Expression of Leukotriene Biosynthetic Enzymes in Tonsillar Tissue of Children With Obstructive Sleep Apnea
A Prospective Nonrandomized Study

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IMPORTANCE Cysteinyl leukotrienes (CysLTs) potentially promote adenotonsillar hypertrophy in children with obstructive sleep apnea (OSA). Previous studies have identified CysLTs and their receptors in tonsillar tissue from children with OSA.

OBJECTIVE To demonstrate expression of the leukotriene biosynthetic enzymes 5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP), leukotriene A4 hydrolase (LTA4H), and leukotriene C4 synthase (LTC4S) in T and B tonsillar lymphocytes from pediatric patients with OSA. It was hypothesized that children with OSA have greater expression of biosynthetic enzymes for CysLTs (5-LO, FLAP, and LTC4S) in their tonsillar tissue than do children with recurrent tonsillitis (RT), who were enrolled as controls.

DESIGN, SETTING, AND PARTICIPANTS This prospective, nonrandomized study was performed at a tertiary care university hospital on 13 children with OSA and adenotonsillar hypertrophy undergoing adenotonsillectomy and 12 children without OSA also undergoing tonsillectomy for RT. Tonsillar tissue from children with OSA or RT was examined for 5-LO, FLAP, LTA4H, and LTC4S expression under real time–quantitative polymerase chain reaction (RT-qPCR), flow cytometry (FC), and confocal laser scanning microscopy (CM).

MAIN OUTCOMES AND MEASURES Expression of biosynthetic enzymes for CysLTs (5-LO, FLAP, and LTC4S) was the main outcome measure. Patients with OSA and control patients with RT were compared for numbers of copies of 5-LO, FLAP, and LTC4S messenger RNA (by RT-qPCR) in T or B tonsillar lymphocytes and proportions of CD3+ or CD19+ tonsillar lymphocytes that expressed 5-LO, FLAP, and LTC4S (by FC).

RESULTS Messenger RNA for all 4 enzymes was detected in T and B lymphocytes from both study groups, and expression of all biosynthetic enzymes was demonstrated in participants with OSA and RT by FC. Patients with OSA differed from controls in the proportions (median [10th-90th percentile]) of LTC4S+ CD3+ T lymphocytes (23.31% [8.64%-50.07%] vs 10.81% [3.48%-23.32%], respectively) (P = .01) and LTC4S+ CD19+ B lymphocytes (20.66% [14.62%-65.77%] vs 12.53% [2.87%-36.64%], respectively) (P = .01) detected by FC. Immunoreactivity for the 4 enzymes was detected by CM in B lymphocytes of mantle zones and T lymphocytes of extrafollicular areas.

CONCLUSIONS AND RELEVANCE Leukotriene biosynthetic enzymes are expressed in tonsillar lymphocytes, and the previously reported detection of CysLTs in tonsillar tissue from children with OSA may be attributed to endogenous synthesis. Enhanced expression of LTC4S is a potential target for pharmacologic interventions in OSA.
Increased upper airway resistance resulting from enlarged adenoid and tonsils is a common predisposing factor for obstructive sleep apnea (OSA) in childhood. In children with snoring, adenotonsillar hypertrophy occurs during preschool years and persists beyond the eighth birthday, and cysteinyl leukotrienes (CysLTs) have been implicated in its pathogenesis. More specifically, tonsillar T and small B lymphocytes express CysLT receptors and the addition of leukotriene D4 to tonsillar cell culture induces a proliferative response. Administration of montelukast, an inhibitor of type 1 CysLT receptors, to children with mild OSA is accompanied by reduction in the size of adenoids and a decrease in the severity of intermittent upper airway obstruction during sleep. Moreover, increased numbers of CysLTs (leukotrienes C4, D4, and E4) have been found in tonsillar tissue excised from children with OSA.

Following stimulation of neutrophils and lymphocytes, 5-lipoxygenase (5-LO) localized in the cytoplasm translocates to the nucleus where it catalyzes the conversion of free arachidonic acid to leukotriene A4. Arachidonic acid is released from the outer nuclear membrane and is presented to 5-LO by the 5-LO activating protein (FLAP). Leukotriene A4 can be transformed to leukotriene B4 by leukotriene A4 hydrolase (LTA4H) or to leukotriene C4 by leukotriene C4 synthase (LTC4S). Both FLAP and LTC4S are proteins embedded in the nuclear membrane. Leukotriene C4 is further converted to leukotrienes D4 and E4 by extracellular enzymes.

Previous studies in adults have detected the presence of 5-LO in tonsillar B cells, but conflicting data have been presented in relation to the presence of 5-LO in T lymphocytes. To our knowledge, there are no published reports on the expression of biosynthetic enzymes for leukotrienes B4 and C4 in tonsillar tissue of children with OSA. Hence, the primary goal of this investigation was to demonstrate expression of enzymes related to the leukotriene biosynthetic pathway (5-LO, FLAP, LTA4H, and LTC4S) in tonsillar T and B lymphocytes. Furthermore, it was hypothesized that children with OSA have greater expression of biosynthetic enzymes for CysLTs (5-LO, FLAP, and LTC4S) than children with recurrent tonsillitis (RT) recruited as controls. Detection of leukotriene biosynthetic enzymes in tonsillar cells is of clinical importance, since the LTC4S catalytic architecture has been elucidated, and an LTC4S inhibitor with potential therapeutic applications has been described recently.

Methods

Participants and Clinical Evaluation

The research protocol was approved by the institutional review board of the Aghia Sophia Children’s Hospital (Scientific Council approval No. 25930/19-11-10), and written informed consent for participation in the study was obtained from the parents of all participants.

Children with OSA and adenotonsillar hypertrophy who underwent adenotonsillectomy after preoperative polysomnography were recruited for the study. OSA was diagnosed when symptoms of a sleep-related breathing disorder (SRBD) were present, and the apnea-hypopnea index in polysomnography was greater than 1 episode per hour. Children without symptoms of OSA who had tonsillectomy for RT (≥7 episodes over the past year) and an SRBD score lower than 0.33 (by the Pediatric Sleep Questionnaire) were recruited as controls. Children with a diagnosis of asthma or history of respiratory infection during the previous 8 weeks were excluded.

The SRBD score was calculated for all participants, and a physical examination was completed. Size of tonsils was graded from 1+ to 4+ by direct inspection of the oropharynx, and tonsillar hypertrophy was diagnosed when tonsils were larger than 2+. The patients’ weight and standing height were measured, and body mass index (BMI) z-score was calculated. Adenoidal hypertrophy was diagnosed by lateral neck radiography.

Polysomnography

Polysomnography was carried out for 1 night (9 hours) at the Sleep Disorders Laboratory using the Somnolar pro Cephalo Pro Amplifier and Software (Viasys Healthcare). A 4-channel electroencephalogram (C3/M2, O2/M1, O1/M2, and F4/M1), 2-channel electrooculogram, submental and tibial electromyograms, and an electrocardiogram were recorded. Airflow was detected by thermocouples at the nose and mouth and by nasal pressure transducer, and respiratory movements were monitored using inductive plethysmography with thoracic and abdominal belts (RespirTrace QDC, RIP module; Viasys Healthcare). The oxygen saturation of hemoglobin was measured by an oximeter. Sleep stages, arousals, and respiratory events were scored using the American Academy of Sleep Medicine recommendations.

Collection and Processing of Tonsillar Tissue

After surgical excision, tonsillar tissue was placed in phosphate-buffered saline and transferred rapidly to the pathology laboratory for further processing and use in real-time quantitative polymerase chain reaction (RT-qPCR), flow cytometry, and confocal laser scanning microscopy (for details, see the eAppendix in the Supplement).

RT-qPCR Analysis

Total RNA was extracted from T or B lymphocyte fractions with the NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH & Co KG), and its concentration and quality were determined using a spectrophotometer (Genova; Jenway). A total of 1.0 μg of RNA was reverse-transcribed into cDNA by Moloney Murine Leukemia Virus Reverse Transcriptase using oligo(dT) as reverse transcription primer (PrimeScript RT-PCR kit; TaKaRa Bio Europe).

The cDNA equivalent to 5.0 ng of total RNA was subjected to RT-qPCR analysis in triplicates in an Mx3005P RT-qPCR system (Stratagene) according to the manufacturer’s protocol (KAPA SYBR Fast Universal qPCR kit, KAPA Biosystems). Expression of 5-LO, FLAP, LTA4H, and LTC4S was measured using Mx3005P soft-
Flow Cytometry
A 3-color experiment was set up. Two-color staining with directly labeled antibodies was applied to identify subpopulations of T and B lymphocytes. More specifically, T and B lymphocytes were defined by a fluorescein isothiocyanate (FITC) mouse antibody against human CD3 and a PerCP-Cy5.5 mouse antibody against human CD19 (BD Biosciences). To quantify the expression of the intracellular leukotriene biosynthetic enzymes (5-LO, FLAP, LTA₄H, and LTC₄S) in tonsillar lymphocytes, indirect immunofluorescence staining was used. Lymphocytes were permeabilized and fixed with the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s protocol. Cells that had undergone fixation and permeabilization were then incubated for 30 minutes in 4°C in the dark with rabbit polyclonal antibodies against 5-LO (Cayman Chemical), FLAP (dilution 1:50; Santa Cruz Biotechnology), LTA₄H (dilution 1:200; Cayman Chemical), or LTC₄S (dilution 1:100, Sigma-Aldrich). Finally, cells were incubated with fluorochrome R-phycoerythrin (PE)–conjugated Fab′(ab′) secondary anti-rabbit antibody (BD Biosciences) for 30 minutes at 4°C in the dark.

During the experimental procedure and along with the test samples, various controls were prepared: (1) unstained cells to check the background fluorescence of the cells; (2) CD3 FITC-stained cells and CD19 PerCP-Cy5.5–stained cells to check the background fluorescence of the double-stained cells, the nonspecific antibody fixation, and the fluorescence spillover in the channel used for quantitation of the intracellular enzymes; and (3) CD3 FITC–, CD19 PerCP-Cy5.5–, and PE–conjugated Fab′(ab′) secondary anti-rabbit antibody–stained cells (secondary control) to check the background fluorescence of the stained cells and the nonspecific antibody fixation. The secondary control sample was used to set the boundaries for the marker delimiting the negative expression for every patient.

Fluorescence was measured on a FACS Calibur cytomter (BD Biosciences) using BD CellQuest Pro software. The analysis of the results was performed with BD FACST Diva software (BD Biosciences).

Confocal Laser Scanning Microscopy
Confocal laser scanning microscopy was used to localize T and B lymphocytes expressing leukotriene biosynthetic enzymes within the tonsillar tissue. After deparaffinization and antigen retrieval, a 2-day, double-stain protocol was applied in adjacent formalin-fixed, paraffin-embedded tissue sections. The same antibodies used for detection of biosynthetic enzymes by flow cytometry were also applied in the tonsillar tissue for examination by confocal laser scanning microscopy (anti-5-LO, dilution 1:100; anti-FLAP, dilution 1:100; anti-LTA₄H, dilution 1:100; and anti-LTC₄S dilution 1:100). The detailed protocol is described in the eAppendix in the Supplement.

Data Analysis
Expression of biosynthetic enzymes for CysLTs (5-LO, FLAP, and LTC₄S) was the main outcome measure. Patients with OSA and control participants with RT were compared as follows: (1) for patient characteristics, the t test was used for continuous variables and the χ² test (with the Yates correction) for categorical variables; (2) for numbers of 5-LO, FLAP, and LTC₄S mRNA copies found by RT-qPCR in T or B lymphocytes, multivariable analysis of variance (MANOVA) was used, followed by univariate F tests; and (3) for proportions of CD3+ or CD19+ lymphocytes that expressed 5-LO, FLAP, and LTC₄S found by flow cytometry, MANOVA was used, followed by univariate F tests.

Results
Participant Characteristics and Polysomnography Findings
Thirteen children who underwent polysomnography and adenotonsillectomy for OSA were enrolled in the study. Twelve children without symptoms of OSA, with SRBD scores lower than 0.33, and who underwent tonsillectomy for RT were also enrolled in the study as controls. The 2 study groups were similar in terms of age at surgery, female-to-male ratio, and BMI -score, but they differed significantly in SRBD score (P < .01) (Table 1). None of the participants had a history of physician-diagnosed allergic rhinitis or atopic dermatitis. Children with OSA had an atopic (10th–90th percentiles) apnea-hypopnea index of 10.6 (3.7–54.1) episodes per hour, a respiratory arousal index of 1.5 (0–6.8) episodes per hour, an oxygen desaturation of hemoglobin index of 11 (1.3–52.8) episodes per hour, and an oxygen saturation of hemoglobin nadir of 86% (74.2%–97.0%).

RT-qPCR Analysis
Messenger RNA for 5-LO, FLAP, LTA₄H, and LTC₄S was detected in both CD3+ T lymphocytes and CD19+ B lymphocytes isolated from children with OSA and controls with RT (Table 2). The 2 study groups did not differ in terms of 5-LO, FLAP, and LTC₄S mRNA copies obtained from CD3+ T or CD19+ B lymphocytes (Table 2).

Flow Cytometry
Both tonsillar CD3+ T lymphocytes and CD19+ B lymphocytes in children with OSA or RT expressed all 4 enzymes of the bio-
Table 2. Summary of RT-qPCR Results for Expression of Leukotriene Biosynthetic Enzymes in Study Participants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Patients With OSA (n = 13)</th>
<th>Control Patients With RT (n = 12)</th>
<th>P Value (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ T lymphocytes (model 1)(c)</td>
<td>1.34 (1.06-1.33)</td>
<td>1.34 (1.24-1.18)</td>
<td>...</td>
</tr>
<tr>
<td>CD19+ B lymphocytes (model 2)(c)</td>
<td>1.97 (1.82-17.89)</td>
<td>1.69 (0.2-5.01)</td>
<td>...</td>
</tr>
<tr>
<td>CD19+ B lymphocytes (model 4), % of CD19(c)</td>
<td>1.35 (1.22-2.00)</td>
<td>1.34 (1.24-2.18)</td>
<td>...</td>
</tr>
</tbody>
</table>

Abbreviations: 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; OSA, obstructive sleep apnea; RT, recurrent tonsillitis; RT-qPCR, real-time polymerase chain reaction.

\(a\) Unless otherwise indicated, data are reported as median (10th-90th percentile) values.
\(b\) Univariate F test.
\(c\) MANOVA Pillai trace, \(P > .05\).

Table 3. Summary of Flow Cytometry Results for Expression of Leukotriene Biosynthetic Enzymes in Study Participants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Patients With OSA (n = 13)</th>
<th>Control Patients With RT (n = 12)</th>
<th>P Value (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ T lymphocytes, % of total cells</td>
<td>20.13 (13.80-32.61)</td>
<td>23.74 (13.90-30.73)</td>
<td>...</td>
</tr>
<tr>
<td>CD19+ B lymphocytes, % of total cells</td>
<td>67.72 (47.44-75.86)</td>
<td>62.68 (54.13-74.10)</td>
<td>...</td>
</tr>
<tr>
<td>CD3+ T lymphocytes (model 3), % of CD3(c)</td>
<td>7.7 (7.08-54.57)</td>
<td>19.79 (7.76-30.98)</td>
<td>...</td>
</tr>
<tr>
<td>CD19+ B lymphocytes (model 4), % of CD19(c)</td>
<td>23.51 (6.46-50.07)</td>
<td>18.81 (3.48-23.32)</td>
<td>...</td>
</tr>
</tbody>
</table>

Abbreviations: 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; OSA, obstructive sleep apnea; RT, recurrent tonsillitis; RT-qPCR, real-time polymerase chain reaction.

\(a\) Unless otherwise indicated, data are reported as median (10th-90th percentile) values.
\(b\) Univariate F test.
\(c\) MANOVA Pillai trace, \(P > .05\).

Confocal Laser Scanning Microscopy

Tonsillar tissue samples from 4 patients with OSA and 3 control participants, randomly selected among children of the current study cohort, were examined by confocal microscopy to localize the expression of leukotriene biosynthetic enzymes. In patients from both groups, the biosynthetic enzymes (5-LO, FLAP, LTA4H, and LTC4S) were expressed mostly by CD3+ T lymphocytes and CD19+ B lymphocytes than participants with RT (\(P = .01\)) (Table 3). The 2 study groups were similar regarding fractions of tonsillar CD3+ and CD19+ lymphocytes expressing 5-LO and FLAP (Table 3).

Discussion

In adults, circulating myeloid cells (neutrophils, eosinophils, basophils, monocytes) and B lymphocytes express 5-LO, and hence they have the potential to synthetize leukotrienes. However, not all reports have provided consistent data about the presence of 5-LO in T lymphocytes. To our knowledge, the present study is the first to demonstrate that both tonsillar T and B lymphocytes from children with OSA express enzymes for the biosynthesis of leukotrienes B4 and C4. This finding is of clinical importance because CysLT1s (leukotriene C4 and its product leukotrienes D4 and E4) have been implicated in the pathogenesis of tonsillar hypertrophy, and LTC4S—a key enzyme for the biosynthesis of leukotriene C4—is soon to become a therapeutic target for a recently developed LTC4S inhibitor.

In the adenotonsillar tissue, germinal centers of the lymphoid follicles are surrounded by the mantle zones of the fol-
Leukotriene Biosynthetic Enzymes in Pediatric OSA

Figure 1. T-Lymphocyte Leukotriene Biosynthetic Enzyme Expression in Tonsillar Tissue From a Patient With OSA Who Underwent Adenotonsillectomy

Images obtained by confocal laser scanning microscopy. EF indicates extrafollicular area; FLAP, 5-lipoxygenase (5-LO)–activating protein; GC, tonsillar germinal center; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; OSA, obstructive sleep apnea. In the upper panels (original magnification ×20), merged confocal laser scanning microscopy images of GCs and EFs with DAPI staining and concurrent immunostaining for CD3 and 5-LO, FLAP, LTA4H, or LTC4S. The lower panels (original magnification ×100) show high-power views of T lymphocytes in the EF with the characteristic large nucleus (stained blue by DAPI) surrounded by a rim (cellular membrane, cytoplasm, and nuclear membrane). The rim is orange stained in several T lymphocytes, coexpressing CD3 (red) and 1 of the enzymes (green); representative cells are marked by arrowheads.

Meaning of leukotrienes

Leukotrienes are derived from the arachidonic acid cascade. They are produced by cells of the immune system, including B lymphocytes, macrophages, and mast cells. The class of enzyme responsible for their production is the leukotriene biosynthetic pathway. This pathway is composed of various enzymes, including 5-lipoxygenase (5-LO), 5-LO–activating protein (FLAP), leukotriene A4 hydrolase (LTA4H), and leukotriene C4 synthase (LTC4S).These enzymes are involved in the conversion of arachidonic acid to leukotrienes, which are known for their pro-inflammatory properties.

The presentation focuses on the expression of leukotriene biosynthetic enzymes in tonsillar tissue from a patient with obstructive sleep apnea (OSA) who underwent adenotonsillectomy. The images show confocal laser scanning microscopy of tonsillar tissue, highlighting the presence of extrafollicular (EF) and germinal center (GC) areas. The images demonstrate the expression of several enzymes involved in the leukotriene biosynthetic pathway, including CD3, 5-LO, FLAP, LTA4H, and LTC4S, in T lymphocytes within the EF areas.

The study suggests that the expression of these enzymes is higher in children with OSA compared to those without, indicating an increased production of leukotrienes in this population. This finding supports the role of leukotrienes in the pathogenesis of OSA, as they contribute to the inflammatory response and airway obstruction.

Implications for treatment

The study highlights the potential role of leukotriene inhibitors in the management of OSA. Since leukotrienes play a significant role in the inflammatory response, targeting their biosynthetic pathway could be a promising therapeutic strategy. Adenotonsillectomy, the standard treatment for OSA in children with adenotonsillar hypertrophy, may lead to an improvement in symptoms by reducing the production of leukotrienes and the associated inflammation. However, further research is needed to explore the efficacy and safety of pharmacologic interventions targeting leukotrienes in the treatment of OSA.

Overall, the study underscores the importance of understanding the role of leukotrienes in the development of OSA, which could lead to new therapeutic approaches for this condition.
troph and OSA. However, in adults with OSA, treatment with nasal continuous positive airway pressure or upper airway surgery is not accompanied by reduction in leukotriene B4 levels.27,28

In accordance with previous studies, children undergoing tonsillectomy for RT were recruited as controls4,29 in the present study, since it is unethical to obtain tonsillar tissue from healthy children. A potential limitation of the present study is that control participants did not undergo polysomnography owing to families’ practical difficulties. Instead, the Pediatric Sleep Questionnaire by Chervin et al16 was used to rule out OSA.

Conclusions

Tonsillar lymphocytes from children with OSA or RT express enzymes of the leukotriene biosynthetic pathway in the tonsillar mantle zones and the extrafollicular areas. Furthermore, enhanced expression of LTC4S in children with OSA may contribute to the pathogenesis of palatine tonsil overgrowth and increased upper airway resistance. These novel findings suggest that inhibition of leukotriene biosynthetic enzymes should be explored as a potential therapeutic intervention for pediatric OSA.

 References

1. Kaditis A, Kheirandish-Gozal L, Gozal D. Algorithm for the diagnosis and treatment of...


