Pneumococcus Activation of the 5-Lipoxygenase Pathway and Production of Glycoproteins in the Middle Ear of Rats

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Pneumococcal otitis media is associated with the production of potent inflammatory mediators (leukotrienes), but the mechanism by which pneumococcus induces production of leukotrienes in the middle ear is poorly understood. In this study, up-regulation of 2 genes that govern the lipoxygenase pathway, cPLA₂ and 5-LOX, was observed in rats following inoculation of pneumococcus into the middle ear cavity. Expression of cPLA₂ was low, and 5-LOX gene expression was not detected in control animals. Up-regulation of cPLA₂ and 5-LOX in middle ear epithelial cells was accompanied by an increase of high-molecular-weight glycoproteins in middle ear fluid and cells. These findings suggest that pneumococcus activates the lipoxygenase pathway by up-regulating expression of the cPLA₂ and 5-LOX genes. This, in turn, may stimulate synthesis and secretion of high-molecular-weight glycoproteins that facilitate production of fluid in the middle ear cleft.

Streptococcus pneumoniae is the most common middle ear pathogen in childhood acute otitis media (OM). Nearly every child in the United States experiences an ear infection by the age of 5 years. Studies indicate that infection of the middle ear cleft is associated with production of potent inflammatory mediators, leukotrienes, which are products of the lipoxygenase pathway [1, 2] involved in induction of middle ear effusions [2] and secretion of high-molecular-weight (HMW) glycoproteins [3], a major component of middle ear effusion. While it is clear that leukotrienes participate in the pathogenesis of OM, the mechanism by which pneumococcus produces leukotrienes in the middle ear is poorly understood.

Leukotrienes are potent chemoattractants that induce infiltration of inflammatory cells; they are vasoactive substances that modulate permeability of the vessels (fluid induction) and are secretagogues for glycoproteins that stimulate secretion of HMW glycoproteins [3, 4]. HMW glycoprotein is a major component of middle ear effusions [5]. It facilitates the formation of fluid in the middle ear and contributes to its viscosity [6], owing to its biologic properties [5, 7]. Excessive production of HMW glycoprotein is a serious problem in middle ear infections because it impairs fluid clearance from the middle ear cavity.

cPLA₂ and 5-LOX genes play a key role in regulation of the lipoxygenase pathway. The gene cPLA₂ is responsible for specific cleavage of arachidonic acid from cellular membranes at the 2-position of phospholipids. Following release of arachidonic acids, several enzymes downstream are responsible for metabolizing arachidonic acids to prostaglandins, leukotrienes, and lipoxins.

The 5-LOX pathway plays an important role in inflammation and is important in disease processes [8, 9]. It was shown recently that inflammation of the middle ear cleft induced by arachidonic acids was markedly reduced in 5-LOX-deficient mice [10]. Mice with knockout of the 5-LOX gene demonstrated resistance to the lethal effects of shock [10]. Products of the 5-LOX pathway determine the extent and duration of the inflammatory reaction [8, 9, 11, 12]. We and others have demonstrated that lipoxygenase products trigger release of mucous glycoproteins and induce OM with effusion (OME) [2, 4]. Several lines of evidence accumulated to date prompted us to examine the expression of genes responsible for activity of the 5-lipoxygenase pathway and production of mucous glycoprotein in pneumococcal OM.

Materials and Methods

Induction of pneumococcal OM and collection of middle ear effusions. Twelve specific germ-free Sprague-Dawley rats weighing 200–250 g each were inoculated with S. pneumoniae type 6A (2.5 × 10⁸ cfu/ear) in 50 μL of PBS via a transbullar approach. Rats were anesthetized with ketamine HCl (40 mg/kg) and xylazine (8 mg/kg). After decontamination of the neck skin, a ventral midline incision was made, and the ventral surface of the bullae was exposed. A tiny hole was made with a needle (gauge 25) to vent the bullae, and another hole was made for the inoculation. Both middle ear cavities of each rat were inoculated, the holes were sealed with sterile bone wax, and the incision was closed. PBS alone (50 μL/ear) was inoculated into both middle ear cavities of another 12 rats as controls. On day 3, all rats were deeply anesthetized with...
ketamine HCl (100 mg/kg) and decapitated. Both bullae of each rat were immediately opened in the bottom of the bullae prior to washing of the middle ear cavity with PBS containing 40 U/mL RNase-inhibitor, 1 mM phenyl-methylsulfonyl fluoride (PMSF), and 15 mM 3-(3-cholamidopropyl)dimethylammonio]-propane-sulfonate. Effusion samples from each group were pooled, supplemented with 4 M guanidine HCl, 50 mM Tris HCl (pH 7.5), 5 mM EDTA (pH 8.0), 2 mM PMSF, and 0.02% sodium azide, and stored at 4°C.

Harvest and isolation of middle ear total RNA. Following collection of effusion samples, the entire layer of the middle ear cleft (including mucosal, submucosal, and infiltrated inflammatory cells) was lysed in situ by lavage [13]. Bullae were repeatedly injected with 4 M guanidine isothiocyanate solution (4–6 times, 75 μL each) for harvest and isolation of total RNA (complete harvest of the middle ear cleft was confirmed by histologic examination). Lyse from the control and experimental bullae were pooled by group. Total RNA was isolated using the one-step method described by Chomczynski et al. [14]. Sodium acetate (3 M, pH 5.2, 1/10 by volume of the lysate) was added to the lyse. An equal volume of a mixture of phenol–chloroform–isoamyl alcohol (245:49:1, vol/vol) was added to the lyse for 15 min on ice. The lyse was centrifuged at 11,900 g for 20 min. The aqueous phase was transferred to a new microtube, and an equal volume of cold isopropanol was added to precipitate RNA at −20°C overnight.

The mixture was centrifuged for 20 min at 11,900 g at 4°C. The RNA pellet was harvested, washed with 70% ethanol, dissolved in water free of RNase (ribonuclease), quantitated by spectrophotometry at 260 nM, and stored at −70°C. After removal of DNA by ethanol, proteins were precipitated using isopropanol, dissolved in 4 M guanidine HCl, 50 mM Tris HCl (pH 7.5), 5 mM EDTA (pH 8.0), 2 mM PMSF, and 0.02% sodium azide, and stored at 4°C.

Examination for expression of the 5-LOX gene. By use of reverse transcription–polymerase chain reaction (RT-PCR) in a quantitative manner, we evaluated the effect of pneumococcus on examination for expression of the 5-LOX gene. For construction of a control repetitive standard of the cPLA2 gene (cPLA2-CRS), we amplified a fragment of the cPLA2 gene (Genbank accession nos. S77829, S76481). Primers 1–4 were as follows: primer 1 (sense), 42 mers, bases 322–340, preceded by T,-sequences 5'-TTATACGACTCATATAGGCCGACACGAGGAAGCGAACAAGA-3'; primer 2 (antisense), 40 mers, bases 1015–1033 and 795–814, 5'-AGTAAAGGTGACAGTGTTGATCCAAATCCCTGATTCAT-3'; primer 3 (sense), 19 mers, bases 322–340, 5'-CAGCAGGAAGCGAACAAGA-3', and primer 4 (antisense), 19 mers, bases 1015–1033, 5'-AGTAAAGGTGACAGTGTTGATCCAAATCCCTGATTCAT-3'; and

Quantitation of expression of the cPLA2 gene. By use of competitive RT-PCR, primer sequences were derived from mRNA for the cPLA2 gene (Genbank accession nos. S77829, S76481). Primers 1–4 were as follows: primer 1 (sense), 42 mers, bases 322–340, preceded by T,-sequences 5'-TTATACGACTCATATAGGCCGACACGAGGAAGCGAACAAGA-3'; primer 2 (antisense), 40 mers, bases 1015–1033 and 795–814, 5'-AGTAAAGGTGACAGTGTTGATCCAAATCCCTGATTCAT-3'; primer 3 (sense), 19 mers, bases 322–340, 5'-CAGCAGGAAGCGAACAAGA-3', and primer 4 (antisense), 19 mers, bases 1015–1033, 5'-AGTAAAGGTGACAGTGTTGATCCAAATCCCTGATTCAT-3'; and

For construction of a control repetitive standard of the cPLA2 gene (cPLA2-CRS), we amplified a fragment of the cPLA2 gene using RT-PCR with primers 1 and 2. The fragment contained the same sequence as that of the template (the wild type), except for a 100-bp deletion between bases 814 and 1015 (figure 1). Samples of RNA isolated from the middle ear were reverse-transcribed into cDNA using oligo dT and MuLV reverse-transcriptase (at 42°C for 25 min). Following the RT, primers 1 and 2 and Taq DNA polymerase were added to the above reaction mixture to amplify a fragment of cDNA (cDNA for cPLA2-CRS, 100-bp deletion compared with the product of the wild type cPLA2). The cDNA for cPLA2-CRS was isolated by electrophoresis on 2% agarose gel, purified by a GenElute spin column (Supelco, Bellefonte, PA), and transcribed into RNA (RNA for cPLA2-CRS) in vitro by T, RNA polymerase using a Megascript kit (Clontech, Palo Alto, CA). The RNA for cPLA2-CRS was quantitated by spectrophotometry at 260 nM.

We performed the competitive RT-PCR by addition of decreasing amounts of cPLA2-CRS, from 30 μg/mL to 0.0096 μg/mL, to

Figure 1. Construction of control repetitive standard of cPLA2 gene. Primer (P) 2 (compound antisense primer) was designed to delete 100 bp from wild type (thick line) between 814 bp and 1015 bp. P1 (compound sense primer) contained promoter area (T,-sequences). cPLA2-CRS cDNA was produced by Taq DNA polymerase using compound sense and antisense primers P1 and P2. cPLA2-CRS RNA was transcribed by use of T, RNA polymerase in vitro.
a constant amount of sample RNA (1 μg/microtube) in six successive microtubes. Each mixture was then subjected to the RT-PCR procedure. RT was done using primer 4 and MuLV reverse transcriptase, and primers 3 and 4 and Taq polymerase were used for PCR. Products of the RT-PCR were run on 2% agarose gels for ~20 min to allow bands from the sample and from the control (CRS) to be separated. Incorporation of ethidium bromide into 2 bands was detected as described by Riedy et al. [15]. The point at which cDNA from the corrected cPLA2-CRS equaled cDNA from the wild type PLA2, provided an indicator of concentration of cPLA2 mRNA transcript.

**Results**

**Quantitation of HMW glycoproteins.** HMW glycoproteins (mucin or mucin-like) were purified using the method described by Dekker and coworkers [16, 17], with minor modifications. Samples from both middle ear fluid (MEF) and cells were sonicated and homogenized for 1 min. Samples were reduced by incubating the homogenate with 100 mM dithiothreitol for 24 h and were carbosylated by incubation with 250 mM iodoamylamide for 24 h under a nitrogen atmosphere. Debris or insoluble substances were removed by centrifugation of samples at 8000 g for 20 min. The solution was then supplemented with cesium chloride (1.4 g/mL) to a final volume of 11 mL, centrifuged for 72 h at 1.6 × 10^6 g with the brake off, and fractionated with 1 mL per tube. Density, protein, and hexose of each fraction were measured. Density was determined by weight. Protein concentration was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Carbohydrate concentration was determined by hexose assay.

**Bicinchoninic acid protein assay.** Ten microliters of sample from each fraction was placed onto an ELISA plate. A series of dilutions of albumin (range, 32.5–2000 μg/mL) were used as standards. A buffer containing 4 M guanidine HCl, 50 mM Tris HCl (pH 7.5), 5 mM EDTA (pH 8.0), and 0.02% NaN3, was used as the blank control. All samples, standards, and controls were run in duplicate. A total of 200 μL of the mixture containing solutions A and B (50:1 from the protein assay kit) was added to each well and incubated at 37°C for 30 min. The plate was loaded onto a kinetic plate reader (Molecule Devices, Menlo Park, CA), and each well was measured at 490 nm. The proteinaceous concentration was calculated using the standard curve with the background subtracted (version 2.35; Softmax, Menlo Park, CA). Results were expressed as micrograms per milliliter.

**Hexose assay.** In a similar manner, samples, standards (galactose with a series of dilutions of 68.125–2500 μg/mL), and blank controls were placed onto ELISA plates (10 μL/well, in duplicate), and 2.3 μL of water-saturated phenol was added, followed by 69.5 μL of concentrated sulfuric acid. The plate was loaded onto the kinetic plate reader (Molecule Devices), and each well was measured at 490 nm. Concentrations of hexose were calculated using the standard curve with the background subtracted. Results were expressed as micrograms per milliliter. The ratio of hexose-to-protein in each fraction was calculated as the concentration of hexose over that of protein. Identification of HMW glycoproteins (mucin or mucin-like) was determined by a density of ~1.4 g/mL overlapped by a ratio of hexose-to-protein of ~2:1.

**Discussion**

Inoculation of 2.5 × 10^6 cfu type 6A S. pneumoniae into the middle ear cavity of Sprague-Dawley rats produced experimental acute OM with an inflammatory reaction in the middle ear and MEF that became apparent on days 2–5 (peaked on
Figure 2. Identification and quantitation of high-molecular-weight glycoproteins (HMW) in middle ear effusions. A, CsCl density gradients of effusion. Density increases with fraction no. (~1.38–1.52 g/mL between fractions 8 and 10). B, Concentrations of hexose after CsCl density-gradient ultracentrifugation of effusions. Hexose peaked at fraction 9. Note differences of hexose concentration between MEEC (middle ear epithelial cells), MEF (middle ear fluid), and Ctrl (control). C, Concentrations of protein in effusion. Note differences in protein concentration between MEEC, MEF, and Ctrl. HMW protein defined as having density of ~1.4 g/mL with ratio of hexose-to-protein of ~2:1. Fractions 8–10, especially fraction 9, show characteristic mucous glycoprotein.

days 3–4) after inoculation and declined thereafter [19]. Development of MEF appears to be closely related to the production of leukotrienes [1] and HMW glycoproteins in the middle ear mucosa [5, 6]. Day 3 after inoculation was chosen as the time point to study activity of the lipoxygenase pathway and the production of mucin glycoprotein for the current study because of maximal inflammatory reaction in the middle ear cleft and maximal effusion in the middle ear cavity, which represents an acute onset of OM.

Haemophilus influenzae and its products also produce OM in rats. How the middle ear pathogens activate production of those metabolites in its host is unclear. Both gram-positive and gram-negative middle ear pathogens can stimulate production of arachidonic metabolites such as prostaglandins and leukotrienes. In addition to arachidonic acid metabolites, other inflammatory cytokines, such as platelet-activating factor (PAF), tumor necrosis factor-α, and interleukin-1β, are present in MEF and contribute to the pathogenesis of OM and production of MEF [20]. Inflammatory cytokines such as PAF are linked to the 5-lipoxygenase pathway in the middle ear in vitro [4, 21].

Products of the 5-lipoxygenase pathway are potent mediators that play an important role in many diseases, including inflammatory, allergic, and immune reactions [9, 22]. Products of the 5-lipoxygenase pathway in the middle ear cleft play a critical role in the pathogenesis of OM [1, 2, 4]. Our study demonstrated expression of cPLA2 and activated 5-LOX genes in pneumococcal OM, suggesting that production of leukotrienes in the middle ear cleft is up-regulated by S. pneumoniae. The activity of this pathway appeared to be very low or null in control animals with low cPLA2 gene expression and no detectable expression of the 5-LOX gene.

cPLA2 and 5-LOX genes encode 2 enzymes that release arachidonic acid and metabolize it to leukotrienes. cPLA2 is an enzyme specifically related to the release of arachidonic acids at the 2 position of phospholipids. This process involves a 2-step reaction and requires the participation of calcium. First, calcium binding to a site on the enzyme causes a conformational change that activates the enzyme. Then the activated enzyme moves to a site on the membrane (i.e., the enzyme is translocated) and forms a reaction complex (enzyme-calcium-phospholipid). This reaction leads to the release of arachidonic acids from the cellular membrane [23–25].

The increased expression of the cPLA2 gene demonstrated in this study suggests an increase in the release of arachidonic acids, which are known precursors of leukotrienes. Consistent with this, Jung and colleagues [1, 2] have demonstrated increased arachidonic acids in effusions of the middle ear subsequent to bacterial infection. Following release of arachidonic acids, the 5-LOX gene catalyzes the peroxidation of arachidonic acids to form 5-hydroperoxycis-9,11-octadecadienoic acid (5-HPETE). The same enzyme catalyzes the conversion of 5-HPETE to leukotriene A4, leading to the subsequent formation of leukotrienes B4, C4, D4, and E4 [9, 22]. Leukotriene C4 is a potent
mediator that is implicated in the production of fluid, infiltration of inflammatory cells, and edema in the middle ear [2]. These responses are believed to occur either through a cellular and tissue contractile process dependent upon actomyosin (vasoconstriction and vasopermeability) or through direct chemical attraction to inflammatory cells (chemoattractance).

Expression of the 5-LOX gene in the middle ear in our study following inoculation with pneumococcus indicates that the lipoxygenase pathway was activated. Jung and colleagues [1, 2] also observed an increase in products of the 5-LOX pathway in middle ear effusion of humans and animals. Moreover, inoculation of the 5-LOX gene products into the middle ear cavity can induce middle ear effusion in animals [2] and secretion of mucous glycoprotein in vitro [21]. Evidence accumulated to date suggests that pneumococcus induces middle ear effusion by up-regulating the 5-LOX pathway and increasing synthesis and secretion of HMW glycoproteins.

How *S. pneumoniae* up-regulates the cPLA₂ and 5-LOX genes in the middle ear in vivo is not understood. Signal transduction for regulation of the lipoxygenase pathway is not clear, and the transcription factor(s) responsible for up-regulating transcription of the cPLA₂ and 5-LOX genes has not been identified. Protein kinase C is involved in activity of PLA₂ in some cells in vitro [26], including epithelial cells in the middle ear [27]. Whether this signal transduction pathway was involved in transcription of these 2 genes in our study is not known.

One of the biologic functions of leukotrienes on target cells is secretion of mucous glycoprotein [4], a major structural component of middle ear effusions that contributes to viscosity [6] and chronicity. Accumulation of viscous fluid in the middle ear cavity compromises its function (reducing hearing) and impairs the mucociliary transport system, which is responsible for fluid clearance from the middle ear cavity. In this study, animals inoculated with *S. pneumoniae* had evidence of increased HMW glycoproteins in cells and effusion (more than half the total mucin or mucin-like glycoprotein was released into the middle ear cavity), suggesting increased synthesis and secretion of mucin or mucin-like glycoproteins in the middle ear.

Leukotrienes may be one of the secretagogues responsible for the mucin or mucin-like glycoprotein in the middle ear cavity of the experimental group, as suggested by this in vivo study and by our previous in vitro study [4]. Further studies using animals with knockout of cPLA₂ and 5-LOX genes are needed to confirm whether expression of cPLA₂ and 5-LOX genes is required for hyperproduction of HMW glycoproteins. Of interest, the control MEEC also produced HMW glycoprotein, although the level of HMW glycoprotein was low compared with ears treated with *S. pneumoniae*. This may be due, in part, to the physical stimulus or trauma caused by the transbular inoculation or it may represent the baseline level of normal MEEC in the middle ear ciliated tract. Since the amino
acid and carbohydrate compositions of HMW glycoproteins are unknown, we assume that the HMW glycoproteins identified in this study are a mixture of mucous glycoproteins or “serous” glycoproteins from goblet cells and “serous” cells. It has long been known that epithelia of the middle ear and eustachian tube contain both mucous and serous types of secretory cells [28, 29]. Because our previous study demonstrated that blockage of the lipoxygenase pathway reduced secretion of mucous glycoproteins in vitro [4], the increase of HMW glycoproteins noted in effusions in this study appears to be due to up-regulation of the cPLA2 and 5-LOX genes. Whether the increased synthesis of HMW glycoproteins in middle ear epithelial cells was due to increased activity of the 5-LOX pathway is not clear from findings in the current study.

The cellular source of cPLA2 and 5-LOX genes is broad and extensive [30, 31], including monocytes [12, 32], lymphocytes [33], neutrophils [34], epithelial cells [35], and fibroblasts [36]. We recently found cPLA2 gene expression in cultured epithelial cells from middle ears of rats with no evidence of inflammatory cells (unpublished data), suggesting that noninflammatory cells may have contributed to the increased activity in the lipoxygenase pathway noted in this study.

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