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DEFICIENCY OF CYCLOOXYGENASES TRANSCRIPTS IN CULTURED PRIMARY BRONCHIAL EPITHELIAL CELLS OF ASPIRIN-SENSITIVE ASTHMATICS

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Background Airway function is actively regulated by epithelium through generating PGE_2 , the production of which depends on cyclooxygenases (COX-1 and COX-2). Analysis of bronchial biopsies and bronchial epithelial cells in culture conducted so far gave conflicting results of expression pattern of these enzymes in healthy subjects and asthmatics patients, with and without aspirin hypersensitivity. **Objective** Our aim was to investigate the expression of COX-1 and COX-2 mRNA in primary human bronchial epithelial cells (HBEC) isolated from asthmatics and non-asthmatics. **Methods** We isolated HBEC from bronchial brushing preparations taken during bronchoscopy of 10 non-asthmatics (NA), 8 aspirin-tolerant asthmatics (ATA) and 9 aspirin-intolerant asthmatics (AIA). HBEC were cultured in serum free medium until 80% confluent. Total cellular RNA was isolated and reversed transcribed using oligo(dT)15 primers. Real time PCR was performed with primers to COX-1, COX-2, GAPDH and β -actin in the presence of SYBR green dye. The cycle threshold (C_T) for COX-1 or COX-2 was normalized using β -actin and GAPDH as the internal standards. **Results** Not only COX-1 but also COX-2 mRNA were expressed by HBEC without any proinflammatory stimulation. We detected the smallest amount of COX-1 mRNA in the AIA group. The same trend was observed for COX-2 mRNA, though it didn't reach the statistical significance. We also analysed the relationship between $\Delta C_{T\text{COX-1}}$ to $\Delta C_{T\text{COX-2}}$ by calculating the difference $\Delta \Delta C_{T\text{COX-1-COX-2}}$. This analysis revealed that AIA group can be characterized by relatively smallest COX-1 mRNA expression in comparison to COX-2. There is a strong positive correlation between $C_{T\text{COX1}}$ and $C_{T\text{COX2}}$ in NA group ($r=0.85$; $p<0.001$). In both groups of asthmatics this correlation is absent (ATA – $r=0.5$, $p>0.1$; AIA – $r=0.43$, $p>0.1$). **Conclusions** Cyclooxygenases transcripts expression is altered in HBEC derived from the asthmatic patients, and this phenomenon is pronounced in case of aspirin hypersensitivity.

Keywords: *asthma, aspirin-induced asthma, human bronchial epithelial cells, real time PCR, cyclooxygenase-1, cyclooxygenase-2, prostaglandin E_2*

INTRODUCTION

The products of cyclooxygenase pathway, prostanoids, are centrally involved in pulmonary physiology and have been implicated in asthma, especially as a potential regulator of the bronchial tone (1). Inflammation and remodelling accompanying the disease are characterized not only by infiltration of immune cells, but also by the activation of epithelial cells and subepithelial fibroblasts and smooth muscle cells.

Leukocytes migrating to the bronchial wall from the bloodstream are the source of such proinflammatory prostanoids as PGD_2 and $\text{PGF}_{2\alpha}$. The cells of mesenchymal origin produce mainly anti-inflammatory PGE_2 and much less PGD_2 (2). Some recent studies showed the potential for the bronchial epithelial cells both *in vivo* and *in vitro* to produce substantial amounts of prostanoids and suggested that airway epithelium actively regulates airway function by generating PGE_2 (3).

Inhalation of PGE_2 can prevent the asthmatic attack evoked by allergen or aspirin challenges in susceptible subjects (4, 5). Both types of genetically modified mice, created with the knockout of PGE_2 catabolic enzyme or with overexpression of a lung specific PGE_2 synthase (mPGES1) transgene were less likely to develop bronchoconstriction (6). Prostanoids production by bronchial epithelium depends on the activity of the both cyclooxygenases enzymes (COX-1 and COX-2) that are fine regulated on the transcriptional and posttranscriptional level.

In about 10% of adult asthmatics ingestion of aspirin or other nonsteroidal anti-inflammatory drugs induces severe asthmatic attacks. It is generally believed that aspirin hypersensitivity is not an immunologically mediated reaction, but results from inhibition of the cyclooxygenases by the drugs. In consequence, the level of protective PGE_2 decreases facilitating the local overproduction of cysteinyl leukotrienes, the potent bronchoconstrictors. Despite general acceptance of "cyclooxygenase theory" (7) it is still obscure why the mechanism operates only in the limited fraction of sensitive individuals rather than all asthmatics or healthy subjects (8). This phenomenon may depend on altered expression of cyclooxygenases in the bronchi of aspirin intolerant asthmatics.

Analysis of bronchial biopsies and bronchial epithelial cells in culture gave conflicting results on COX-2 expression level in healthy subjects as compared to asthmatic patients (9-12). In this study we addressed the question whether cultured human bronchial epithelial cells derived from aspirin tolerant (ATA), aspirin-intolerant asthmatics (AIA) and non-asthmatics (NA) differ in the respect to COX-1 and COX-2 mRNA expression.

MATERIALS AND METHODS

Subjects

The study was performed in three groups of subjects comprising a total number of 27 individuals. The first group consisted of 10 non-asthmatic patients (NA), the second of 8 aspirin-

tolerant asthmatics (ATA) and the third, of 9 aspirin-intolerant asthmatics (AIA). The last group of patients gave a clear history of bronchospasm following ingestion of aspirin or other NSAIDs. The clinical characteristics of the patients' groups are given in *Table 1*. All the patients were treated in the Department of Medicine of the Jagiellonian University Medical School and were in a stable clinical condition. Within the six months preceding the bronchoscopy all asthmatic patients underwent oral aspirin challenge test; the results were positive in AIA patients, but negative in ATA. The test was performed as previously described (13). The NA group included patients in whom diagnostic bronchoscopy ruled out any serious airway pathology, including asthma, fibrotic lung disease, sarcoidosis or cancer. All NA patients were asked specifically and responded that they used aspirin or other nonsteroidal anti-inflammatory drugs without any adverse effects. The study protocol was approved by Jagiellonian University Bioethical Committee.

Isolation and culture of bronchial epithelial cells (HBEC)

The bronchial brushing samples were obtained from the segmental bronchi during bronchoscopy, using fiberoptic bronchoscope (Olympus, Japan). The brush was immediately immersed in the tube containing 25 ml of ice-cold phosphate buffered saline and kept on ice. Within a few minutes the tube was filled up to 50 ml with Dulbecco's Modified Eagle's Medium (DMEM – Sigma) containing 10% of Foetal Calf Serum (FCS-Gibco BRL). The cells were centrifuged (100 g, 5 minutes) and resuspended in 5 ml of Bronchial Epithelial Cell Growth Medium supplemented with bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin /amphotericin-B and retinoic acid according to manufacturer's instruction (CC-3170 BEGM® Cambrex). The cell suspension was divided into two and the cells were seeded into 25 cm² culture flask and into 6-well plate (Falcon, Primaria™, Becton Dickinson, Heidelberg, Germany). After 24 hours the medium was changed in both vessels containing primary culture and the same procedure was repeated every other day. In 2-3 weeks time the primary cultures of epithelial cells reached about 80% of confluence. The 6-well plate was used for PGE₂ production measurements and the flask was used for the isolation of total cellular RNA.

PGE₂ and PGD₂ measurements

The cells seeded into 6-well plates were cultured until 80% confluent. Then the wells were washed once in PBS and the medium was replaced with BEGM without additives, but containing 0.1% of bovine serum albumin (BSA, endotoxin free, cell culture tested, Sigma). The cells were stimulated with cytomix composed of IL-1β (human recombinant, BD-Pharmingen, Heidelberg, Germany) - 5 ng/ml, TNF-α and IFNγ (both 10 ng/ml, human recombinant, Calbiochem, La Jolla, CA, USA) or with single stimulants; IL-1β (5ng/ml), TNF-α, IFNγ (10 ng/ml), IL-4 (20 ng/ml), IL-

Table 1. Patients characteristics.

	NA	ATA	AIA
Number of subjects	10	8	9
Sex (male/female)	4/6	1/7	4/5
Age (year), mean, range	50 (27-81)	40(18-63)	44 (24-59)
FEV ₁ initial value (% predicted), mean, range	102 (80-123)	89 (68-104)	83 (67-108)
Use of inhaled steroids (treated subjects /all subjects)	0/10	9/9	7/7
Use of systemic steroids (treated subjects/all subjects)	0/10	0/8	0/9

13 (20 ng/ml). One well was incubated in non-supplemented medium (non-stimulated control). After 18 hours the medium was changed for 1 ml of DMEM and exogenous arachidonic acid (Sigma) was added into each well to the final concentration of 20 μ M. Following 30 minutes of incubation, the cells were washed twice with DMEM, and then incubated in 1 ml of serum-free DMEM (without albumin). After 90 minutes of incubation, the supernatants were harvested into tubes already containing deuterated internal standards of PGE₂ and PGD₂ and immediately frozen in -80°C.

PGE₂ was measured in culture supernatants (following the extraction step, derivatisation to pentafluorobenzyl ester, TLC purification, and derivatisation with methoxyamine and trimethylsilylation) by gas chromatography - negative ion chemical ionization - mass spectrometry (Hewlett Packard 5989 B). Deuterium labeled prostaglandins ([²H₄]-PGD₂) and ([²H₄]-PGE₂) (Cayman Chemicals, Ann Arbor, USA) added at the collection time were used for the compensation of the analytes loss. For the PGE₂ quantification two ions were recorded, m/z 524 for prostaglandin and m/z 528 for standard. PGD₂ was also measured, but in most samples the concentration of this metabolite was around 10 pg/ml which is the limit of the detection sensitivity of the system.

RNA isolation and RT-PCR

To assess the level of cyclooxygenases transcripts we used the comparative C_T method. Total cellular RNA was isolated using TriZOL reagent (TriZOL® Invitrogen) according to the manufacturer's protocol and RNA (1 μ g) was reverse transcribed using Promega Reverse Transcription system (AMV, Oligo(dT)₁₅ Primers). The PCR reaction was performed using iCycler Real Time PCR equipment (Biorad, Hercules CA, USA). SYBR Green (Amresco, Solon, OH) was added into each sample to the concentration of 50 nM. For each primary culture tested the reaction was performed with specific primers for β -actin (5'-AGC GGG AAA TCG TGC GTG-3', 5'-GGG TAC ATG GTG GTG CCG-3'); GAPDH (5'-ACA GTC AGC CGC ATC TTC-3'; 5'-GTC CTT CCA CGA TAC CA-3'); COX2 (5'-AAT TGA CCC AGA ACT ACT TT-3', 5'-AAT GAT TCA TAG GGC TTC AG-3'); COX1 (5'-CTC TTG CGG TAC TCA TTG AAG-3'; 5'-GAG CTG CTG TTC GGT GTC-3'). The relative abundance of the specific mRNA transcripts was referred as the cycle threshold value (C_T) - a PCR cycle resulting in significant (10 times from background deviation) increase in the product fluorescence. The comparison between samples was performed using β -actin and GAPDH genes as internal standards. First we calculated the normalized C_T value for GAPDH and β -actin in each sample. We averaged C_{T β -actin} from all sample tested and calculated how much the C_T for the particular sample differs from the mean (the parameter C'_{T(β -actin)} = C_{T β -actin} - MEAN_{C_{T β -actin}} was calculated for each sample). The same calculations were made for GAPDH. Then we averaged the corrected C_T values for both normalizing genes and this parameter - (C'_{T β -actin} + C'_{TGAPDH})/2 - was subtracted from the C_{T_{COX-1}} or C_{T_{COX-2}} obtained for the same sample to get Δ C_{T_{COX}} value. The smaller Δ C_T value corresponded to the bigger amount of specific mRNA in the studied sample. To compare the amount of mRNA for COX-1 and COX-2 in each sample we calculated the difference $\Delta\Delta$ C_{T_{COX-1-COX-2}}. We decided to use two types of transcripts (GAPDH and β -actin) as standards to minimize a bias related to change in expression of these housekeeping genes (14). The fact that in all groups the strong correlation exists between C_{T β -actin} and C_{TGAPDH} (p<0.0001) made us believe that all the steps in the RT-PCR procedure were done correctly (data not shown).

Statistical analysis

The statistical analysis of the difference between grouped subjects was performed using non-parametric Mann-Whitney U-test, the correlation between standardized C_T for COX-1 and COX-2 was analysed by calculating Spearman's rank correlation coefficient. The p value below 0.05 was considered significant.

RESULTS

Release of PGE₂ and PGD₂ by HBEC

We measured the amount of prostaglandins produced by HBEC *in vitro* using cell culture isolated from 5 different non-asthmatic subjects and 5 asthmatic patients (3 from ATA and 2 from AIA group) randomly chosen from the studied groups. One million of non-stimulated cells produced about 0.5-1 ng of PGE₂ during 90 minutes of incubation. The amount of PGD₂ was about 50 times lower. No significant differences between groups were observed. From all stimulants tested only cytomix and IFN γ gave moderate but significant increase in PGE₂ production ($p < 0.01$) (Fig. 1).

Expression of COX-1 and COX-2 mRNA in HBEC in culture

In contrast to other cell types of the bronchial wall and in contrast to blood-derived cells, not only COX-1 but also COX-2 mRNA could be easily detected in

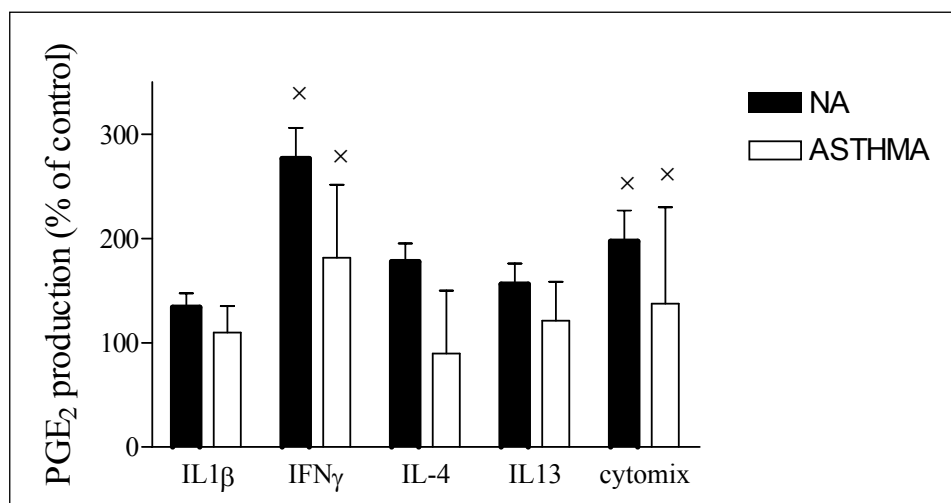


Fig. 1. PGE₂ production by HBEC is moderately enhanced by pro-inflammatory cytokines. The cells were stimulated with single stimulants: IL-1 β (5 ng/ml), TNF- α , IFN γ (10 ng/ml), IL-4 (20 ng/ml), IL-13 (20 ng/ml) or with cytomix composed of IL-1 β (5 ng/ml), TNF- α and IFN γ (both 10 ng/ml) for 24 h in 6-well plates. Then the cells were incubated for 30 min with arachidonic acid (20 μ M), washed twice and incubated for 90 min in 1 ml of DMEM. The PGE₂ concentration in the collected supernatants were measured by gas chromatography and mass spectrometry as described in Materials and Methods. The results were presented as a percent of the production from cultures incubated in control conditions in the non-supplemented medium. The presented values are the mean of independent measurements performed on cells isolated from 5 healthy subjects (solid bars) and 5 asthmatics (open bars) $\pm$ SEM. Only IFN γ (in cytomix and alone) gave statistically significant increase in PGE₂ production in all cell lines tested (The Wilcoxon Matched-Pairs Signed-Ranks Test, significant increase - $p < 0.01$ - was marked by "x").

HBEC incubated in bronchial epithelial cells growth medium, despite absence of any proinflammatory factor in the medium (Fig. 2).

We compared the abundance of COX-1 and COX-2 mRNA in subconfluent primary cultures of HBEC kept in growth medium before seeding them into the second flask.

We detected the smallest amount of COX-1 mRNA in the AIA group (mean $\Delta C_T \pm SD$: NA – 23.7 ± 4.8 ; ATA – 26.2 ± 4.03 ; AIA – 28.1 ± 1.4 (Fig. 3A). The significant difference was only detected while comparing NA with AIA group (NA vs. AIA $p < 0.05$). The same tendency was observed for COX-2 mRNA but it didn't reach the statistical significance (mean $\Delta C_T \pm SD$: NA – 23.2 ± 3.8 ; ATA – 25.2 ± 2.4 ; AIA – 23.1 ± 4.6) (Fig. 3B).

We also analysed the relationship between ΔC_{TCOX-1} and ΔC_{TCOX-2} in our samples by calculating the $\Delta \Delta C_{TCOX-1-COX-2}$. This type of analysis revealed that AIA group was characterized by relatively smallest COX-1 mRNA expression in comparison to COX-2 mRNA (NA vs. AIA - $p < 0.05$; ATA vs. AIA - $p < 0.1$) (Fig. 3C). Such outcome can result either from COX-1 deficiency or COX-2 upregulation.

Another analysis of the same data revealed that there was a strong positive correlation between ΔC_{TCOX-1} and ΔC_{TCOX-2} in the control group (NA, $r = 0.85$;

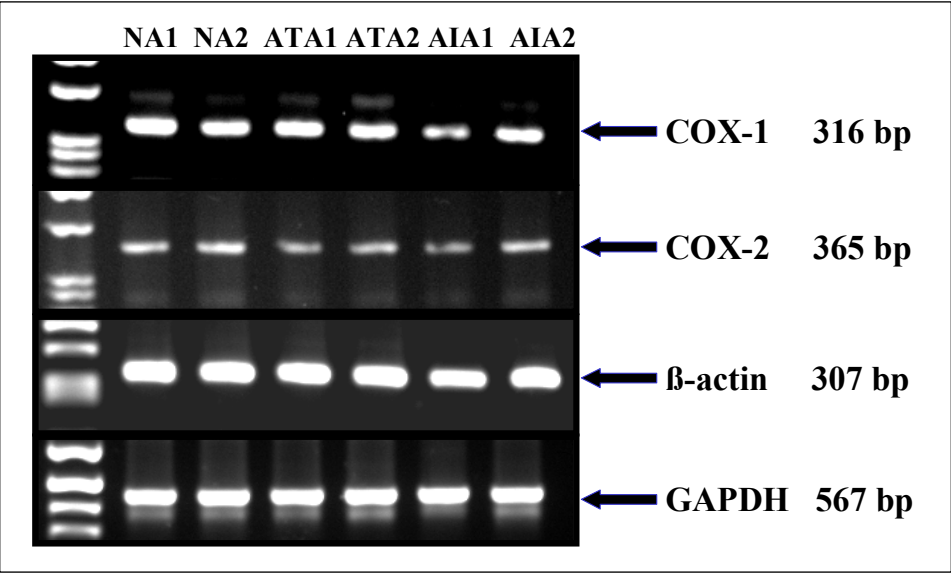


Fig. 2. Both COX-1 and COX-2 transcripts are expressed in cultured HBEC. Total cellular RNA (1 μ g) was reverse transcribed using oligo(dT)₁₅ primers. The PCR reactions were performed using 1 μ l of RT-PCR reaction products and specific primers to COX-1, COX-2, GAPDH, or β -actin as described in Materials and Methods. The agarose gels were run to visualize specific bands in each sample tested. The figure shows the results for 6 representative reactions.

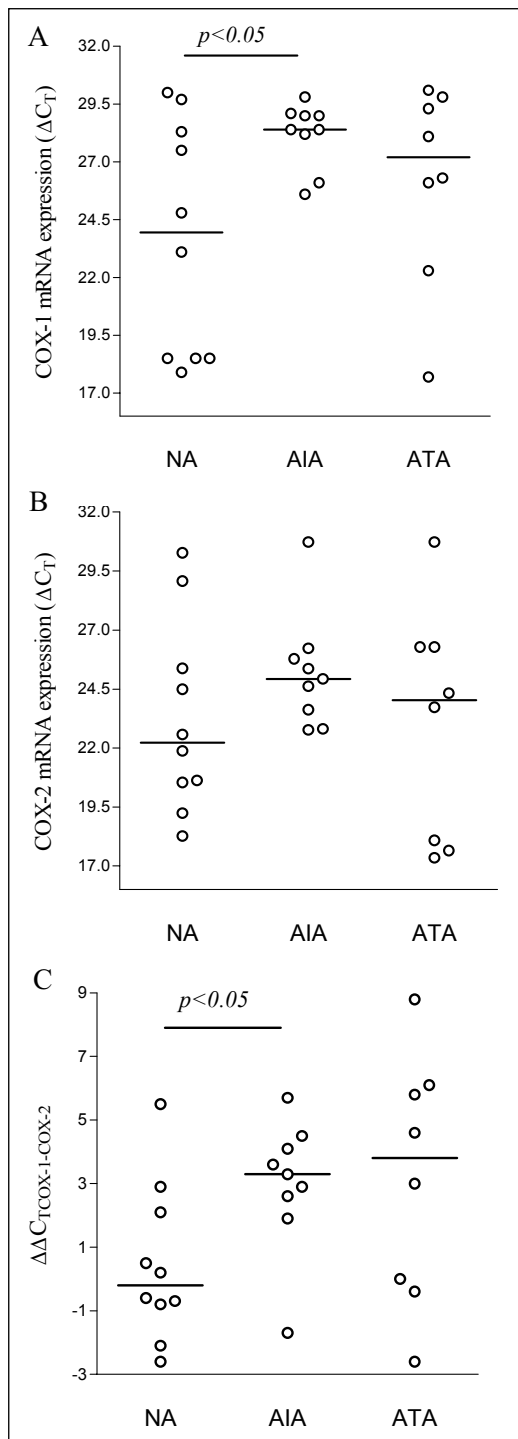


Fig. 3. Expression of cyclooxygenases' mRNA in cultured HBEC. A COX-1, B - COX-2, C relationship between COX-1 and COX. Cells were isolated from 3 groups of subjects. The points represent $\Delta C_{T_{COX}}$ values normalized using GAPDH and β -actin genes as internal standards as described in Material and Methods (panel A and B) or the ΔC_T value for COX-2 was subtracted from ΔC_T value obtained for COX-1 ($\Delta \Delta C_{T_{COX-1-COX-2}}$) (panel C). Bars represent median values. The statistical analysis was performed using Mann-Whitney U-test and the expression of COX-1 turned out to be lower in AIA group as compared with NA group (the bigger C_T value reflects the situation when less mRNA copies are present in the sample). $\Delta \Delta C_{T_{COX-1-COX-2}}$ parameter was the lowest in NA group as compared with both groups of asthmatics (the bigger $\Delta \Delta C_T$ value observed in AIA group reflects the situation when less COX-1 mRNA or more COX-2 mRNA copies are present in the sample).

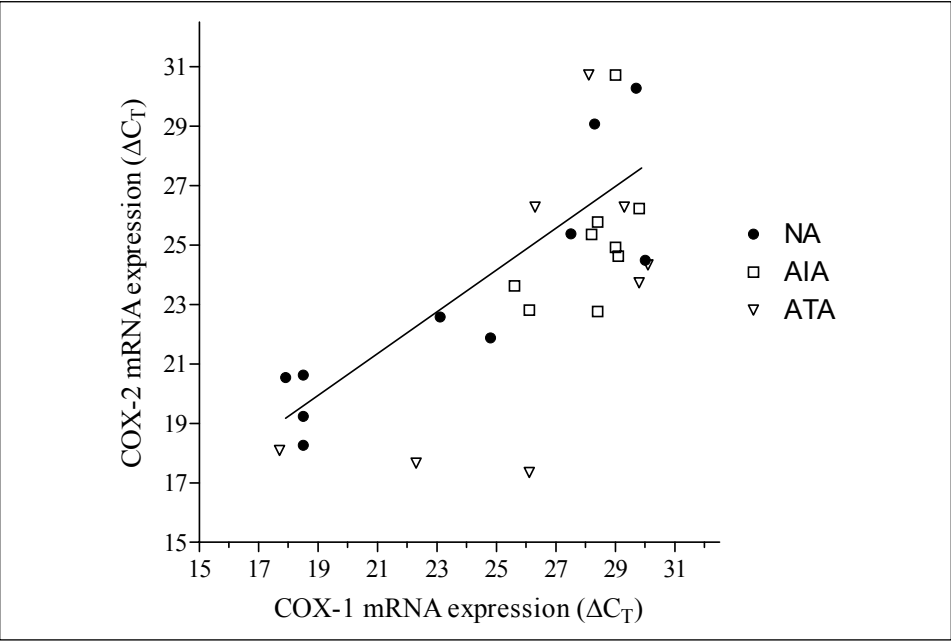


Fig. 4. Correlation between ΔC_T values for COX-1 and COX-2 mRNAs. The figures showed normalized $\Delta C_{T\text{COX-1}}$ and $\Delta C_{T\text{COX-2}}$ in NA (circles), ATA (triangles), AIA (squares) groups. The line represents the positive correlation between ΔC_T of COX-1 and COX-2 calculated in NA group (Spearman's coefficient $r = 0.85$, $p < 0.001$). In the case of both groups of asthmatic patients $r < 0.5$ and the correlation is not statistically significant.

$p < 0.001$). In both groups of asthmatics the correlation is much weaker and not statistically significant (ATA – $r = 0.5$, $p > 0.1$; AIA – $r = 0.43$, $p > 0.1$) (Fig. 4).

DISCUSSION

Previous investigations gave confusing results of the cyclooxygenases' expression in human bronchial epithelium in healthy subjects or asthmatics, and in cultured cells isolated from their airways. We investigated the COX-1 and COX-2 mRNA expression in bronchial epithelial cells *in vitro* and our results confirmed the studies indicating that both types of the enzyme are present in non-stimulated cells cultured in the standard culture conditions (15 - 17).

It seems that a similar pattern exists *in situ* since several studies showed by using immunostaining of bronchial biopsies, that COX-2 is present in the airway epithelial cells of rats and healthy humans (18 - 20). All these results corroborate the hypothesis that the airway epithelium – in similarity to epithelium of gastrointestinal tract - expresses COX-2 as a constitutive enzyme needed for the normal function of the tissue despite the lack of inflammation. That would

explain why the production of PGE₂ increased only moderately under stimulation by pro-inflammatory cytokines. In our experiments the cytotoxic combination of TNF α , IFN γ and IL1 β gave only two-fold increase of PGE₂ production in some cultures. This is in agreement with the observation of Asano *et al.* (21) who found that, in contrast to A594 cells (alveolar type-II epithelial cell line), primary tracheal epithelial cells do not increase PGE₂ production and COX-2 mRNA level upon IL-1 β or TNF α stimulation; only IFN γ caused delayed and moderate response in this type of cells. In other cell types, like fibroblasts or monocytes, these cytokines can cause an increase of PGE₂ production up to 100 times in comparison to nonstimulated cells. Recently Trudeau *et al.* have shown that IL-13 downregulates PGE₂-related pathways in the differentiated HBEC cultured in the air-liquid interface. We did not observe this effect but this discrepancy might be due to the different culture conditions (22).

PGE₂ synthesis can be triggered in lung epithelial cells by the activation of PAR2 receptor *e.g.* by trypsin (23). These observations led us to the conclusion that the comparison of growing, non-stimulated and not-subcultivated by trypsinization bronchial epithelial cells from healthy subjects and asthmatics will be the most valuable tool to investigate their ability to express cyclooxygenases mRNA. As the cultures from which we isolated RNA were still in the phase of intensive growth, the *in vitro* model used by us corresponds partly to the state of injured and self-repairing epithelium which is characteristic of asthmatic airways.

The analysis of real time RT-PCR for COX-1 and COX-2 indicates that there is a strong tendency towards the lower amount of cyclooxygenases' transcripts in asthmatics. In the case of COX-1 this difference reached the statistical significance when AIA group was compared to NA.

Although some authors showed in asthmatics an increase in COX-2 expression in bronchial epithelium (8), or lack of difference between asthmatics and healthy controls (24), most of *in vitro* studies indicated an opposite phenomenon especially in the case of aspirin-induced asthma. Picado *et al.* demonstrated that COX-2 is downexpressed in nasal polyps of aspirin sensitive asthmatics (12). The same authors suggested in their next papers that the COX-1 and COX-2 are abnormally regulated in human nasal polyps in comparison to the normal nasal mucosa (25, 26). Kowalski *et al.* reported in epithelial cells cultured from nasal polyps of aspirin-sensitive asthmatics diminished production of PGE₂ in comparison to aspirin-tolerant patients (27). The work presented here dealt for the first time with cultured epithelial cells isolated from the bronchi of aspirin-induced asthmatics. While studying the cells in culture, we concentrated more on the ability of the patients' cells to express cyclooxygenases, a property determined mainly by genetic factors. In contrast, the biopsies studies reflected also the state of inflammation in asthmatic airways and could be influenced by the drugs used, including steroids.

It is evident that quite big variation exists inside the NA group (Fig. 3A and 3B). This seems remarkable in case of COX-1, as expression level of its mRNA

usually does not vary greatly. This fact interfered with statistical analysis and limited significance of differences between relatively small groups.

Exogenous and endogenous PGE₂ was shown to enhance COX-2 mRNA upregulation evoked by pro-inflammatory stimulants in synovial fibroblasts and human airway smooth muscle cells (28,29). If the same mechanism exists in the epithelial cells which expressed substantial amount of COX-2 constitutively, this phenomenon could explain the strong positive correlation between the levels of cyclooxygenases transcripts observed by us in the NA group. Such correlation was absent in case of both asthmatic groups (*Fig. 4*). This situation could mirror the fact that a positive feedback loop between PGE₂ production and the COX-2 mRNA expression doesn't function effectively in the asthmatics cells. Recently Jinnai et al reported that the polymorphic variant located in regulatory region of EP2 gene, coding one of PGE₂ receptors, is significantly associated with AIA and is functional by reducing transcription of EP2 gene. The EP2 receptors are also expressed in bronchial epithelial cells (30). The possible downexpression of EP2 in epithelial cells of aspirin-induced asthmatics could, therefore explain lack of correlation between COX-1 and COX-2 mRNA expression levels described above.

The relationship between COX-1 and COX-2 mRNA indicates that in HBEC from aspirin-induced asthmatics either COX-1 is downexpressed or COX-2 is upregulated (*Fig. 3C*). The first phenomenon would be in agreement with the results obtained by us using cultured bronchial fibroblasts in which COX-1 protein level seemed to be lower in AIA group than in ATA (31). Downregulation of COX-1 could be a disadvantage upon NSAIDs administration as these drugs block PGE₂ production driven mainly by this form of the enzyme. This is in agreement with the clinical studies demonstrating that selective COX-2 inhibitors, contrary to COX-1 inhibitors, are very well tolerated by aspirin-sensitive asthmatics and evoke no alterations in their eicosanoid metabolism (32, 33). The second possibility – COX-2 upregulation – is less likely in the light of the results obtained by others who used nasal polyps' epithelial cells from aspirin-induced asthmatics (12).

In conclusion, we have shown for the first time that cyclooxygenases' mRNA expression is altered in primary bronchial epithelial cells cultures derived from the asthmatic patients and this phenomenon is mainly pronounced in the case of aspirin hypersensitivity. Our observation could be helpful in explaining the mechanisms of bronchial asthma, as bronchoprotective PGE₂, produced locally by a great surface of airways epithelium, affects the function of both residual and infiltrating cells of the airways.

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