

Reduced iNOS expression in adenoids from children with otitis media with effusion

Granath A, Norrby-Teglund A, Uddman R, Cardell L-O. Reduced iNOS expression in adenoids from children with otitis media with effusion.

Pediatr Allergy Immunol 2010; 21: 1151–1156.

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Nitric oxide (NO) is a key mediator in the local immune response of human airways. Inducible NO-synthases (iNOS), and endothelial NO-synthases (eNOS) are two enzymes known to regulate its production. The role of NO in middle ear disease is not fully known. Previous studies suggest that NO might have a dual role, both promoting and suppressing middle ear inflammation. The aim of the present study was to compare the eNOS and iNOS expression in adenoids obtained from children with otitis media with effusion (OME) with the expression seen in adenoids derived from children without middle ear disease. In addition, the expression of IL-1 β and TNF- α were analyzed, because of their role in the iNOS-induction pathway. The iNOS and eNOS expression were analyzed with real-time PCR in 8 OME and 11 control adenoids. The corresponding proteins were demonstrated by immunohistochemical staining of adenoid tissue. A Luminex[®] assay was performed to analyze IL-1 β and TNF- α in nasopharyngeal secretion in 10 OME and 8 controls, and immunohistochemistry was performed on adenoid tissue and imprints from the adenoid surface. Children with OME exhibited lower levels of iNOS than controls without middle ear disease. No such difference was seen for eNOS. The corresponding proteins were found mainly in conjunction with surface epithelium. No significant changes were seen among the cytokines tested. The present results indicate that local induction of iNOS in adenoids might be of importance for preventing development of OME.

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Key words: Adenoids; inducible NO-synthases; endothelial NO-synthases; otitis media with effusion; IL-1 β ; TNF- α

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Accepted 4 February 2010

Otitis media with effusion (OME) is a common condition in childhood and infancy. It is characterized by longstanding non-purulent fluid in the middle ear. The main symptom leading to medical or surgical intervention is mild to moderate conductive hearing loss. OME is considered to be the result of an inflammatory response of the middle ear, but the etiology is not fully understood. Viral or bacterial infections have been suggested to play a role along with a dysfunction of the Eustachian tube (1, 2). The middle ear has no aggregated lymphoid tissue of its own and is not considered as an immune induction site (3). Therefore, the nasopharynx might not only be implicated as the source of organisms causing the disease, but also as the origin of the inflammation characterizing it (3, 4).

The present study was designed to investigate the presence of inflammatory mediators in adenoids, focusing on enzymes responsible for nitric oxide (NO) production. Increased NO production is closely linked to local airway inflammation (5–7). NO also constitutes a key mediator in the local immune response, with a role in neutrophils recruitment and activation as well as T-lymphocyte differentiation (8–10).

NO is synthesized from the amino acid L-arginine with NO synthases (NOS) as rate-limiting steps. It is released in trace quantities by neurons, endothelial cells, platelets, and neutrophils in response to homeostatic and pathologic stimuli. There are three isoforms of NOS in humans; neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial

NOS (eNOS or NOS-3) (11). We and other groups have in previous middle ear studies focused on iNOS and eNOS (12, 13). Hence, the present study was designed to compare the expression of eNOS and iNOS in adenoids from children suffering from chronic OME with the expression found in hypertrophic adenoids from children without middle ear disease. In addition to NOS, we mapped and quantified cytokines in different compartments of the adenoid with emphasis on IL-1 β and TNF- α because of their role in the iNOS activation pathways.

Subjects and methods

Patients studied

The study subjects were recruited among children scheduled for adenoidectomy because of either chronic OME or symptoms of adenoid hypertrophy (mainly snoring and sleep apnoea). Adenoids were obtained from 12 children (11 boys, 2–13 yr old) with OME and 14 children (9 boys, 2–9 yr old) with adenoid hypertrophy. The children in the first group had an OME duration exceeding 3 months and a history of hearing loss with impact on daily social interactions. The hearing impairment was confirmed by audiometry and found to be 31.25 and 31.36 dB (for both ears at 250–3000 Hz), median and mean, respectively. The control group consisted of children without any history of recurrent middle ear disease or hearing impairment, and they all exhibited a faultless ear-examination upon the day of surgery.

We used hypertrophic adenoids from children without middle ear disease as controls, as tonsils from healthy children for obvious reasons could not be obtained. Even though we consider adenoid hypertrophy as an age-related physiologic condition, it cannot be entirely ruled out that it might represent a condition with an ongoing low-grade inflammation. However, other studies have used hypertrophic tonsils as controls and found that these exhibit a lower iNOS expression than lymphatic tissue from patients with chronic TB-infections (14). This supports our use of hypertrophic adenoids, the healthiest matched tissue that can be obtained, as control.

The study was approved by the local ethics committee, and an informed consent was obtained from all parents involved.

Sampling techniques

During anesthesia, prior to adenoidectomy, samples of the nasopharyngeal surface secretion were

collected using a Juhn-Tym-Tap (Xomed-Treace, Jacksonville, FL, USA) suction device. The volumes of the surface secretions were very small, and the mucus membrane on the surface of the adenoid is delicate which, in some cases, resulted in bleeding during the sampling procedure. Secretions that contained blood were rejected, resulting in 10 samples from the OME group and eight samples from the control group. Imprints were taken during anesthesia before surgical intervention and were performed by pressing a piece of sterilized plastic foam gently to the adenoid surface. The prints were then transferred to adhesion glass slides and were fixed in formaldehyde. Imprints were obtained from all children included in the study. Adenoidectomy was performed according to standard procedure using cold steel instruments, and tissue samples were obtained during surgery. The main portion of the samples was earmarked for cytokine analysis. After snap freezing in liquid nitrogen, they were stored in -80°C . The remaining portion of the samples was reserved for mRNA-analysis and was frozen and stored in the same way. Because of medical considerations the surgeon refrained from adenoidectomy in a few cases, resulting in 11 tissue samples from the OME group and 10 tissue samples from the control group.

RNA extraction and real-time PCR

The NOS expression was quantified with real-time PCR (RT-PCR). Tissue samples from eight children in the OME-group and 11 controls were eligible for this analysis. RNA from adenoid tissue was extracted using RNeasy (Qiagen, GmbH, Hilden, Germany), including an optional DNaseI following the protocol of the manufacturer. RNA quantity was measured using a spectrophotometer, and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 in all preparations. Reverse transcription to cDNA was carried out with OmniscriptTM reverse transcriptase kit (Qiagen) with oligo-dT primer in a final volume of 20 μl using the Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany) at 37°C for 1 h. The obtained cDNA was diluted with water and 18 ng was used for amplification. The real-time PCR was performed on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) using TaqMan Universal PCR Master Mix, No AmpErase UNG and Assay-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA), containing unlabeled primers and MGB probe (FAMTM

dye-labeled). The thermal cycler was programmed to perform an initial set-up (95°C, 10 min) and 45 cycles of denaturation (95°C, 15 s) followed by annealing/extension (60°C, 1 min). The relative amounts of mRNA for the NOS were determined by subtracting threshold cycle (Ct) values for these genes from the Ct value for the internal control gene β -actin (Δ Ct). Data are depicted as 2Δ Ct $\times$ 10⁵ and presented as mean values $\pm$ s.e.m.

Immunohistochemical staining

For the localization of eNOS and iNOS in adenoids, tissue samples were embedded in paraffin, cut into 3- μ m thick sections, mounted on glass slides and stored in -80°C until use. Prior to visualization, the sections were treated with xylene to remove the paraffin and rehydrated using ethanol. The eNOS and iNOS antibodies (Upstate Biotechnology Inc, Lake Placid, Millipore Billerica, MA, USA) were raised in rabbit and used in a dilution of 1:20. To facilitate binding of the antibodies, the sections were treated with Target retrieval solution (DakoCytomation, Copenhagen, Denmark) for 20 min in a microwave oven, followed by Triton $\times$ 100 (1%) for increased membrane permeability. To quench endogenous peroxidase activity, sections were incubated in 0.03% hydrogen peroxide for 10–15 min. If needed, sections were incubated for 20 min in PBS containing 2% FCS to block non-specific binding. Subsequently, HRP-labeled goat anti-mouse or goat anti-rabbit polymer was incubated with the sections for 30 min, followed by 3, 3'-diaminobenzidine (DAB) (DakoCytomation EnVision+ System-HRP kits, Glostrup, Denmark). The sections were dehydrated with increasing concentrations of ethanol, rinsed in xylene and mounted in Pertex (Histolab: Gothenburg, Sweden). TBS (pH 7.6) supplemented with 0.05% Tween 20 was used for all washing steps.

To visualize IL-1 β and TNF- α in tissue samples and imprints frozen adenoid, tissue samples were embedded in OCT compound (Tissue-TEK; Mites, Elkhart, IN, USA) and sectioned to 8 μ m in a cryostat, and mounted on glass slides (Novakemi, Sweden). Sections and imprints were fixed and stained as previously described (15). In this procedure, 0.1% saponin was used as a permeabilizing agent to allow intracellular staining for cytokines. The following antibodies were used at a concentration of 2 μ g/ml: IL-1 β MAB 201 (mouse IgG1; R&D Systems MN, USA), TNF- α : MAB I+ MAB II (mouse IgG1; BD-Sciences-Pharmingen, Franklin Lakes, NS, USA), and for isotypic controls IgG1 mouse

negative control (DAKO). Tissue sections were incubated with primary antibody at room temperature over night, whereas 30 min at 37°C were used for imprints. Secondary biotinylated goat-anti-mouse IgG1 (Caltag Laboratories, Burlingame, CA, USA) were used. Stainings were detected by use of avidin-biotin horseradish peroxidase (Vectastain, Vector Lab, Burlingame, CA, USA) followed by the substrate DAB and counterstained with hematoxylin.

Luminex assessments of secreted cytokines

Secreted cytokines in nasopharyngeal secretion samples were assessed using the Luminex instrument (Luminex, Austin, TX, USA). To enable assessment of the samples, which were highly viscous, they were weighed and diluted in PBS (1:10) after which they were centrifuged and the supernatant was collected for analysis.

IL1 β - and TNF α -levels were determined by Luminex cytokine kits (R&D systems, MN, USA) according to instructions from the manufacturer. All samples were analyzed in duplicate in two different dilutions: 1/10 and 1/50 respectively and the mean values from these were calculated and corrections were made for the diluting steps. The corrected mean value was considered the final result. The detection range was 400–13000 pg/ml for IL-1 β and 30–1000 pg/ml for TNF- α .

Statistical analysis

All data are presented as boxplots showing the median value, the upper and lower quartile and regular minimum and maximum values. Outliers are specially marked ($\odot$) and n equals the number of subjects. Mann-Whitney test was used to determine statistical differences between unpaired observations (GraphPad Prism 5, San Diego, CA, USA). p-values < 0.05 were considered statistically significant.

Results

RT-PCR analysis of the sampled adenoids revealed that children with OME causing clinically significant hearing loss exhibited lower levels of iNOS expression than controls without signs of hearing loss or other middle ear disease. No such difference was seen for eNOS (Fig. 1). The corresponding proteins for eNOS and iNOS were demonstrated in tissue samples (Fig. 2), and they were mainly localized in and around the surface epithelium. Immunohistochemical staining showed IL-1 β and TNF- α -producing cells in

adenoid tissue, but there was no difference in frequencies of positive cells between the two study groups (Fig. 2 and data not shown). In both study groups, IL-1 β -positive cells were mainly found in and around the epithelium. Clusters of positively stained cells were also found in adjacent tissue close to the adenoid surface. There was a clear interindividual variation in frequencies of IL-1 β -producing cells, ranging from very few cells (sample considered negative) to samples with more abundant expression. TNF- α -positive cells appeared as single-cells and generally in fewer numbers when compared to IL-1 β -producing cells (Fig. 2). They were also more evenly spread throughout the tissue layers. A semi quantitative survey of both cytokines in the tissue revealed an apparent preponderance of IL-1 β compared to TNF- α . Even so, the entire area positive for IL-1 β in each sample was small in relation to the total area of the tissue section. When Luminex-analysis was performed for IL-1 β and TNF- α in secretion samples from the adenoid surface, IL-1 β was detected at higher levels than TNF- α , but there was no difference between the two study groups (Fig. 3). Immunohistochemical analysis of imprints from adenoid surface secretions confirmed the local production of both IL-1 β and TNF- α . The Luminex data were therefore interpreted as a reflection of events at the actual site (adenoid surface).

Discussion

The present study reveals that children with OME exhibit lower levels of iNOS in adenoids than controls. No such difference was seen for eNOS. Proteins corresponding to iNOS and eNOS were demonstrated in tissue samples along with the presence of IL-1 β and TNF- α . The down regulation of iNOS did not correlate with changes in the cytokine levels.

It is well established that NO can be produced and released in the human upper airways (16). In line with the present data, it has been demonstrated that the human middle ear mucosa can express iNOS (12, 13), although the actual release of NO into the middle ear cavity is yet to be proven. Forseni and colleagues have reported low expression rates of iNOS in middle ear mucosa from children with OME compared to patients with tympanosclerosis (13) which agrees with the results of the present study. iNOS is of particular interest as it constitutes a part of the innate immune defence (17). The adenoid in young children is frequently colonized by potentially pathogenic airway-derived bacteria. It also is exposed to viruses that invade the upper airways. A well-functioning innate immune defence in this niche therefore seems to be a necessity. iNOS is primarily regulated at the expression level by transcriptional and post-transcriptional mechanisms. Activation of iNOS is pre-translational and depends mainly on the NF κ B- and STAT-1 α -pathways in human cells, pathways known to be activated by IL-1 β , IFN- γ and TNF- α via agents such as bacterial lipopolysaccharides (18). NO can act as an autoregulating factor of iNOS-expression, causing either increased or decreased expression. We have reported that adenoids from children in this age group usually are colonized with airway-derived bacteria such as *Pneumococci* and non-typable *Haemophilus Influenzae* (19). These bacteria are known to induce IL-1 β and TNF- α production. In the present study, we could demonstrate clear presence of these cytokines both in the adenoid tissue and adenoid surface secretion. However, we found no difference in cytokine levels between OME and healthy children. Such a difference has previously been shown by Lindberg et al. (20), who found lower IL-1 β levels in nasopharyngeal secretions from children with recurrent acute otitis media compared to healthy indivi-

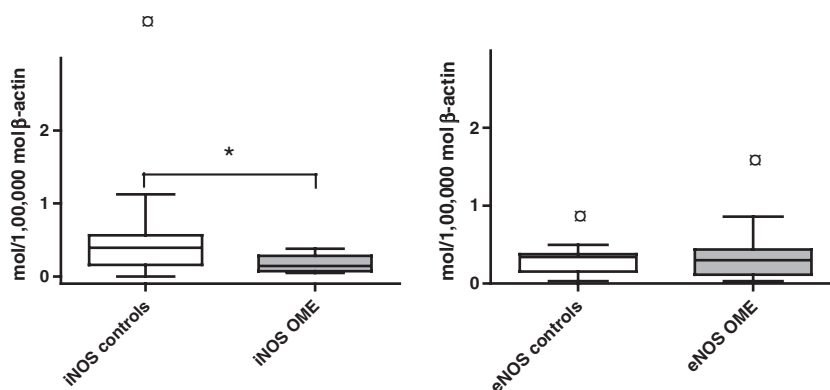


Fig. 1. mRNA expression of iNOS and eNOS in adenoids from children with otitis media with effusion (grey columns, $n = 8$) and from healthy controls (white columns, $n = 11$). The expression is shown in relation to 100 000 mol mRNA β -actin, (* $p < 0.05$). iNOS, inducible NO-synthase; eNOS, endothelial NO-synthase.

Fig. 2. Immunohistochemical staining of adenoids. Sections were stained with antibodies against eNOS (a), iNOS (b), IL-1 β (d) and TNF- α (e). C is an isotypic control. In (a) a strong immunostaining is seen in the epithelium and in the endothelium of small blood vessels. In (b) a moderate staining is seen in the surface epithelium. In (d) IL-1 β positive cells are found in clusters adjacent to the epithelium of crypts (arrows). In (e) TNF- α producing cells deep to the surface are demonstrated (arrows). Magnification a and b $\times 200$, c and d $\times 100$, E $\times 400$.

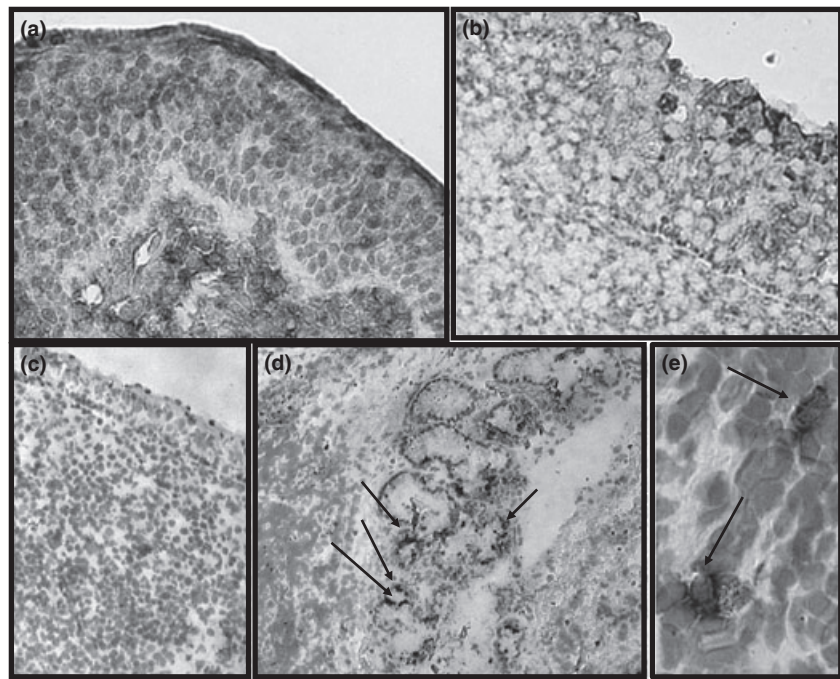
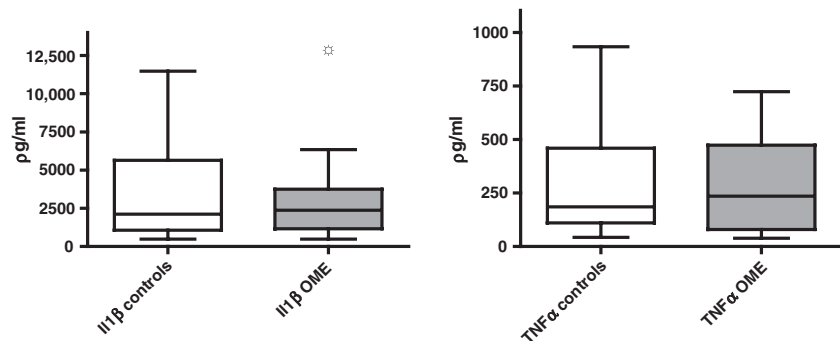


Fig. 3. TNF- α and IL-1 β levels in nasopharyngeal secretion from children with otitis media with effusion (grey columns, $n = 10$) and from healthy controls (white columns, $n = 8$).



duals. Thus, it could not be ruled out that the immunology of the OME condition might differ from that found during recurrent acute otitis media.

The beneficial effect of adenoidectomy on OME has been demonstrated in a clinical setting (21). One reason for this might be that adenoidectomy leads to a drastic reduction of the microbial load close to the Eustacian tube entrance of the middle ear. It is intriguing to speculate that the antimicrobial role of locally produced NO may play a part in clearing infections in this area during normal conditions. However, it is important to recognize that the role of NO in infection remains unclear and that it is still debated whether NO protects from or promotes development of middle ear infections (22, 23). If we assume NO to be protective, the reduced iNOS levels presently found among OME-patients might reflect a reduced ability to respond to microbes by

increasing the NO production. A child with such an inadequate innate immune response might have reduced NO levels at the entrance of the Eustacian tube, making this specific site a weak spot. This could result in proneness for local infection and middle ear disease. The idea that a local infection involving lymphoid tissue normally should be accompanied by an increase in iNOS can be derived from recent experiments, in which chronically infected lymph glands (tuberculosis) showed higher levels of iNOS compared to tonsillar tissue from uninfected children (14).

To conclude, the reduced levels of iNOS in adenoids presently seen among children with OME might indicate a role for NO in preventing the development of OME. Hence, it is tempting to speculate that children with a reduced ability to respond to microbial infections with increased local NO production might be more at risk for developing OME.

Acknowledgments

We acknowledge the support from the Swedish Medical Research Council, Karolinska Institute and the Acta-Otolaryngologica Research Foundation. We further thank Ingegerd Larsson for contributing with expert laboratory work.

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