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Endocannabinoid receptor CB2R is significantly expressed in aspirin-exacerbated respiratory disease: a pilot study

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Abstract

Background: The endocannabinoid system represents a highly conserved, innate signaling network with direct and indirect control of eicosanoid-mediated inflammation. Activation of the type 2 cannabinoid receptor (CB2R) leads to decreased type 2 inflammation and reduced production of arachidonic acid (AA). Given that altered AA metabolism is associated with aspirin-exacerbated respiratory disease (AERD), we hypothesized that expression of the CB2R gene *CNR2* is increased in AERD.

Methods: Nasal polyps from consecutive patients under-going endoscopic sinus surgery for AERD or allergic fungal rhinosinusitis (AFRS) were prospectively evaluated. Control sphenoid mucosa was collected from patients undergoing endoscopic skull base procedures. Expression and localization of endocannabinoid receptors were evaluated by quantitative reverse transcript–polymerase chain reaction (qRT-PCR) and immunohistochemistry. A 2-group unpaired *t* test with unequal variances was used to evaluate group differences.

Results: Thirteen subjects were included in this pilot study, including 5 controls, 5 AFRS patients, and 3 AERD patients. Upregulated expression of *CNR2* was detected in subjects with AERD vs both AFRS ($p = 0.049$) and controls ($p = 0.047$), with a mean increase of 5.2-fold. No significant differences in expression of the CB1R gene *CNR1* were detected between control and AFRS groups. Immunohistochemistry predominantly localized CB1R and CB2R expression to the surface epithelium in all subjects.

Conclusion: The endocannabinoid system is an emerging immunomodulatory network that may be involved in AERD. This is the first study of CB2R in sinonasal disease, showing significantly

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increased transcription in nasa lpolyps from subjects with AERD. Additional study is warranted to further evaluate the contribution and therapeutic potential of this novel finding in chronic rhinosinusitis.

Keywords

sinusitis; chronic disease; aspirin-induced asthma; endocannabinoid

Aspirin-exacerbated respiratory disease (AERD) is a severe inflammatory syndrome with inadequate diagnostic and treatment options for the >2 million Americans and 10–20% of asthmatics affected by this disease.[1,2] AERD is characterized by a triad of moderate to severe asthma, chronic rhinosinusitis with nasal polyposis (CRSwNP), and hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). Symptoms initially present in the second to third decade and follow a progressive course marked by increasing airway inflammation and life-threatening pulmonary exacerbations. Uncontrolled symptoms are associated with >$4.5 billion in national annual healthcare expenditures.[2,3] Despite recommended therapies, patients have significant upper and lower airway inflammation, resulting in 90% receiving multiple sinus surgeries within 10 years of diagnosis.[4] Although aspirin desensitization represents an emerging, disease-specific therapy, utilization remains limited by heterogeneous outcome studies, provider availability, and concerns of adverse reactions associated with acute and long-term NSAID exposure.[5] Aspirin desensitization is therefore not recommended until after revision sinus surgery, leaving a significant need for additional treatment options for early intervention.[3]

Dysregulated arachidonic acid (AA) metabolism represents a potential source of airway inflammation in AERD. Increased expression of cysteinyl leukotriene receptors and synthetic enzymes shunts AA toward the pro-inflammatory 5-lipoxygenase pathway, increasing production of inflammatory leukotrienes both at rest and after aspirin exposure.[6] Altered function of anti-inflammatory prostaglandin E2 (PGE2) has also been demonstrated, potentially accounting for the failure of leukotriene-modifying drugs such as montelukast and zileuton to control patient symptoms.[3,7] Dysregulated AA metabolism represents a disease-specific target for drug discovery.

The endocannabinoid (eCA) system is a recently discovered immunomodulatory network that may be involved in AERD via the direct production of AA and regulation of adaptive immunity.[8] Activation of the type 2 cannabinoid receptor (CB2R) results in decreased leukotriene synthesis and leukocyte migration, as well as a reduction of T-helper 2 (Th2)-cytokine profiles.[9,10] AA is also produced by cleavage of the endocannabinoid and CB2R ligand 2-arachidonoylglycerol (2-AG) by fatty acid amid hydrolase (FAAH) or monoacylglycerol lipase (MAGL), directly increasing the production of inflammatory leukotrienes in AERD.[8,9] In this pilot study, we hypothesized that altered CB2R expression is associated with AERD and represents a novel target for future discovery.

Patients and methods

Subject identification and tissue collection

All aspects of this human subjects research have been approved by the Emory University institutional review board (IRB00066294). Adult patients electing to undergo endoscopic sinus surgery for the management of AERD, allergic fungal rhinosinusitis (AFRS), or noninflammatory skull base pathology were recruited from the Sinus, Nasal & Allergy Center at Emory University, between 2015 and 2017. AERD subjects were identified by a history of CR-SwNP, moderate to severe asthma, and a reported history of NSAID intolerance.[3] AFRS subjects were identified by CRSwNP, type 1 hypersensitivity, fungus, and eosinophilic mucin on pathology and characteristic findings on computed tomography(CT)scan.[11] Patients were excuded from the study if they were from a vulnerable population; unable to provide informed consent; or had a concurrent diagnosis of a corticosteroid-dependent condition, cystic fibrosis, granulomatous sinonasal disorder (granulomatosis with polyangiitis, sarcoidosis), or another immunosuppressive diagnosis (human immunodeficiency virus, hematologic malignancy or transplant history). Informed consent was obtained at the time of study enrollment according to the Declaration of Helsinki. Ad hoc power analysis was not completed for this exploratory pilot study.

AFRS and AERD subjects completed initial medical therapy with nasal saline irrigations, topical corticosteroids, and at least 1 trial of antibiotics and oral corticosteroids, before electing sinus surgery.[12] Preoperative corticosteroids were defined as any oral corticosteroid given within 14 days of surgery. Recreational or medicinal use of cannabis was queried in all patients. Nasal polyps were collected from AERD and AFRS subjects as part of their planned sinus surgery. Noninflamed sphenoid mucosa was collected from control subjects as part of their planned sinonasal approach to access nonfunctional pituitary tumors. Collected tissues were transferred in saline according to an established protocol, for further processing and evaluation. Additional samples (nasal polyps and sinus mucosa) were formalin-fixed at the time of tissue harvest and provided to the Winship Research Pathology Core Lab at Emory University for slide preparation.

Tissue processing

Tissue samples (nasal polyps and sinus mucosa) were weighed and mechanically homogenized with a scalpel (Integra Miltex 4–511; Plainsboro, NJ). The resulting tissue was placed in a digestion solution with Dulbecco's modified Eagle medium (DMEM) + Liberase TL 125 μg/mL (5401020001; Sigma-Aldrich, St. Louis, MO) + DNAse I 100 μg/mL (1128432001; Sigma Aldrich) + antibiotic/antimycotic (15240062; Gibco/Thermo Fisher Scientific, Waltham, MA) at a ratio of 5 mL of solution per gram tissue. The tissues were incubated at 37°C for 45 minutes and then vortexed for 10 minutes to release cellular contents. The tissues were then filtered through a 70-μm Falcon cell strainer (08–771-2; Fisher Scientific). The obtained cells were washed twice in RPMI + 10% fetal calf serum (FCS) and then stored at −80°C in RNA lysis buffer (79216 Buffer RTL; Qiagen, Valencia, CA)) with <5 million cells in 350 μL.

Quantitative reverse transcript polymerase chain reaction

Established protocols with previously published primers were used to evaluate transcription levels of the eCA receptor genes *CNR1* and *CNR2* in collected tissues (Table 1).[13] Total RNA from prepared patient samples was isolated using the RNeasy Mini Kit (Qiagen), following the manufacturer's protocol. After RNA purification, cDNA was produced from approximately 0.1 μg of RNA per sample using a cDNA synthesis kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA), followed by real-time PCR with the SYBR Green method (iQ SYBR Green Supermix; Bio-Rad). All samples were run in triplicate. Resulting cycle threshold (Ct) values were normalized to the housekeeping gene RPS18 and compared with the average expression in harvested control mucosa. Differential expression was calculated with the 2^{-ddCt} method and reported as fold-change.

Immunohistochemistry

Established immunohistochemistry protocols were utilized to evaluate the tissue localization of eCA components in AERD polyp tissues.[10] Serial 4- μm sections of 4% paraformaldehyde fixed, paraffin-embedded tissues were incubated overnight with primary antibodies against the eCA receptors CB1R (ab23703; Abcam, Cambridge, MA) and CB2R (ab3561; Abcam) antigens.[14] Samples were then incubated with secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) and visualized with peroxidase activity using 3,30-diaminobenzidine with hematoxylin and eosin counterstaining. Isotype control for both the CB1R and CB2R antibodies was obtained using nonspecific rabbit IgG. Sequential tissue samples were stained with hematoxylin and eosin to demonstrate tissue morphology and Alcian blue/periodic acid-Schiff to mark mucus-producing cells.

Statistical analysis

A 2-group unpaired *t* test with unequal variances was used to evaluate group differences in eCA transcription. Due to limited enrollment, subject comparisons between study groups were not made.

Results

Thirteen subjects were included in this pilot study from April 2015 to November 2017. Distribution of enrolled subjects included 5 controls, 5 AFRS patients, and 3 AERD subjects. Demographics and patient factors are presented in Table 2. All subjects with AERD or AFRS underwent elective endoscopic sinus surgery with bilateral nasal polypectomy, maxillary antrostomy, total sphenoethmoidectomy, and frontal sinusotomy. No subjects underwent concurrent septoplasty or submucosal reduction of the inferior turbinates. One (33%) subject from the AERD cohort underwent a bilateral partial middle turbinate resection. All subjects enrolled in the control group underwent an elective endoscopic transsphenoidal approach to the sella to access a nonfunctional pituitary tumor.

Relative to control tissues, transcription of the CB1R gene *CNR1* appears to be increased in AFRS (ddCt = 1.35-fold ± 0.50, $p = 0.113$) and AERD (ddC_t = 1.90-fold ± 0.88, $p = 0.165$) subjects, despite failing to meet statistical significance (Fig. 1A). No significant differences

in *CNR1* expression was detected between AFRS and AERD cohorts (ddCt = 0.55-fold ± 1.01, $p = 0.623$). Upregulated transcription of the CB2R gene *CNR2* was detected in subjects with AERD vs both controls (ddCt = 5.29-fold ± 1.19, $p = 0.047$) and AFRS (ddCt = 5.15-fold ± 1.19, $p = 0.049$) (Fig. 1B). No significant differences in *CNR2* were detected between control and AFRS cohorts (ddCt = 0.14-fold ± 0.08, $p = 0.180$).

Immunohistochemistry of nasal polyps revealed structural findings consistent with the underlying disease state. Consistent with eosinophilic CRSwNP, AFRS, and AERD samples displayed a hypertrophic epithelium marked by excessive basal cells and locations of dense, mucus-producing cells vs control mucosa.[15] Both CB1R and CB2R eCA receptors predominantly localized to the well-differentiated mucosal epithelium in all samples (Fig. 2). Some positive CB1R and CB2R staining was observed in the underlying polyp tissue in AFRS and AERD, which was not observed in the control tissue.

Discussion

This pilot study is the first to evaluate eCA expression in AERD. Findings include a specific 5.2-fold upregulation in the transcription of the immunomodulatory CB2 receptor, but not CB1R, with a discrete epithelial localization that has not been described previously. Expression of the CB1R gene *CNR1* may also be increased in both AERD and AFRS cohorts relative to control mucosa without inflammation, although a level of significance change was not reached in this pilot study.

It is possible to hypothesize that eCA expression is increased as a nonspecific marker of tissue inflammation in CRSwNP. AFRS patients with eosinophilic nasal polyps, but not aspirin sensitivity, were therefore included to control for mucosal inflammation, allowing for the specific evaluation of eCA expression in aspirin-sensitive polyp disease (AERD). AFRS is a good control for AERD, as both represent eosinophilic forms of CRSwNP with similar type 2 inflammatory profiles and a lack of neutrophilic infiltration found in the heterogeneous group of patients with CRSwNP.[16] The findings suggest that, although CB1R may be increased as a nonspecific marker of sinonasal inflammation or polyposis, expression of CB2R appears specific to AERD.

The upregulation of CB2R receptors is a surprising finding as one may hypothesize that suppression of this emerging immunomodulatory system would be related to uncontrolled airway inflammation in AERD. One potential explanation for this finding relates to the functional inactivation of the CB2R receptor with secondary compensatory upregulation to reestablish physiologic eicosanoid homeostasis. Although the functional activity of CB2R in AERD is not within the scope of this pilot investigation, a nonsense CB2R Q63R single-nucleotide polymorphism has been previously described and associated with advanced cirrhosis among patients with viral hepatitis.[17,18] Additional study is necessary to evaluate native CB2R function, relative amounts of eCA ligands, and the prevalence of inactivating polymorphisms in AERD.

The role of the eCA system in the regulation of acute and chronic inflammation is slowly emerging. Earlier work has identified CB2R as a therapeutic target for the treatment of

multiple inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, and colitis.[8,16–19] Although effects of eCA modulation on sinonasal inflammation in chronic rhinosinusitis have yet to be evaluated, insight may be gained from studies of the lower airways. Braun et al.[19] utilized a murine model of ova-induced allergic asthma to evaluate the role of CB2R and CB1R in allergic sensitization and response. Findings included a significant reduction in pulmonary and systemic markers of inflammation after eCA activation in wild-type mice, a response that was not found in either $CB1R^{-/-}$ or $CB2R^{-/-}$ knockouts. Further, Motwani et al.[20] examined a selective CB2R agonist in a human model of acute dermal inflammation and identified potent anti-inflammatory activity mediated by inhibition of the leukocyte chemoattractant leukotriene B4, and the antiphagocytic prostanoids PGE2, thromboxane B2, and prostaglandin F2α. The therapeutic potential of CB2R and other eCA modulators for recalcitrant airway inflammation in AERD deserves further study.

This exploratory pilot study was designed to screen for eCA dysregulation in AERD and was not powered to exclude statistical differences in study cohorts. The external validity of the findings presented is therefore limited, which highlights the need for an additional, appropriately powered study to establish the validity of the data. In addition, enrolled subjects were not screened for the presence of exogenous cannabis metabolites that were not disclosed through the initial patient questionnaire. Future study should include objective testing to screen for this potential confounding factor. Further, our study is limited to the evaluation of eCA transcription and did not evaluate relative protein expression, ligand metabolism, or receptor function. Although *CNR2* transcription appears elevated in AERD, the expression and functional evaluation of CB2 receptors remains to be investigated.

Conclusion

The endocannabinoid system is an emerging immunomodulatory network that may be involved in AERD. This is the first study of eCA expression in sinonasal disease, showing significantly increased *CNR2* transcription with a predominantly epithelial expression pattern in subjects with AERD. Additional study is warranted to further understand the contribution and therapeutic potential of this emerging immunomodulatory system in chronic rhinosinusitis.

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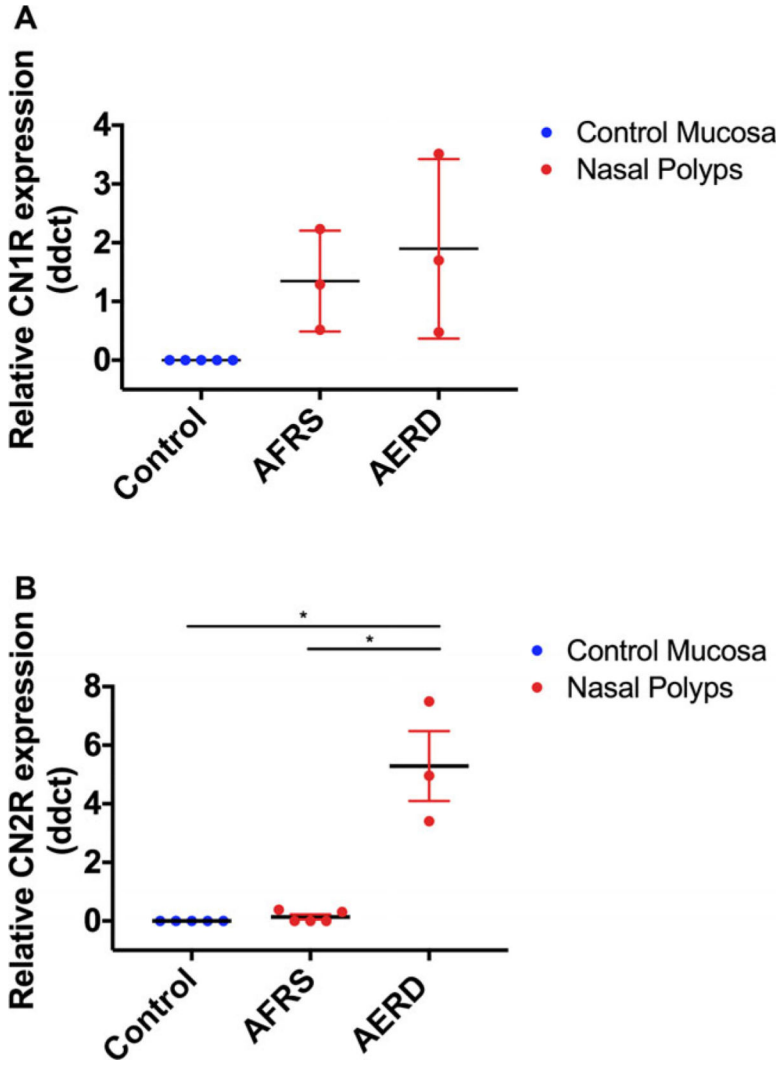

FIGURE 1.

(A) *CNR1* mRNA expression is elevated in polyp tissues from AERD or AFRS patients compared with control mucosa without inflammation. Patient-derived nasal polyp tissues were enzymatically and mechanically dissociated, then separated into a single cell suspension before isolating the mRNA, followed by creating a cDNA library for qRT-PCR analysis. (B) *CNR2* mRNA expression is 5.2-fold higher in polyp tissue from AERD patients compared with either AFRS polyp tissue or control mucosa without inflammation. AERD patient polyps demonstrated 5.2-fold higher expression of *CNR2* cannabinoid receptor than control and AFRS patient polyps, indicating an upregulation of expression. *$p < 0.05$. AERD = aspirin-exacerbated respiratory disease; AFRS = allergic fungal rhinosinusitis; mRNA = messenger RNA; qRT-PCR = quatititative reverse transcript polymerase chain reaction.

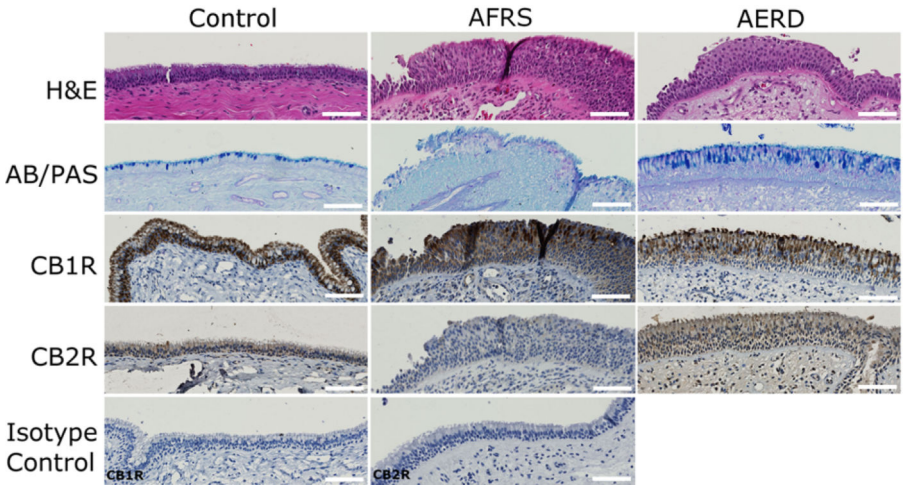

FIGURE 2.

CB2R protein localizes to the epithelial cell layer in polyp tissue from AERD patients. Formalin-fixed patient-derived tissues were processed by immunohistochemistry to detect CB1R and CB2R protein localization. The epithelial cell layer is marked by hematoxylin-and-eosin stain. Both CB1R and CB2R localize to the polyp epithelia and displayed diffuse expression in the polyp tissue underlying the epithelial cell layer at 1:200 (CB1R) and 1:25 (CB2R) dilutions of primary antibody. Isotype controls were completed at the same concentration as the CB1R and CB2R antibodies. AERD = aspirin-exacerbated respiratorydisease.

TABLE 1.

Primer sequences used for qRT-PCR

Gene	Accession number	Basepairs	Sequence name	Primers
CNR1	NM_001160226	363	CB1 F4961	5′-CCT TTT GCT GCC TAA ATC CAC-3′
			CB1 R5323	5′-CCA CTG CTC AAA CAT CTG AC-3′
CNR2	NM_001841	353	CB2 F1008	5′-TCA ACC CTG TCA TCT ATG CTC-3′
			CB2 R1360	5′-AGT CAG TCC CAA CAC TCA TC-3′
RPS18	NM_022551	170	RPS18 F171	5′-TGT GGT GTT GAG GAA AGC AG-3′
			RPS18 R340	5′-GGA CCT GGC TGT ATT TTC CA-3′

CNR1 = type 1 cannabinoid receptor; *CNR2* = type 2 cannabinoid receptor; qRT-PCR = quantitative reverse transcript-polymerase chain reaction; RPS18 = ribosomal protein S18.

TABLE 2.

Demographics and patients' characteristics of enrolled subjects

	Control	AFRS	AERD
Number	5	5	3
Age, in years[a]	55 ± 13.84	23 ± 4.69	36 ± 19.6
White, n (%)	5 (100)	0 (0)	2 (67)
Hispanic/Latino, n (%)	1 (20)	0 (0)	0 (0)
Male, n (%)	4 (80)	3 (60)	2 (67)
Allergies (skin prick/RAST), n (%)	0 (0)	5 (100)	3 (100)
Asthma, n (%)	0 (0)	2 (20)	3 (100)
Depression, n (%)	0 (0)	0 (0)	0 (0)
Current tobacco use, n (%)	0 (0)	4 (80)	0 (0)
Current cannabis use, n (%)	0 (0)	0 (0)	0 (0)
Preoperative steroids, n (%)	0 (0)	1 (20)	0 (0)
Previous sinus surgeries, n (range)	0 (0)	0.8 (0–2)	2.3 (1–3)
CT score[a]	0 ± 0	16.2 ± 1.30	16.7 ± 1.53

[a]Data expressed as mean ± standard deviation.

AFRS = allergic fungal rhinosinusitis; AERD = aspirin-exacerbated respiratory disease; CT = computed tomography; RAST = radioallergosorbent test.