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Eicosanoids from biopsy of normal and polypous nasal mucosa*

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SUMMARY

In order to clarify the influence of inflammatory mediators of the arachidonic acid cascade in the mechanism of nasal polyp growth, peptido-leukotriene (pLT), prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) synthesis was investigated. In addition to several stimuli, functionally intact human biopsy specimens of polypous and normal tissue were incubated. Especially remarkable was the significantly increased release of pLT by polypous tissue upon arachidonic acid stimulation, in contrast to only slightly elevated PGE_2 release compared to normal tissue. Basic release of pLT and PGE_2 was similar for polypous and normal tissue. Examining TXB_2 release, no significant difference was observed with regard to the origin of tissues. These data support an altered pattern of the lipoxygenase and cyclo-oxygenase pathways when tissue becomes irritated and suggest their involvement in the aetiopathogenesis of nasal polyps.

Key words: biopsy, nasal polyps, peptido-leukotrienes, prostaglandin E_2, thromboxane B_2

INTRODUCTION

Nasal polyps are still an enigma with regard to their aetiology and biological meaning. A number of systemic as well as local diseases including cystic fibrosis, asthma and disorders of ciliary motility, perennial rhinitis and chronic sinusitis (Settipane, 1987; Schramm and Effron, 1988) occur in association with nasal polyps. Much attention has been paid to IgE-mediated allergy as a causal factor for polyp formation (Calenoff et al., 1970; Bunnag et al., 1983; Frenkiel et al., 1985; Ohashi et al., 1986). This is confirmed by a significant increase in the release of histamine only in connection with different bacterial antigens from the mucosa of polyps (Baenkler et al., 1983, 1987). Determination of arachidonic acid (AA) metabolites refers also to other potent mediators of inflammation with numerous physiological reactions. One study on eicosanoids has focussed on the role of arachidonic acid metabolites in the pathogenesis of nasal polyps and nasal mucosa, especially in aspirin-sensitive patients using homogenized tissue samples after incubation with [^{14}C]arachidonic acid (Jung et al., 1987). However, an altered arachidonic-acid metabolism has been postulated, based upon data referring to eicosanoid synthesis but without measurement of leukotriene C_4 in nasal mucosa.

The present study was undertaken to assess the involvement of the basal and arachidonic-acid-induced eicosanoid release patterns of vasoconstrictive peptido-leukotrienes (pLT), and the vasodilatory and permeability-altering mediators, prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2), in normal mucosa and in nasal polyps from patients without history of aspirin-triad especially in view of the involvement of PGE_2 and pLT in nasal polyp formation. Therefore, histopathological evaluation and measurement of pLT, PGE_2 and the biologically inactive but stable conversion product of TXA_2, thromboxane B_2 (TXB_2), were carried out.

For this purpose, competitive immuno-assays were established using monoclonal antibodies. The capacity of synthesis and secretion of pLT, PGE_2 and TXB_2 from non-homogenized and functionally intact biopsy specimens were examined *in vitro*.

MATERIAL AND METHODS

Patients

The study was performed on 30 patients, of whom 12 suffered from *polyposis nasi et sinuum*. Aspirin intolerance was excluded. The control group of 15 individuals was undergoing medical care because of non-inflammatory diseases, such as deviation of the septum. None of these patients had been given corticosteroids or other immune-modulating drugs. Separately, two patients with polyposis were studied during medication with steroids; additionally one patient suffered from eosinophilia of the tissue.

Since the experiments were performed using material obtained during surgical intervention, there was no additional risk or inconvenience for the patients.

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Reagents

All chemicals were purchased from pharmaceutical distributors. Monoclonal antibodies to the eicosanoids were kindly donated by Dr. M. Reinke (Erlangen, Germany).

Biopsy processing

Specimens of polypous and normal tissue were obtained during surgical intervention. Mucosal specimens of the inferior turbinate, in this text described as "normal tissue", were taken from patients of the control group. Specimens of polypous tissue were acquired from patients with *polyposis nasi et sinuum*. The biopsy specimens were immediately placed in cold (4°C) modified Hanks' balanced salt solution (HBBS), containing 50 mM L-serine to prevent oxidative decomposition of pLT (Slivka et al., 1980). Each specimen was weighed and transfered separately into tubes with 500 µl HBSS alone or HBBS containing either 10^{-5} M arachidonic acid (AA), 10^{-5} M acetylsalicylic acid (ASA) or 10^{-5} M nor-dihydroguaiaretic acid (NDGA). Samples were performed in duplicates. The tubes were placed in a shaking incubator and aerated with carbogen (95% O_2, 5% CO_2) for 30 min at 37°C. Next, biopsy specimens were removed, shock-frozen (Wollenberger et al., 1960) and stored at -84°C. Also, the supernatants of the incubation medium were stored at -84°C until eicosanoid determination was performed. The frozen specimens were homogenized mechanically and ultrasonically. Protein concentration was determined (Bradford, 1976) using microplates.

Determination of eicosanoids

Immunoreactive pLT (pLT-IR), PGE_2 (PGE_2-IR) and TXB_2 (TXB_2-IR) in the incubation medium supernatants were determined simultaneously for each sample in duplicate. This was done using monoclonal antibodies for the pLT radioimmunoassay (RIA) as well as for the PGE_2- and TXB_2 enzyme-immunoassay (EIA).

In the case of pLT-IR, a conventional fluid-phase RIA was applied with a total sample volume of 1 ml, containing 2.8 µl of monoclonal antibody solution (1 mg/ml LT-mAB (1A-LDR1) in PBS-BSA (1%); Reinke et al., 1991). As standards, 10 different concentrations of LTD_4 (0.98-500 pg) were employed. Treatment with 100 µl of a 3% charcoal suspension separated the bound from the unbound [^{3}H]LTD_4 with a specific activity of 168.4 Ci/mM after incubation for 18 h at 4°C. The rate of recovery for pLT-IR, incubated under the same conditions as the specimens, was 92.3%. The limit of detection was 3 pg per total sample volume. The intra-assay and interassay coefficient of variation (CV) for pLT-RIA standards were 7.4% and 8.6%, the CV for the biological samples were 18.7% and 20.4%, respectively.

PGE_2- and TXB_2-EIA were carried out using the same procedure. Concisely, a 96-well microtiter plate was coated with PGE_2 or TXB_2 coupled to bovine serum albumin (BSA) in 15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.6) for 18 h. Subsequently, the remaining binding sites were blocked with 1% BSA in phosphate-buffered saline (PBS-BSA). As standards, PGE_2 was used in 10 different concentrations (0.98-500 pg) in HBSS, in duplicate. The supernatants were diluted in HBSS at a ratio of 1:4. These samples were incubated with a highly-specific mouse anti-PGE_2 monoclonal antibody (5 µg/ml in PBS-BSA (1%); mA-PGE_2 (E_2R_1); Brune et al., 1985) or mouse anti-TXB_2 monclonal antibody (2.3 µg/ml in PBS-BSA (1%); MAB-TXB_2 (4E-TBR_1); Reinke et al., 1989) at 4°C for 18 h. Next, biotinylated goat anti-mouse antibody (diluted 1:2,000 in PBS-BSA) was added for 2 h at 37°C, followed by an incubation with a streptavidin-biotin complex coupled to horseradish peroxidase (diluted 1:1,000 in PBS-BSA) for 1 h at 37°C. Finally, ABTS solution (2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonate], 1 mg/ml in 70 mM Na_2HPO_4; 6 mM sodium perborate; 40 mM citric acid, pH 4.3-4.6) as the chromogenic substrate was added. Absorption was measured at 405 nm (reference filter: 490 nm). Each incubation step was followed by a washing step. The detection limit was 5 pg per well for PGE_2- and TXB_2-EIA. The rate of recovery for PGE_2 and TXB_2 was >95%. The intra-assay and interassay CV for PGE_2-EIA standards were 8.1 % and 9.4%, for TXB_2-EIA standards 7.2% and 8.9%. The CV of the biological samples for PGE_2-EIA reached 13.2% and 18.7%, for TXB_2-EIA 14.7% and 16.3%.

Histology

A further biopsy specimen was taken from each patient for histological examination. Sections (5 mm) were prepared and stained with haematoxylin-eosin (HE), azan trichome and trichrome according to Giemsa (Romeis, 1982).

Data analysis

Data are expressed as mean±SD. The statistical analysis of the results was performed by the analysis of variance using the paired Students' t-test. For each stimulation two specimens were obtained from the patient.

RESULTS

Correlation of tissue protein content and fresh weight

Fresh weight and protein content of 150 specimens were plotted, resulting in a correlation coefficient of r=0.965. This was correct with regard to normal mucosa and polypous mucosa (Figure 1).

Specificity of eicosanoid determination

Typical standard curves for pLT-RIA, PGE_2- and TXB_2-EIA are presented in Figure 2. The intra-assay and interassay CV was <10% for the standards. Taking all data into consideration the intra- and interassay CV of the biological samples for pLT-IR (18.7% and 20.4%) were higher than those for PGE_2 (13.2% and 18.7%) and TXB_2 (14.7% and 16.3%). Analysing the CV data in detail, 83.2% of the pLT-IR data, 91.4% of the PGE_2-IR data and 88.7% of TXB_2-IR data were <10%. This will be discussed subsequently.

Histological findings

In all specimens the structure was typical for normal or polypous tissue. There were no signs of cellular inflammation or destruction in normal tissue. In the polypous tissue, eosinophilia was not obvious. The same result was found in the two patients who underwent steroid treatment. The patient with eosinophilia was excluded from the polypous group.

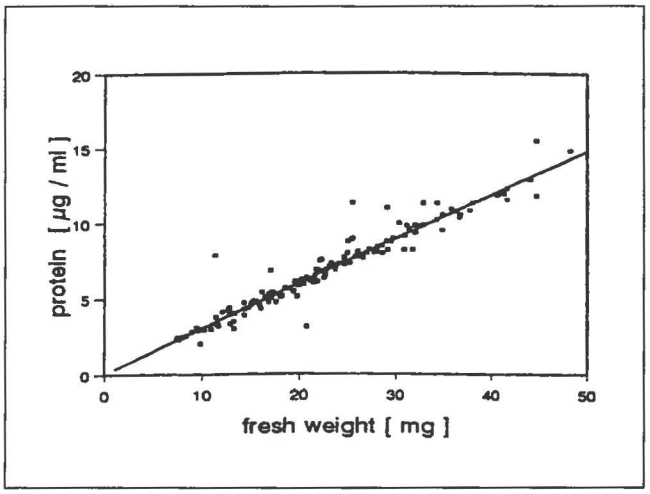

Figure 1: Correlation of protein concentration and fresh weight (FW). Fresh weight and protein concentration of 150 biopsy specimens were determined, each in triplicate. Range of FW was from 7.65 to 48.52 mg. The protein concentration had values between 2.1 and 15.9 µg, as calculated by a bovine serum albumin standard.

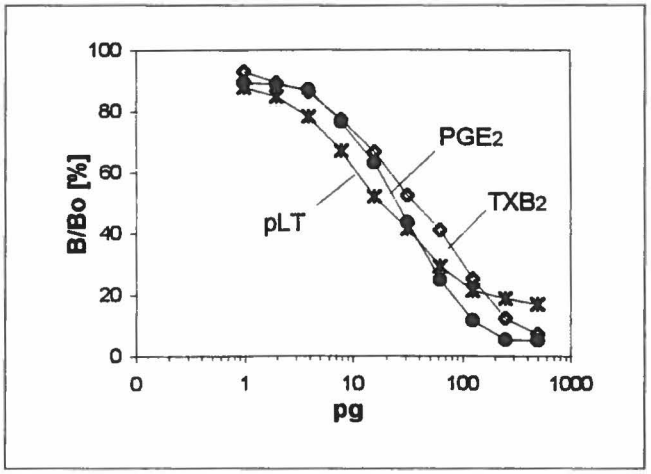

Figure 2: Typical standard curves for pLT-RIA, PGE_2-EIA and TXB_2-EIA. Limit of detection for pLT-RIA: 3 pg per total sample volume; for PGE_2-EIA and TXB_2-EIA: 5 pg per well (pLT: peptido-leukotrienes; PGE_2: prostaglandin E_2; TXB_2: thromboxane B_2).

Release of peptido-leukotrienes

The basic release of pLT-IR from polypous mucosa did not vary significantly as compared to normal mucosa (4.7±1.7 *versus* 3.3±1.4 pg/mg fresh weight). Upon stimulation with arachidonic acid, polypous tissue released considerably more pLT-IR ($p<0.05$) than normal mucosa (14.2±3.5 *versus* 8.9±3.2 pg/mg fresh weight). The corresponding basic and arachidonic-acid-induced release of pLT-IR was significant ($p<0.01$) in both pairs of specimen.

The specific cyclo-oxygenase inhibitor, acetylsalicylic acid, had no significant inhibitory effect in the arachidonic-acid-induced pLT-IR release of the specimens of normal mucosa and polypous tissue. However, incubation with NDGA, a non-specific 5-lipoxygenase inhibitor, blocked the pLT-IR release from both tissues significantly ($p<0.01$; Figure 3a). In a separate experiment, NDGA was tested in various concentrations (10^{-3}-10^{-9} M; data not shown). A predominant effect on 5-lipoxygenase was measured at a NDGA concentration of 10^{-5} M, whereas the effect on cyclo-oxygenase at this concentration was negligible.

Release of PGE_2

Polypous tissue and normal mucosa had no noteworthy differences in basic PGE_2-IR release (33.2±10.2 *versus* 35.1±8.7 pg/mg fresh weight). But the arachidonic-acid-induced release was substantially lower ($p<0.05$) in polypous than in normal tissue (97.5±23.6 *versus* 45.9±13.1 pg/mg fresh weight). The 2-fold increase of PGE_2-IR in polypous tissue upon arachidonic acid stimulation was not significant to the corresponding basic level, whereas the 3-fold increase of normal mucosa was significant ($p<0.01$). Acetylsalicylic acid had a distinct inhibitory effect on PGE_2-IR release ($p<0.05$). No significant difference could be observed comparing both tissues. NDGA had a very poor inhibitory effect which was not of importance towards arachidonic-acid-stimulated specimens (Figure 3b).

Release of TXB_2

No noteworthy difference between both groups could be seen at the basic level of TXB_2-IR (58.7±20.8 *versus* 62.9±27.3 pg/mg fresh weight) as well as in arachidonic-acid-induced TXB_2-IR release (160.1±57.9 *versus* 132.3±54.3 pg/mg fresh weight). However, arachidonic-acid-stimulated TXB_2-IR releases were significantly higher ($p<0.05$) than their corresponding basic values. In normal mucosa, the release of TXB_2-IR was inhibited more by acetylsalicylic acid than in polypous tissue ($p<0.05$). NDGA had a minor inhibitory effect (Figure 3c).

DISCUSSION

The experiments demonstrate the practicability of the use of singular small biopsy specimens for the investigation of eicosanoid synthesis and secretion, even with regard to the metabolic pathway. Actually, this was supported by the strict fresh weight/protein correlation. Thus, requirements have been met to detect functional differences between normal and polypous tissue of the nasal mucosa. Until now, methods used to collect and analyze eicosanoids have included local lavage, brush, imprint-smear technique, and homogenization of tissue (Smith et al. 1981; Whorton et al., 1983; Jung et al., 1987; Pipkorn and Karlsson, 1988; Hedqvist et al., 1990). This meant restricted relevance of data to the pathophysiology because of the considerable destruction and irritation of the tissue as well as unrepresentative distribution of cells. Intact mucosa, however, enables investigation under more physiological conditions. In addition, the experiments using enzyme inhibitors underline the reliability of our techniques and methods. Although more selective 5-lipoxygenase inhibitors - such as A64077 (zileuton; Carter et al., 1989) and MK-886, blocking the translocation and docking of the enzyme (Evans et al., 1991) - are now available, experimental inhibition of the enzyme could be demonstrated. Further experiments will have to prove the potency of these inhibitors in the experimental procedure on human biopsy specimens.

For 9-17% of all analyzed eicosanoid data the intra- and interassay CV exceeded 10%, whereas the standard CVs were generally <10%. Using these data the difficulties in sample handling over a longer period (approximately 1 year) were shown. This was especially true for pLT, which are most sensitive to oxida-

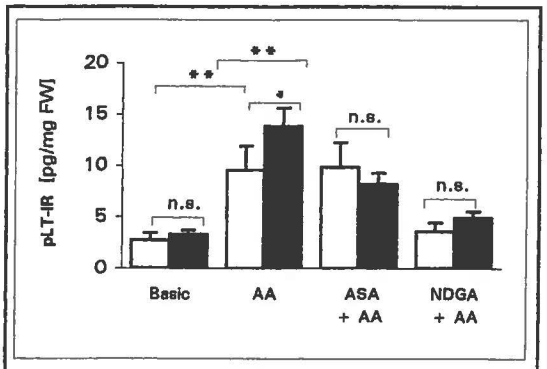

Figure 3a: Release of peptido-leukotrienes

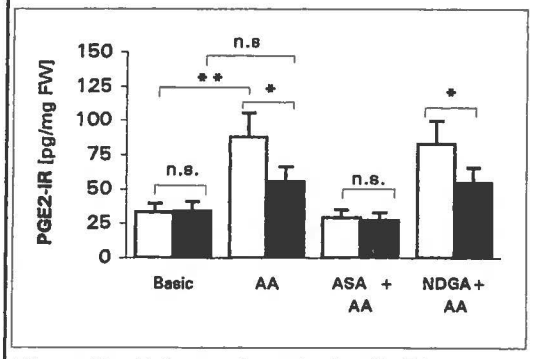

Figure 3b: Release of prostaglandin E2

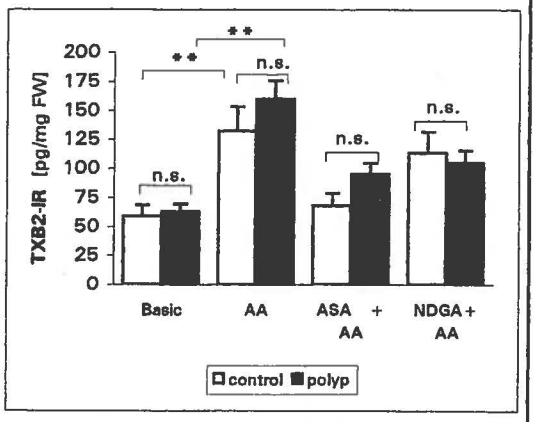

Figure 3c: Release of thromboxane B2

Figure 3: Release of eicosanoids from normal and polypous tissue (3a: release of peptido-leukotrienes; 3b: release of prostaglandin E_2; 3c: release of thromboxane B_2). Control: normal mucosa (n=15; s=30); polyp: polypous tissue (n=12, s=24). Each stimulation was performed by incubation of 2 separate biopsy specimens of each patient for 30 min at 37°C. pLT-, PGE_2- and TXB_2-release was determined simultaneously in duplicate for each sample (B: basic release without any addition; AA: release upon stimulation with 10-5 M arachidonic acid; ASA+AA: release upon pre-incubation with 10-5 M aspirin, 5 min before addition of 10-5 M arachidonic acid; NDGA+AA: release upon pre-incubation with 10-5 M nor-dihydroguaiaretic acid, 5 min before addition of 10-5 M arachidonic acid (n: number of patients; s: number of samples per stimulation; FW: fresh weight). Data are expressed as mean±SD (n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$).

tion, even though L-serine was added to the incubation medium (Slivka et al., 1980). Therefore, in order to analyse eicosanoids in biological samples, improvements in sample handling and biochemical procedure will be necessary to obtain more reliable results in future experiments. Nevertheless, comparative data are now available, even for pLT release in normal mucosa and polypous tissue in comparison to former studies (Smith et al., 1981; Jung et al., 1987).

Data separate from this study were obtained from two patients who were treated with corticosteroids. In spite of the inhibitory action of steroids on eicosanoid synthesis via induction of calcium-binding proteins (Flower, 1988), the release of pLT-IR, PGE_2-IR and TXB_2-IR could be increased to a higher level using arachidonic acid. However, overall eicosanoid release was shifted to a lower level (about 2-fold), consistent with former findings (Jung et al., 1987). This effect can be explained by the calcium dependence of 5-lipoxygenase and cyclo-oxygenase as well as by induction of a post-transcriptional inhibitor of cyclo-oxygenase synthesis (Zakar et al., 1994). In one patient with eosinophilia in polypous tissue differing from patients with *polyposis nasi et sinuum* in our study, a greater release of pLT-IR competent to the potency of those cells was observed (Weller et al., 1983).

Comparing eicosanoid release in normal mucosa and polypous tissue, the release of metabolites of the cyclo-oxygenase pathway is approximately 10 times higher than those of the 5-lipoxygenase pathway. This is true with regard to normal and polypous tissue as well as non-stimulated and arachidonic-acid-exposed specimens. This relation has also been reported in a study using homogenized tissue samples incubated with [^{14}C]arachidonic acid (Jung et al., 1987). In the present study, the basic release of pLT-IR, PGE_2-IR and TXB_2-IR is similar in normal and polypous tissue. However, tissue of nasal polyps synthesize significantly higher amounts of pLT-IR and TXB_2-IR upon arachidonic acid stimulation, but there was no substantial increase of PGE_2-IR. This is in contrast to normal mucosa, which synthesizes significantly higher amounts of PGE_2-IR upon arachidonic acid stimulation. The above-mentioned study has shown similar results for PGE_2 and TXB_2, but only a few patients have been examined, and no information about the size of specimens and leukotriene data for nasal mucosa has been reported (Jung et al., 1987).

The results of our study demonstrate several important differences between human polypous and normal mucosa. There is similar behaviour of all tissues without incubation, whereas upon stimulation with arachidonic acid, polypous tissue differs from normal mucosa by a less pronounced increase in PGE_2-IR release and an elevated pLT-IR release. The observed effect is in accordance with the conception of a shifted pattern of eicosanoid metabolites in polypous tissue (Brandtzaeg, 1984; Frenkiel et al., 1985; Jung et al., 1987; Larocca et al., 1989; Stoop, 1992). In contrast to these studies, our data show no shunt from lowered basal cyclo-oxygenase to elevated basal lipoxygenase metabolites, but a shifting to lipoxygenase metabolites by induced eicosanoid synthesis. Thus, naturally occurring agents (e.g., bacterial antigens or pollen in allergic patients) might direct eicosanoid synthesis to lipoxygenase metabolites and therefore promote the development of polyps. The phenomenon of similar basic eicosanoid release in polypous and normal mucosa remains unexplained. Taking into account that

PGE_2 also has several inhibitory effects – i.e., inhibition of interleukin-1 and interleukin-2 synthesis (Minakuchi et al., 1990), immunosuppressive effects via elevation of the cAMP level (McConcey et al., 1990) or inhibition of leukotriene-B_4 synthesis, a potent chemo-attractant (Ham et al., 1983) – this shift of metabolite pattern, the decreased inducible synthesis of PGE_2 (a potent endogenous inhibitor for many cellular effects) may support the growth of polyps.

Current methods are not able to answer this question, since they rely on material that is not representative because of tissue disintegration or the presence of cells from outside the mucosa. However, the functional experiments in our study, using intact human biopsy specimens, mimick actual events and promote evidence for a metabolic shift in polyps from cyclo-oxygenase to lipoxygenase metabolites when eicosanoid synthesis was induced. Further experiments will be necessary to determine if a decrease in PGE_2 is a concomitant phenomenon or the actual cause of nasal polyp growth. The presented technique can also be used to investigate the influence of drugs, infectious agents and regulatory factors and might promote the investigation of future specific drugs for treatment of chronic polypous sinusitis. In conclusion, functional tests using specimens of the mucosa are of major importance. They elucidate differences in the metabolism and reveal local pathophysiological peculiarities. Moreover, this can be accomplished during routine investigation of out-patients.

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