Antimicrobial lipids: Novel innate defense molecules are elevated in sinus secretions of patients with chronic rhinosinusitis

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ABSTRACT

Background: *Airway secretions possess intrinsic antimicrobial properties that contribute to the innate host defense of the respiratory tract. These microbicidal capabilities have largely been attributed to the presence of antibacterial polypeptides. However, recent investigation has indicated that host-derived lipids including cholesteryl esters also exhibit antimicrobial properties. The purpose of this study was to determine whether sinus secretions contain such antimicrobial lipids and to compare the lipid composition in patients with and without chronic rhinosinusitis (CRS).*

Methods: *Maxillary sinus fluid was obtained* via *antral lavage from subjects with (seven patients) and without (nine patients) a history of CRS. After specimen collection, total lipid was extracted according to Bligh and Dyer (Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification, Can J Biochem Physiol 37:911–918, 1959) and lipid profiles were obtained by reverse phase high-performance liquid chromatography on an amide-embedded C18 column. In addition, the neutrophil-specific antimicrobial peptides human neutrophil peptides 1–3 (HNP1–3) were quantified by Western immunoblotting.*

Results: *Lipids, including cholesteryl esters, were identified in the maxillary sinus secretions of patients with and without CRS. However, levels of lipid composition differed between the two groups with CRS patients exhibiting greater amounts of all classes of lipids, reaching over 10-fold higher concentration when compared with non-CRS patients. This increase was independent of HNP1–3 content.*

Conclusion: *Sinus secretions of patients with CRS appear to show elevated levels of antimicrobial lipids compared with controls independent from neutrophil influx. This up-regulation suggests that host-derived lipids act as mediators of mucosal immunity in CRS. Further study is necessary to determine if such antimicrobial lipids function alone or synergistically with antibacterial peptides in conferring such inherent microbicidal properties.*

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The innate immune system has been increasingly found to play a role in the pathogenesis of airway inflammatory disease including asthma and chronic rhinosinusitis (CRS). This inherent host response provides initial protection of mucosal surfaces against infection using multiple effector molecules to achieve early recognition of microbial pathogens, preliminary bactericidal activity, and activation of the adaptive immune response.[1–4] Toll-like receptors (TLRs), surfactant proteins, and antimicrobial peptides represent significant mediators of this intrinsic defense mechanism, with the latter believed to be predominantly responsible for the innate microbicidal properties of airway surface fluid including nasal and bronchoalveolar secretions.[5–10] The cathelicidin LL-37 and defensins comprise the majority of these antimicrobial polypeptides and have been found to exhibit broad-spectrum antibacterial activity.[1–2,10]

Cathelicidins are prepropeptides that are cleaved to produce a distinct C-terminal peptide.[11,12] In humans only one cathelicidin has been described, LL-37, which possesses not only antimicrobial capabilities but also immunomodulatory activities.[11,12] Human defensins are small cationic peptides of 29–40 AA containing six cysteine residues linked by three disulfide bonds.[1,2] The defensins have been subdivided into two categories according to their distinguishing molecular structure characteristics: the α- and β-defensins.[1,2] Human neutrophil peptides 1–4 (HNP1–4) are α-defensins located in neutrophils and are associated with inflammation.[1,2] Although HNP4 is present only in minute amounts, HNP1–3 are abundantly expressed.[1,2] HNP1–3 differ only by a single amino acid and polyclonal antibodies of HNP1- 3 are cross-reactive.[1,2] β-Defensins are predominant on mucosal epithelial surfaces, *i.e.*, skin, lungs, and gastrointestinal tract.[1,2] The presence of antibacterial peptides in airway surface fluid and their contributions to intrinsic mucosal immunity of the respiratory tract have been well established.[1,2]

However, recent studies indicate that host-derived lipids, particularly cholesteryl esters, also possess antimicrobial properties and may constitute a novel defense component of the innate immune system.[13,14] Both cholesteryl linoleate (CL) and cholesteryl arachidonate (CA) show microbicidal effects against various microbes *in vitro*, including *Pseudomonas aeruginosa*.[14] In addition, antibacterial lipids have been known to participate in the inherent host defense of the skin and play a pivotal role in the protection of neonates from infection through their presence in breast milk and the vernix caseosa (the newborn coating).[15–23] In the upper airway, nasal secretions have been reported to contain all major classes of lipids as well as lipoproteins.[14] Selective removal of lipids from nasal fluid has been described to cause diminished antibacterial activity.[14] Conversely, their reintroduction into lipid-depleted nasal fluid partially restores its bactericidal properties.[14]

Although a lipid-mediated component of the innate immune system is still an emerging concept, such findings suggest that antimicrobial lipids may contribute to the instrinsic host defense of the upper airway and, potentially, the paranasal sinuses. The purpose of this study was to determine if sinus secretions contain such antimicrobial lipids, and if the lipid composition differed in patients with and without CRS. In addition, HNP1–3 levels of CRS and non-CRS patients were analyzed to compare defensin and antimicrobial lipid response to infection and to ascertain whether antimicrobial lipid production was indigenous to the epithelia or secondary to neutrophil delivery during inflammation.

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MATERIALS AND METHODS

Specimen Collection

Maxillary sinus fluid was obtained from nine subjects without a history of sinus disease *via* antral lavage through the canine fossa under institutional board review approval. All patients denied having any previous history of sinus infection and showed no radiographic evidence of sinus pathology either on CT scan or MRI. Similar specimens were also procured from seven subjects who fulfilled the diagnostic criteria for adult CRS as defined by the 2007 American Academy of Otolaryngology–Head and Neck Surgery Foundation multispecialty panel clinical practice guidelines.[24] Nasal endoscopy revealed either mucopurulent discharge and/or edema of the middle meatal mucosa in these patients. CT showed either partial (2/7) or complete (5/7) opacification of the maxillary sinus from which the specimens were obtained. All of the CRS patients were treated with at least a 3-week course of antibiotics in conjunction with saline rinses and steroid therapy. Only when patients continued to have persistent symptoms after medical therapy along with abnormal nasal endoscopic and/or CT findings were they considered for functional endoscopic sinus surgery. None of the seven CRS patients had asthma or nasal polyposis or had undergone previous sinus surgery. No perioperative antibiotics were administered. Intraoperatively, the maxillary sinuses from which the specimens were acquired showed mucopurulent secretions with edema of the mucosa lining. Cultures obtained from the maxillary sinuses were positive for *Staphylococcus aureus* (4 cases), *P. aeruginosa* (2 cases), and *Klebsiella pneumoniae* (1 case), respectively. Pathology reports from the maxillary sinus tissue that was resected showed evidence of chronic inflammation. Fragments of respiratory epithelium were seen with areas of metaplastic squamous mucosa overlying inflamed stroma and a slightly thickened basement membrane. No eosinophils were identified in any of the specimens. Antral lavage was performed immediately before functional endoscopic sinus surgery. After specimen collection samples were centrifuged to eliminate cellular material and the resulting supernatants were stored at −70°C.

Lipid Standards

Lipid standards were purchased from Sigma-Aldrich (St. Louis, MO). These were dissolved in dichloromethane in glass vials, overlaid with nitrogen gas, and stored at −20°C. Free fatty acids used were as follows: myristic acid (C14:0, defining the number of C atoms to the number of double bonds), palmitic acid (C16:0), heptadecanoic acid (C17:0, plant fatty acid absent in humans and used as internal standard), stearic acid (C18:0), and docosahexaenoic acid (DA; C22:6). The glycerolipid used was tripalmitic acid. Sterols used include cholesterol, and the cholesteryl esters were cholesteryl palmitate, cholesteryl stearate, CL, and CA.

Sinus Fluid Processing

Sinus fluid was processed similar to the method used by Do *et al.* (2008).[14] Briefly, sinus fluid was heat inactivated for 10 minutes at 60°C and then sonicated three times for 10 seconds using a Fisher Dismembrator (Fisher Scientific International, Hampton, NH) at power setting 2. The samples were then centrifuged for 3 minutes at 50 × *g* (15°C), and the supernatants were aspirated and further centrifuged for 30 minutes at 16,100 × *g* (4°C). The resulting supernatants were stored under N_2 gas at − 20°C until further use for lipid extraction, total protein, and HNP1–3 quantification.

Lipid Extraction

Lipid extractions were based on the Bligh and Dyer method.[25] Briefly, 1.25 mL of methanol and 0.625 mL of chloroform were added to 0.5 mL of sample, flushed with N_2 gas, and vigorously vortexed for 1 minute. Then, 0.625 mL of chloroform was added, the sample was flushed with N_2 gas, and was vortexed for 1 minute. The procedure was repeated with 0.625 mL of dH_2O. Samples were then incubated on a rotary shaker for 20 minutes at power setting 6 (Lab-Line Maxi Rotator; Barnstead International, Dubuque, IA) and centrifuged (652 × *g*, 15°C, 15 minutes). The lower phase containing lipids was collected and a volume of chloroform equal to the volume of lower phase was added to the upper (aqueous) phase to repeat the lipid extraction. The chloroform layers from both steps were combined and the organic solvent was removed under a gentle stream of N_2 gas at 37°C. To control for lipid extraction efficiency, heptadecanoic acid was added (15 μg) to all samples before lipid extraction. For pooled samples, 0.5-mL volumes were extracted, and for individual samples typically 150 μL was extracted.

Reverse-Phase High-Performance Liquid Chromatography (HPLC)

Separation and quantification of lipids was performed with a low-pressure quarternary gradient system (Summit HPLC System, with Dionex PCS1Chromeleon software; Dionex Corp., Sunyvale, CA) on a reverse-phase column (Dionex Polar Advantage 2 [Dionex Corp.]; particle size, 3 μm; 150 mm length × 2.1 mm ID; preequilibrated in acetonitrile ([olvent A], reagent grade alcohol [solvent B], and water [solvent C], 68.6/16.4/15, respectively; column temperature, 25°C) with evaporative light scattering detection (ELSD 800, operated at 45°C, 1.9 bar N_2; Alltech Associates, Inc., Deerfield, IL). The gradient was as follows: from 0 to 5 minutes, water, 15–0%; alcohol, 16.4–19.4%; and acetonitrile, 68.6–80.6%; from 5 to 6.1 minutes, alcohol, 19.4–50%, and acetonitrile, 80.6–50%; from 6.2 to 33.1 minutes, alcohol, 50%, and acetonitrile, 50%. Lipid extracts were resuspended in 10 μL of dichloromethane and the entire aliquot was injected and eluted (0.307 mL/minute). Response curves were established for the sterols and CL. All solvents used were HPLC grade.

Total Protein Quantification

NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE) was used to quantify the total proteins according to the manufacturer's instructions. The sample volume measured was 2 μL. Individual samples were measured twice and averages were calculated. PBS was used as blank. From the NanoDrop software (NanoDrop Products) the A280 application was used to determine the total protein concentration.

HNP1–3 Quantification

To quantify HNP1–3 in individual samples, 10 μL each were subjected to acetic acid-urea PAGE followed by Western immunoblotting with polyclonal rabbit anti–HNP1–3 (1:400; kindly provided by Tomas Ganz, University of California, Los Angeles), alkaline phosphatase–conjugated goat anti-rabbit polyclonal antibodies (Pierce Biotechnology, Inc., Rockfort, IL, 1:2000), and a BCIP/NBT colorimetric detection system (Research Products International Corp., Mount Prospect, IL) as described previously.[26] Developed blots were scanned and sample HNP1–3 concentrations were calculated based on a standard curve derived from purified HNP2 peptide (kindly provided from Tomas Ganz, University of California, Los Angeles) using the Versadoc imaging system and Quantity One software (Bio-Rad, Hercules, CA). Samples that did not reveal any bands for HNP1–3 were subjected to the more sensitive dot blot to confirm absence of HNP1–3 following a protocol described by Shen *et al.* (2005), but using the polyclonal rabbit anti–HNP1–3, standard HNP2 peptide and imaging analysis as described previously.[27]

Data Analysis

Data analysis was conducted with SPSS Version 16.0 (SPSS Inc., Chicago, IL). Data values for the variables assessed spanned several orders of magnitude (*i.e.*, nonpolar lipids ranged from a minimum of

135 mV × minute to a maximum of 159,359 mV × minute). To control for heteroskedasticity among treatment groups, a variance stabilizing logarithmic transformation, $\log_{10}(1 + X)$, was used. Dependent variables were analyzed using a two-factor analysis of variance. Graphs were prepared and statistical significance was calculated with SigmaPlot Version 9.0 (Systat Software Inc., Chicago IL).

RESULTS

Nonpolar Lipids (NPLs), Cholesteryl Esters, Are Elevated in Sinus Secretions of CRS Patients

Maxillary sinus secretions acquired from non-CRS and CRS patients were subjected to lipid analysis using HPLC as described previously. Corresponding representative chromatograms are shown in Fig. 1 *A*. Although non-CRS samples contained minimal amounts of all classes of lipids in both individual and pooled specimens, a marked elevation of NPL was evident, particularly with respect to cholesteryl esters, in maxillary sinus secretions from CRS patients. Among the cholesteryl esters, the most prominent peak was shown for CL. Comparison of total NPL (Fig. 1 *B*) and CL levels (Fig. 1 *C*) in non-CRS and CRS specimens revealed at least 10-fold and 5-fold increases in the CRS samples, respectively ($p = 0.000$ for NPL and 0.004 for CL in univariate ANOVA). Cholesteryl ester levels reached over 30-μg/mL concentrations in CRS patients. This finding suggests that antimicrobial lipid production is inducible in response to infection.

Up-Regulation of Antimicrobial Lipids Is Independent of HNP Amplification

A hallmark of inflammation is neutrophil influx accompanied by elevated HNP levels. To further corroborate that the observed increased lipid levels in CRS patients are a product of mucosal epithelial cells and not simply a by-product of inflammation contributed by neutrophils or blood contamination acquired during sample aspiration, we quantified HNP1–3 of sinus secretions from non-CRS and CRS patients. In addition, we determined total protein concentrations to address possible variations in sample density. The mean total protein (Fig. 2 *A*) and HNP1–3 content (Fig. 2 *B*, representative Western immunoblot, and Fig. 2 *C*) were not statistically significantly elevated in CRS samples when compared with non-CRS specimens.

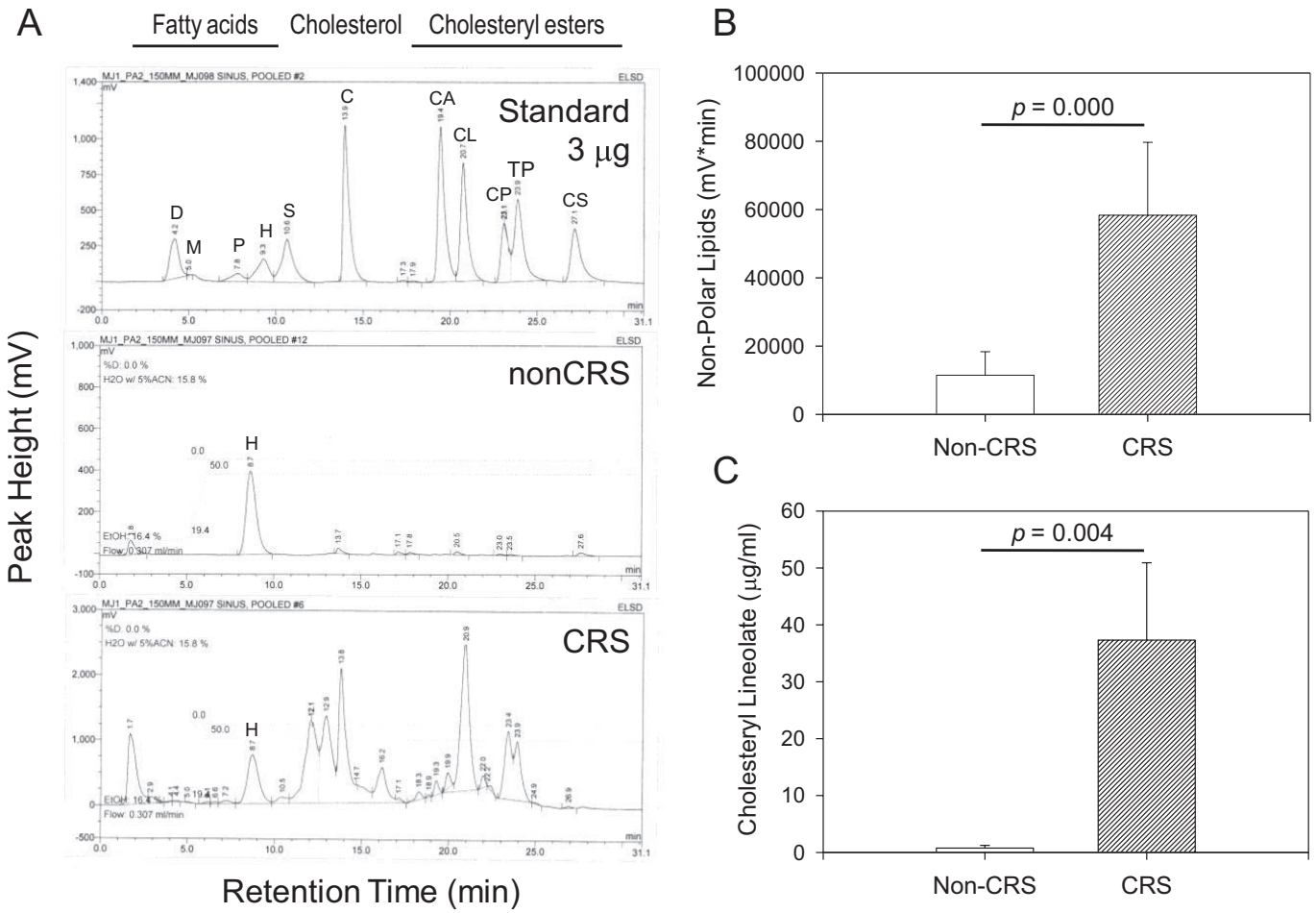

Figure 1. Nonpolar lipids including cholesteryl esters are increased in chronic rhinosinusitis. (A) Representative HPLC chromatograms from standard lipids (standard, 3 μg each) and lipid extracts prepared from 0.5-mL pooled sinus samples obtained from patients with (CRS) and without (non-CRS) CRS, respectively. (B) Quantification of nonpolar lipids consisting of cholesterol, cholesteryl esters, and triglycerides. Shown are the means + SEM of the area under the curves of the HPLC chromatograms expressed as millivolts × minutes for non-CRS and CRS specimens. (C) Cholesteryl linoleate concentrations shown in panel B were calculated according to the equation derived from standard curves. Shown are means + SEM, n = 9, for non-CRS and n = 7 for CRS. Statistical significance was calculated by univariate ANOVA. CRS, chronic rhinosinusitis; D, docosahexaenoic acid; M, myristic acid; P, palmitic acid; H, heptadecanoic acid (internal standard); S, stearic acid; C, cholesterol; CA, cholesteryl arachidonate; CL, cholesteryl linoleate; CP, cholesteryl palmitate; TP, tri-palmitin; CS, cholesteryl stearate; HPLC, high-performance liquid chromatography.

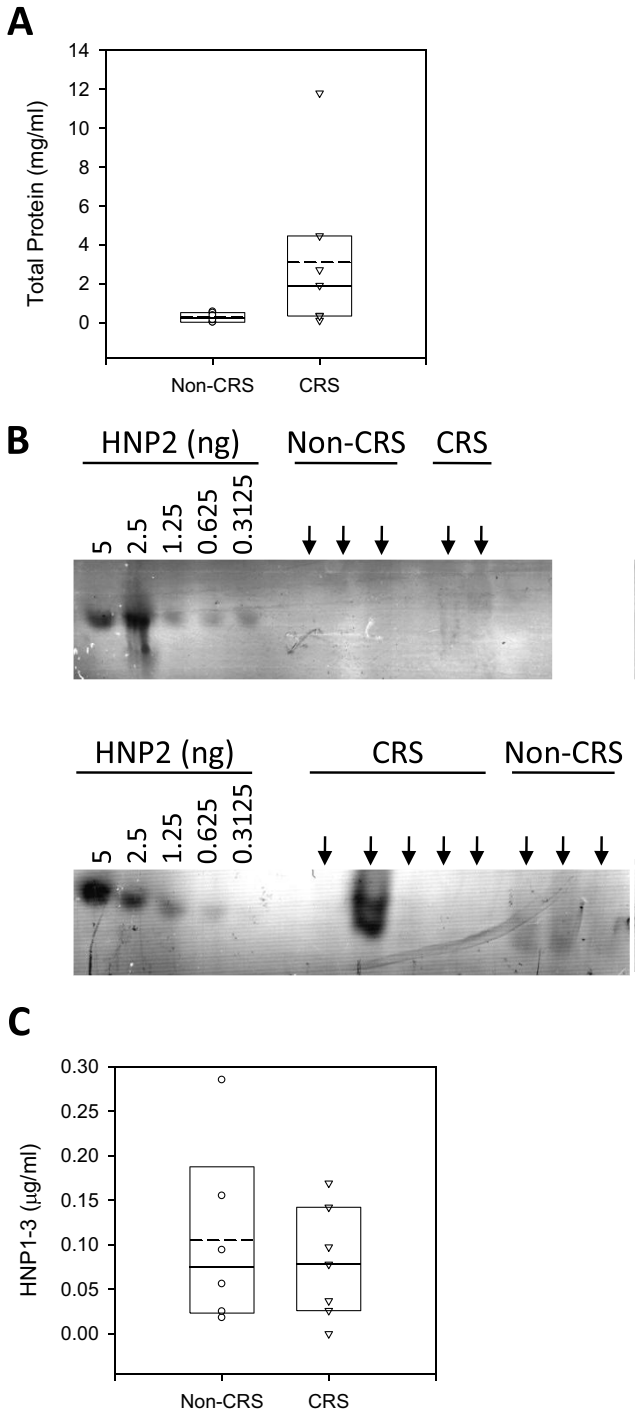

Figure 2. Quantification of total protein and HNP1–3 in sinus samples. (A) Total protein concentrations from non-CRS (n = 9) and CRS (n = 7) patients are depicted. Shown are individual measurements and a summary box plot. For the box plot the boundary of the box closest to and farthest from zero indicates the 25th and 75th percentile, respectively; the straight line and the dashed line within the box indicate the median and the mean, respectively. The error bar above the box indicates the 90th percentile. (B) Representative Western immunoblots for HNP1–3 quantification. Purified HNP2 standard peptide and 10 μL of each sinus sample (arrows) were subjected to acetic acid-urea PAGE followed by Western immunoblotting probing for HNP1–3 with a polyclonal antibody. (C) HNP1–3 concentrations from non-CRS (n = 9) and CRS (n = 7) patients were derived from Western immunoblotting assay. Shown are individual measurements and a summary box plot (see panel A). CRS, chronic rhinosinusitis; HNP1–3, human neutrophil peptides 1–3.

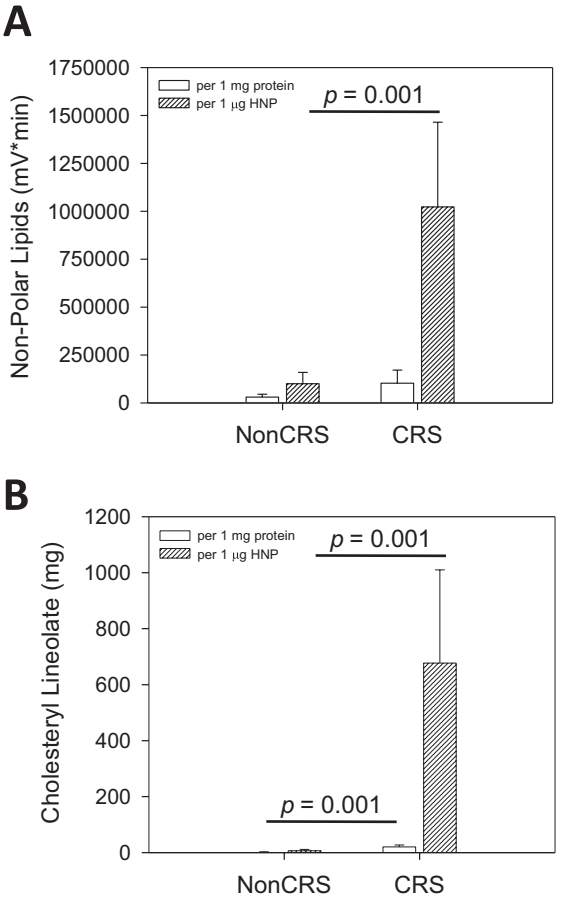

Figure 3. Nonpolar lipids including cholesteryl esters are increased in sinus samples from CRS patients independent from total protein and HNP1–3 levels. Nonpolar lipid contents expressed as (A) millivolts × minutes and (B) cholesteryl linoleate concentrations in lipid extracts from individual non-CRS and CRS samples calculated per 1 mg of total protein and 1 μg of HNP1–3. Means + SEM are shown. Statistical significance was calculated with univariate ANOVA. CRS, chronic rhinosinusitis; HNP1–3, human neutrophil peptides 1–3.

However, antimicrobial lipids were found to be significantly higher in CRS samples (Fig. 3) when adjusted to both total protein and HNP concentrations. NPLs showed a statistically significant increase in CRS patients when compared with non-CRS patients after adjustment to HNP1–3 content ($p = 0.001$ in univariate ANOVA). Likewise, CL was also statistically significantly increased in CRS samples when adjusted to both HNP1–3 and total protein content ($p = 0.001$ for both in univariate ANOVA). These findings suggest that antibiotic lipid synthesis is independent from HNP up-regulation and potentially indigenous to the epithelia rather than exclusively delivered by neutrophil infiltration during the inflammatory process.

DISCUSSION

The innate immune system plays a pivotal role in the initial defense of mucosal surfaces of the respiratory tract. TLR, surfactant proteins, and antimicrobial peptides represent distinct, integral components of the intrinsic host response and their respective contributions have been well delineated.[1,2,6,7,9,10] However, recent studies have shown that host-derived lipids may also contribute to the nascent protection of mucosal surfaces against infection.

In the skin, antimicrobial lipids have already been documented to assist in combating microbial infiltration. Free fatty acids in the stra-

tum corneum possess antimicrobial activity against *S. aureus, Streptococcus pyogene*s, and *Staphylococcus epidermidis*.[15] Mutations in the enzyme responsible for the synthesis of oleic and palmitoleic acids have resulted in failure to clear Gram-positive bacterial skin infections in murine models.[28] Antimicrobial lipids also serve an integral role in the protection of neonates against infection.[17–23] Lipids present in breast milk (sphingolipids and triacylglycerides) are hydrolyzed by lipases in the gastrointestinal tract into breakdown products (medium-chain saturated, long-chain unsaturated fatty acids, and monoglycerides) that exhibit innate antibacterial activity against *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Salmonella enteritidis* that are believed to help reduce the incidence of gastrointestinal infection in infants.[17–23,29] In addition, free fatty acids (palmitic and linolenic) in the vernix caseosa (the newborn coating) have been found to exert antimicrobial activity against *Bacillus megaterium*, which is synergistic with the antibacterial actions of the cathelicidin LL-37.[16]

Antimicrobial lipids may also play a role in the intrinsic host defense of the airway. Both bronchoalveolar lavage fluid and nasal fluid contain all major classes of lipids, with cholesterol and cholesteryl esters being the most predominant NPLs.[14] In addition, CL and CA have been described to exert dose-dependent bactericidal and bacteriostatic activity, respectively, against *P. aeruginosa* at concentrations similar to that seen physiologically in nasal secretions.[14] Removal of such nonpolar antimicrobial lipids from nasal fluid (while taking care to still maintain the same antimicrobial peptide content) diminished its bactericidal activity against *P. aeruginosa*; while reintroduction of lipid extracts into lipid-depleted nasal secretions resulted in partial restoration of its microbicidal properties.[14]

No previous studies in the literature, to the best of our knowledge, have evaluated sinus secretions for the presence of antimicrobial lipids. We hypothesized that antibacterial lipids, due to their potential role as mediators of innate immunity, would be up-regulated in sinus secretions in response to infection. HPLC analysis of maxillary sinus fluid in both CRS and non-CRS patients revealed a lipid profile that was comparable with nasal secretions. All major lipid classes were evident, including more polar fatty acids and nonpolar cholesterol and cholesteryl esters. However, the amounts of lipids varied dramatically between the CRS and non-CRS patients.

Low levels of all lipids were detected in sinus secretions of non-CRS patients when examined collectively. In contrast, when the sinus secretions of CRS patients were analyzed, marked up-regulation of NPLs was evident both in individual and in pooled samples. This amplification was statistically significant for CL, which reached concentrations even greater than that needed for antibacterial activity. Such augmentation is consistent with its potential role as an effector molecule of mucosal innate defense. Fatty acids also appeared to be up-regulated in CRS patients. However, because fatty acids (unlike cholesteryl esters) are also found in bacteria, it is unclear whether this increase was secondary to microbial infiltration or from the epithelia itself.

This enhanced response of antimicrobial lipids, especially CL, is similar to what has been observed in the behavior of other intrinsic defense molecules in the context of infection. Multiple mediators of innate immunity have been found to be up-regulated in CRS.[7,30–34] TLR-2 mRNA expression, human β-defensins 1–2, and cathelicidins (LL-37) have all been reported to be elevated in CRS patients when compared with controls.[7,30–34] Likewise, amplified levels of human defensins 1–3, human β-defensins 1–2, and LL-37 have also been described in patients with CRS and CRS with nasal polyposis (CRSwNP).[31,33–34] However, down-regulation of antimicrobial immune marker expression (TLR-9) has also been reported in CRSwNP patients.[35–37] In our study, none of the CRS patients possessed concurrent nasal polyposis. Consequently, further investigation is necessary to determine whether antimicrobial lipids are elevated or diminished in patients with CRSwNP.

The degree of up-regulation of antimicrobial lipids observed in our CRS specimens was greater than that observed for HNP1–3. When NPL amounts for non-CRS and CRS samples were adjusted to the total protein concentration and HNP1–3 levels of each group, there was a pronounced increase in the NPL/protein ratio and a statistically significant increase in the NPL/HNP, CL/protein, and CL/HNP ratios in CRS patients. These findings suggest that antibiotic lipid production is (1) inducible, similar to the defensin response, and (2) potentially originates from the epithelia itself rather than exclusively delivered to the site of infection by neutrophils. Murine models have illustrated that synthesis of certain skin antimicrobial lipids are TLR responsive, corroborating the concept that production of antibacterial lipids in the upper airways is an inducible reaction and part of the innate immune response.[16]

Abnormalities in antimicrobial lipids have been previously implicated in the development of other inflammatory diseases. Atopic dermatitis and dermal *S. aureus* colonization has been associated with decreased levels of sphingosine in the stratum corneum.[38] Irregularities in fatty acid metabolism has been reported in cystic fibrosis with diminished amounts of linoleic acid and DA seen in the saliva and serum.[39–41] Dietary supplementation of DA has been reported to ameliorate the clinical condition of cystic fibrosis patients.[42] Aberrancies in antibacterial cholesteryl esters may play a role in the pathogenesis of CRS as well. Antimicrobial lipid responses may vary in the sinus epithelia of healthy subjects versus CRS patients in response to bacterial challenge, allowing microbial colonization in the latter. Additional research is warranted to elucidate and compare cholesteryl ester production of sinus mucosa in CRS and non-CRS patients to corroborate this hypothesis.

The precise bactericidal mechanism of antimicrobial lipids is still under investigation. Membrane disruption from insertion of hydrophobic chains into the lipid bilayer similar to what is seen in defensins has been the primary hypothesis proposed.[43] Antimicrobial lipids also work in concert with antibacterial peptides to eliminate offending pathogens. DA, palmitic acid, and CL, have been described to act synergistically with lysozyme, LL-37, and HNP2, respectively, in terms of their microbicidal effects.[14,16,43] However, it is unclear what the relative contributions of antimicrobial lipids and antibacterial peptides are to mucosal host defense. Further investigation is needed to delineate the complex interactions of antimicrobial lipids with other effectors of the innate immune response in providing comprehensive nascent protection against microbial invasion.

CONCLUSION

The concept of a host-derived lipid–mediated component of innate immunity is an emerging phenomenon. Endogenous antibiotic lipids have already been determined to contribute to the intrinsic mucosal defense of the skin, neonate, and the upper and lower airways. Their dramatic up-regulation in patients with CRS independent from HNP amplification suggests that antibacterial lipids, particularly cholesteryl esters, may also play a role in the inherent mucosal host resistance and microbial pathogenesis of this disease. These findings support the contribution of a novel, distinct, lipid-based antimicrobial effector pathway in the innate immune response to infection. However, further investigation is necessary to determine the relationship between such antimicrobial lipids and other mediators of mucosal immunity in the context of CRS.

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