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Glucocorticoid Receptor Subunit Expression in Adenotonsillar Tissue of Children with Obstructive Sleep Apnea

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ABSTRACT

Tonsillectomy and adenoidectomy (T&A) is a frequent surgical procedure in children with obstructive sleep apnea (OSA). Many symptomatic children who do not fulfill the currently recommended criteria for T&A may benefit from topical intranasal steroid therapy. However, the expression of glucocorticoid receptor (GCR) expression in adenoid and tonsillar tissue is currently unknown. The objective of this study was to assess and compare expression patterns of the human GCR in children who undergo T&A for either recurrent throat infections (RI) or OSA. Adenotonsillar tissues from 36 children with OSA or RI were subjected to quantitative PCR using specific primers for GCR-α and GCR-β and to immunohistochemistry and Western blotting for protein expression of GCR isoforms. mRNA encoding for expression of both GCR-α and GCR-β was detected in the tonsils and adenoids of all children, with markedly higher relative abundance of the GCR-α. Furthermore, GCR-α mRNA expression was increased in OSA-derived adenoid and tonsil tissues compared with RI, whereas no differences emerged for GCR-β. Immunoblots confirmed these findings for the protein transcripts of these genes, and immunohistochemistry showed a specific topographic pattern of distribution for both receptors in tonsillar tissue. GCR-α and GCR-β are expressed in pediatric adenotonsillar tissue, are more abundant in OSA patients, and demonstrate a specific topographic pattern of expression. These findings along with the high GCR-α:GCR-β ratio suggest a favorable profile for topical steroid therapy in snoring children with adenotonsillar hypertrophy. **(*Pediatr Res* 57: 232–236, 2005)**

Abbreviations

GCR, glucocorticoid receptor
OSA, obstructive sleep apnea
RI, recurrent throat infections
T&A, tonsillectomy and adenoidectomy

Obstructive sleep apnea (OSA) syndrome has emerged as a frequent and serious condition, affecting at least 2–3% of all children (1), and is associated with substantial cardiovascular, metabolic, and neurocognitive morbidities (2–6). In children, hypertrophy of the tonsils and adenoids is the most frequent and preeminent cause of OSA, such that surgical extirpation of the enlarged lymphoid tissue is usually the initial management approach [tonsillectomy and adenoidectomy (T&A)] (7,8). In addition, T&A is frequently performed in children who experience recurrent upper airway infections (RI) and who require frequent and sustained treatment with antibiotics (9). However, many children who snore, exhibit symptoms suggestive of OSA, and clearly present with adenotonsillar hypertrophy may not fulfill the polysomnographic criteria that would support surgical treatment. Thus, these children are usually left unattended, even though a significant proportion may manifest neurocognitive and behavioral dysfunction (10–12).

The use of corticosteroid therapy as a potential nonsurgical alternative in the management of OSA in children has been advanced by some investigators. Indeed, Brouillette *et al.* (13) initially showed that a short course of systemic prednisone was ineffective in modifying the size of either tonsillar or adenoid tissue, such that no changes in the polysomnographic abnormalities occurred. In contrast, a 6-wk course of the topical steroid fluticasone markedly improved the severity of the gas exchange abnormalities during sleep in 12 children with OSA, whereas a mild but significant deterioration in those sleep measures emerged in the placebo-treated group (14). Furthermore, T&A was more likely prevented after sustained low-dose treatment with nasal beclomethasone (24-wk duration), especially in those who showed improved symptoms after 4 wk

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(15), suggesting that the presence or absence of an initial response to steroids may be a major determinant of whether topical steroids have a role in the treatment of children with symptomatic snoring. Two human isoforms of glucocorticoid receptors (GCR) have been identified and termed GCR-α and GCR-β. Both receptors originate from the same gene through alternative splicing, and in fact GCR-β may represent a decoy GCR and play a role in glucocorticoid ligand efficacy (16). Indeed, although reduced ligand binding to corticosteroids or defects in GCR translocation to the nucleus and binding to the glucocorticoid-binding response element can explain some cases of corticosteroid-resistant asthma (17), altered relationships in the expression of GCR-α and -β in lung tissue have emerged as the most likely and major determinant of corticosteroid insensitivity (18–22). We therefore conducted this study to assess whether GCR-α and GCR-β are expressed in tonsils and adenoids and to determine whether differences in expression of these receptors occur between children with OSA and those with RI.

METHODS

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant. Consecutive children who underwent tonsillectomy for either OSA or RI were identified before surgery and recruited into the study. The diagnosis of OSA syndrome was established by overnight polysomnography in the sleep laboratory and required the presence of apnea-hypopnea index >5 events/h of sleep (23). Patients who were referred for RI were selected on the basis of a history of at least five tonsillar infections in <6 mo, and because the absence of any symptoms suggestive of OSA essentially negates the presence of this condition (11), they were not evaluated by an overnight polysomnogram, because our questionnaire-based evaluation is highly sensitive and specific in ruling out sleep-disordered breathing in children (11). For inclusion, children with RI were required to have received their last dose of antibiotic therapy 6 wk or longer from the day of surgery. Children who had known conditions such as asthma, allergic rhinitis, or history of allergies and/or had received corticosteroid or leukotriene modifier therapy within 1 y from surgery were excluded from the study.

Tissue collection and processing. After both palatine tonsils and adenoids were removed by a pediatric ears, nose, and throat specialist, a portion of each tissue was snap-frozen in liquid nitrogen and stored at -80°C, and another portion was fixed in 4% formalin, cryoprotected with 30% sucrose, and kept at 4°C.

Quantitative (real time) PCR. Total RNA was prepared from either tonsillar or adenoidal tissues using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Isolated total RNA was quantified using a spectrophotometer (Amersham Biosciences Ultraspec 3100 Pro). Aliquots of total RNA (1 μg) were reverse-transcribed using random primers and Superscript II-Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA equivalent to 20 ng of total RNA was subjected to real-time PCR analysis (MX4000; Stratagene, La Jolla, CA) following standard protocols. PCR Primers (Invitrogen) and Taqman probes (Biosearch Technologies, Novato, CA) for GCR-α, GCR-β, and β-actin were designed by Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). The primer and probe for GCR-α were as follows: forward primer 5'-GGCAGCGGTTTTATCAACTGA-3', reverse primer 5'-AATGTTTGGAAGCAATAGTTAAGGAGA-3', Taqman probe 5'-FAM-TTTCAACCACTTCATGCATAGAATCCAAGAGTTT-BHQ-1-3'. The primer and probe for GCR-β were as follows: forward primer 5'-AACTGGCAGCGGTTTTATCAA-3', reverse primer 5'-TGTGAGATGTGCTTTCTGGTTTAAA-3', Taqman probe 5'-(FAM)-CATAACATTTTCATGCATAGAATCCAAGAGTTTTGTCA-(BHQ-1)-3'. The primer and probe for β-actin were as follows: forward primer 5'-GACTACCTCATGAAGATCCTCACC-3', reverse primer 5'-TCTCCTTAATGTCACGCACGATT-3', Taqman probe 5'-FAM-CGGCTACAGCTTCACCACCACGG-BHQ-1–3'. Each reaction (25 μL) contained 2.5 μL of reaction buffer (10×), 6 mM of $MgCl_2$, 0.2 μM of dNTP, 0.6 μM of each primer, 0.25 μL of SureStar *Taq*DNA Polymerase, and 2 μL of cDNA dilutions. The cycling condition consisted of 1 cycle at 95°C for 10 min and 40 two-segment cycles (95°C for 30 s and 55°C for 60 s). Standard curves for target genes (GCR-α and GCR-β) and the housekeeping gene (β-actin) were performed for each assay. Briefly, 10-fold serial dilutions of control cDNA were amplified by the MX-4000 PCR machine (Stratagene). C_T Value (initial amplification cycle) of each standard dilution was plotted against standard cDNA copy numbers. On the basis of the standard curves for each gene, the sample cDNA copy number was calculated according to the sample C_T value. Finally, each of the calculated copy numbers for either GCR-α or GCR-β was normalized against the corresponding β-actin copy numbers. Standard curves and PCR results were analyzed using MX4000 software (Stratagene).

Immunohistochemistry. Coronal sections (30 μm) were initially incubated in 0.3% H_2O_2 for 30 min, washed several times in PBS, and blocked with a PBS/0.4% Triton X-100/0.5% TSA (Tyramide Signal Amplification; Perkin Elmer Life Sciences, Boston, MA) blocking reagent/10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h. Sections then were serially incubated with GRC-α antibody (17) (1:750; Affinity Bioreagents, Golden, CO) or with GCR-β antibody (1:7000; Affinity Bioreagents) at 4°C for 24 h and then washed in PBS six times for 5 min each wash. Sections then were incubated at room temperature for 1 h in biotinylated anti-rabbit antibody (Vectastain Elite ABC kit; Burlingame, CA; 1:600) in a PBS/0.5% TSA blocking reagent/10% goat serum solution. After three 5-min washes, sections were incubated at room temperature with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with tetramethyl rhodamine tyramide diluted 1:50 in amplification diluent (Perkin Elmer, Boston, MA) for 2 min (Perkin Elmer Life Sciences). Sections then were washed in PBS and mounted onto glass slides. Negative controls were prepared by omitting either the primary or the secondary antibody for both receptors. Sections were prepared from five sets of tonsils from either OSA or RI groups and were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Western blotting. Tonsils were homogenized in a lysis buffer [50 mM of Tris (pH 7.5), 0.4% NP-40, 10% glycerol, 150 mM of NaCl, 10 mg/mL of aprotinin, 20 mg/mL of leupeptin, 10 mM of EDTA, 1 mM of sodium orthovanadate, and 100 mM of sodium fluoride], and the protein concentration was determined using the Bradford method (Bio-Rad DC). Samples (40 μg of protein) were resolved on 12% SDS–polyacrylamide gels using electrophoresis (Novex/Invitrogen, Carlsbad, CA) at 120 V for 2 h and electroblotted onto 0.2-μm nitrocellulose membranes for 90 min at 30 V. Membranes were blocked with 5% nonfat dry milk in TBS-T (TBS + 0.05% Tween 20) and then were incubated overnight at 4°C with commercially available primary antibodies that recognize the GRC-α (1:1000; Affinity Bioreagents) or the GCR-β (1:2000; Affinity Bioreagents) and later with anti–β-actin (1:20,000; Sigma Chemical Co., St. Louis, MO) both diluted in 5% milk. Lanes were also incubated with a mixture of the primary antibody and receptor blocking peptide (PEP 221 for GRC-α and PEP 222 for GCR-β, both in 1:2 dilution; Affinity Bioreagents) as competition assay. Membranes then were washed with TBS-T and incubated with either horseradish peroxidase–linked anti-rabbit or anti-mouse antibodies (for GCR and β-actin, respectively). Proteins were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ). The intensities of the bands corresponding to the protein of interest were quantified using scanning densitometry.

Statistical analysis. Results are presented as mean $\pm$ SD. Numerical data were subjected to statistical analysis using either *t* tests or ANOVA followed by post hoc tests, as appropriate. A $p < 0.05$ was considered statistically significant.

RESULTS

Thirty-six children who were recruited for the study included 20 OSA patients and 16 RI patients. Mean age was 6.1 $\pm$ 2.7 y (range: 2–12 y; 19 boys and all prepubertal on the basis of Tanner staging), and 89% were white with the remaining 11% being black. GCR-α and GCR-β mRNA was present in all tonsillar (n = 20) and adenoid (n = 16) tissues studied. No

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differences were present in body mass index among the two groups with only two children in each group fulfilling the criteria for obesity (body mass index >95% for age and sex). As in many other tissues, GCR-α mRNA was markedly more abundant than GCR-β mRNA in both tonsils and adenoids. Furthermore, OSA patients consistently had higher GCR-α mRNA expression in upper airway lymphoid tissues when compared with those derived from children with RI (0.93 ± 0.06 *versus* 0.70 ± 0.04; $p < 0.01$; Fig. 1). In contrast, no differences in GCR-β mRNA emerged in the two patient groups (1.27 ± 0.13 *versus* 1.58 ± 0.25; NS).

In the palatine tonsils excised from OSA and RI patients, both GCR-α and GCR-β immunoreactivities were found and were located primarily within the epithelial layers and the extrafollicular areas within the parenchyma. No staining was detected in the tonsillar germinal centers (Fig. 2). To compare between OSA and RI patients, we performed immunoblots of tonsillar and adenoidal tissue lysates for GCR-α and GCR-β. The identity bands were confirmed using a neutralizing peptide that attenuated the intensity of the band at a molecular weight of 94 kd for GCR-α and 90 kd for GCR-β. GCR-α protein expression was significantly greater in the OSA group compared with the RI group ($n = 12$/group; 1.17 ± 0.07 *versus* 0.63 ± 0.03; $p < 0.001$; Fig. 1). In contrast, no differences in GCR-β protein appeared ($n = 12$; 0.32 ± 0.03 *versus* 0.30 ± 0.04; NS; Fig. 1). Although the relative abundance of GCR-α mRNA was >100,000-fold than that of GCR-β expression, GCR-α:GCR-β protein mean ratios were 3.65 and 1.98 in OSA and RI, respectively ($n = 12$/group; $p < 0.001$).

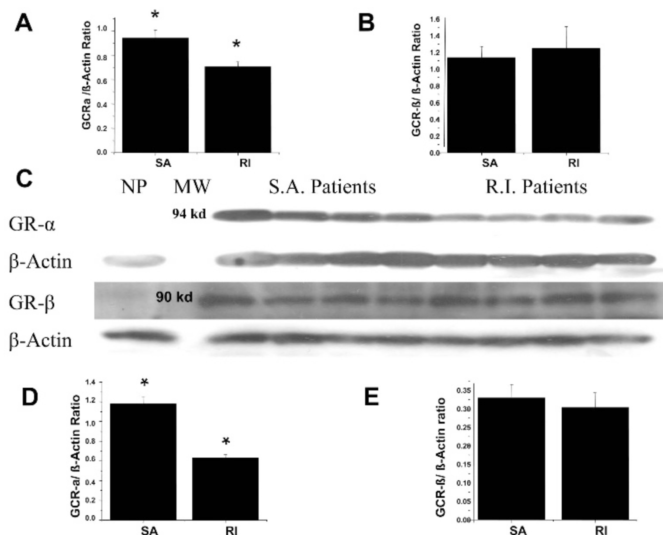

Figure 1. GCR gene and protein expression in upper airway lymphoid tissues. (*A* and *B*) GRC-α and GCR-β mRNA expression in tonsillar and adenoid tissues from pediatric patients with RI and those with sleep apnea (SA). Significantly increased expression in SA compared with RI for GRC-α ($n = 20$) but not for GCR-β emerged ($n = 16$; $p < 0.01$). (*C*) Representative immunoblots of GCR-α (detected at 94 kD) and β-actin, and GCR-β (detected at 90 kd) and β-actin from patients with SA and RI. NP, neutralizing peptide; MW, molecular weight. (*D* and *E*) GCR-α/β-actin and GCR-β/β-actin ratios showed significantly higher GCR-α expression in SA patients ($n = 12$) compared with RI patients ($n = 12$; $p < 0.001$) but no significant differences for GCR-β protein expression.

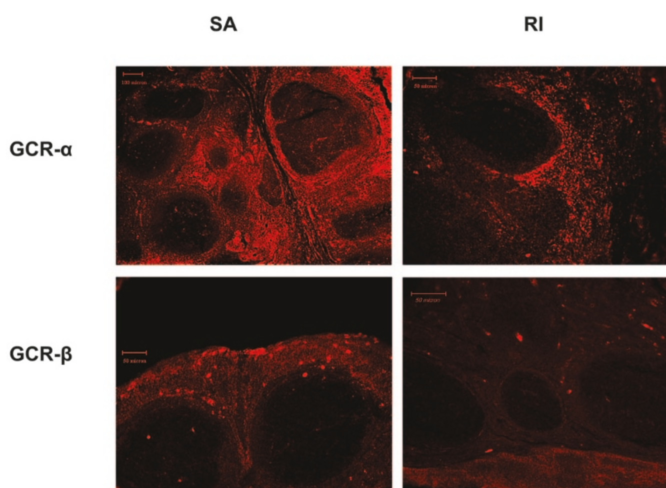

Figure 2. Topographic pattern of GCR expression in tonsillar tissues. Representative immunohistochemical assessment for GCR-α and GCR-β in tonsils from a patient with SA and a patient with RI. Higher abundance of GCR-α receptors is apparent in the SA patient as well as a different topographic distribution of the receptors. Similar findings occurred in five sets of tonsils that were examined blindly for each of the two patient categories.

DISCUSSION

This study shows that both GCR-α and GCR-β mRNA transcripts and immunoreactivities are present and expressed in lymphoid tissues derived from the upper airways of children with either OSA or RI and that the overall abundance of GCR-α is substantially greater than that of GCR-β. Furthermore, we show that GCR-α expression is greater in OSA patients compared with children with RI, whereas such differential expression is absent for GCR-β.

Recent studies have characterized the expression of GCR-α and GCR-β in several human tissues at both the gene and the protein levels (18,19,21,22,24–36). Although the two receptors were found and identified in both normal and inflamed human nasal mucosa leading to polyp formation (28,33), it seems that, as found in the present study, the relative abundance of GCR-α was markedly greater than that of GCR-β. It is interesting that these studies found increased expression of GCR-β when the tissues exhibited inflammatory changes, and this pattern was particularly correlated to the number of mast cells present (33). The increased expression of GCR-β in nasal polyp inflammatory cells, particularly T cells and eosinophils, was associated with increased resistance to the suppression of inflammatory cell counts in the tissues by topical steroids (28). Despite clear differences in the presence of myeloperoxidase-expressing cells in OSA and RI upper airway lymphoid tissues, with markedly greater myeloperoxidase immunoreactivity found in OSA patients (data not shown), GCR-α expression but not GCR-β expression was increased in OSA. These findings suggest that despite the presence of inflammatory processes most probably triggered by the presence of mechanical vibration in the upper airway lymphoid tissue during snoring, the resultant increase in the number of inflammatory cell numbers does not adversely affect the putative therapeutic response profile of these tissues (as might be anticipated if increased GCR-β had been found) but rather suggest a favorable likeli-

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hood that reductions in adenoid and tonsillar tissue mass will occur in response to the intranasal application of topical steroids (35).

The mechanisms associated with the differentially increased expression of GCR-α in OSA tonsils and adenoids are currently unknown. As mentioned above, there is increasing evidence that inflammatory processes are activated and perpetuated in the retropalatal region and pharyngeal introitus by the presence of snoring and repeated upper airway occlusions as seen in OSA (37–39). It therefore is possible that the increased release of inflammatory mediators such as cytokines in the upper airways of snoring children (but not in children with RI) may play a role in the regulation of GRC-α gene expression (40). However, we cannot rule out the possibility that the inverse may occur, *i.e.* that the expression of GRC-α may be down-regulated. Notwithstanding such eventuality, the presence of episodic hypoxia in OSA could also contribute to the discrepant expression of GRC-α and GCR-β in patients with OSA. Indeed, induction of hypoxia-related transcription factors could interact with GRC-dependent transcriptional regulation and affect the expression of downstream target genes, including GRC-α (41).

A substantial number of topical corticosteroid preparations are widely used in the treatment of asthma and allergic rhinitis in children. However, the implications of these compounds for the clinical treatment of children with sleep apnea primarily attributable to enlarged adenotonsillar tissue remains unknown and clearly merits further investigation. The cloning of GCR and generation of isoform-specific antibodies that specifically recognize GCRs enabled us to explore the levels of gene and protein expression in tonsillar and adenoid tissues of children. We could not obtain such tissues from normal children for obvious ethical reasons and therefore are precluded from comparing expression levels in children who are devoid of any medical history. Nevertheless, current findings indicate that both GCRs are expressed in human tonsils and adenoids and that different conditions such as RI and OSA alter the patterns of expression in these tissues, particularly that of GRC-α. We postulate that the increased expression of GRC-α in OSA may reflect a consequence rather than a cause in the pathophysiologic mechanisms that link the enlargement of the lymphoid tissue in the upper airway to the emergence of sleep apnea in snoring children. Surprising, lower expression levels were present in children with recurrently infected tonsils considering that these children sustain episodic infectious processes in their tonsils. However, it should be stressed that surgical removal of the tissues was performed in children with RI only during periods of quiescence in which no evidence for any ongoing inflammatory processes was present.

In summary, we have delineated the patterns of expression and tissue distribution of GRC-α and GCR-β in developing human tonsils and adenoids and shown that these receptors are regulated differentially in two frequent disease conditions that lead to the need for their surgical removal, namely RI and OSA. The cumulative, albeit very preliminary evidence, suggests a favorable response profile to the use of topical steroids in OSA and the likely absence of corticosteroid insensitivity when considering the GRC-α and GCR-β expression ratios found in this study. Thus, randomized controlled trials of intranasal steroids should be conducted in children who have symptomatic snoring with adenotonsillar hypertrophy to delineate better the role, response pattern as a function of GCR expression, and optimal duration of this therapeutic modality.

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