can decrease IL-6 secretion,[6] MMP-9 expression,[53] and VEGF expression[54] and can block ROS generation.[55]

Various LX analogues have developed because of the rapid inactivation and short half-life of natural LXs.[12] Promising therapeutic bioactions of LX analogues have been determined at several experimental disease models, including atopic dermatitis, contact dermatitis, and asthma.[12,19,56] Additionally, clinical studies of allergic dermatoses have reported comparable therapeutic efficacy of topical corticosteroids but without any side effects.[19,23] If further studies also demonstrate reduced LXA_4 levels in CSU patients, this disease may be another research area for investigating the possible therapeutic importance of these agents.

In conclusion, plasma LXA_4 levels are significantly decreased in pediatric patients with CSU compared to healthy controls. Additionally, ASST-positive patients have even lower values than ASST-negative patients. Deficient LXA_4 bioactions at the cellular level can explain many aspects of the CSU pathogenesis puzzle that we know but we cannot combine. There are many lipoxin analogues offering new and attractive treatment options. We emphasize that this is the first study conducted on this topic, and that further studies are needed. Nevertheless, proresolving eicosanoids may be the answer to many questions about the etiopathogenesis of CSU.

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