



Changes in NADPH oxidase mRNA level can be detected in blood at inhaled corticosteroid treated asthmatic children

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ARTICLE INFO

Article history:

Received 5 April 2012

Accepted 30 August 2012

Keywords:

NADPH oxidase
Inhaled corticosteroids
Bronchial asthma
Blood

ABSTRACT

Aim: Oxidative stress, observed in the asthmatic airways, is not localized only to the bronchial system. It would be a great advantage to monitor the oxidative stress markers from blood especially in childhood asthma following the inflammation. Our aim was to measure the levels of antioxidants and the oxidatively damaged biomolecules. We were also interested in the gene expression alterations of the free radical source gp91^{phox} subunit (CYBB) of the NADPH oxidase system, and the antioxidant heme oxygenase-1 (HMOX-1) isoenzyme in the blood. Our findings were also examined in the context of medical treatment.

Main methods: Oxidative stress parameters via photometric methods, CYBB and HMOX-1 expressions via real-time PCR were measured in 58 asthmatic and 30 healthy children.

Key findings: Higher blood thiobarbituric acid reactive substances (TBARS) ($p < 0.03$) and carbonylated protein ($p < 0.05$) levels were found in the asthmatic children than in the controls. The relative expression of CYBB was significantly lower ($p < 0.05$) in patients treated with a low daily dose of inhaled corticosteroid (ICS), than in asthmatics not receiving ICS therapy. Higher ICS doses alone or combined with long acting β_2 -receptor agonists did not influence the expression significantly. No similar tendency was found as regards to HMOX-1 expression.

Significance: Elevated levels of damaged lipid (TBARS) and protein (carbonylated) products corroborate the presence of oxidative stress in the blood during bronchial asthma and suggest the presence of chronic oxidative overload. Our findings also suggest that ICS treatment can influence the relative CYBB mRNA expression in circulating leukocytes in a dose dependent manner.

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Introduction

Airway inflammation and oxidative stress play major roles in the pathophysiology of bronchial asthma (Rahman et al., 2006; Mak and Chan-Yeung, 2006). Structural and functional alterations can be observed as a result of the excessive accumulation of free radicals and reactive oxygen or nitrogen species (ROS/RNS), produced by activated eosinophilic and neutrophilic granulocytes and macrophages. Constitutive airway cells, such as epithelial cells, are also considered to participate in ROS overproduction and in the antioxidant defense (Rochelle et al., 1998; Dworsky, 2000). Even if the inflammation is not localized only to the respiratory system, but also involves the systemic circulation, the available data concerning oxidative markers from the blood are limited and conflicting (Shanmugasundaram et al., 2001; Perišić et al., 2007; Comhair et al., 2005; Nadeem et al., 2003; Vural et al.,

2005). So far numerous data have emerged from studies using the bronchoalveolar lavage fluid (BALF) in animal or human experiments (Park et al., 2009; Novak et al., 2006; Schock et al., 2003; Ozaras et al., 2000). In certain patient groups, such as asthmatic children, it would be a great advantage to develop protocols to monitor the oxidative stress before and during treatment using peripheral blood samples rather than collecting BALF or tissues, or to analyze exhaled breath condensates (Grob et al., 2008; Baraldi and Carraro, 2006; Busse et al., 2005).

Phagocytic NADPH oxidase, a multiprotein enzyme complex, is one of the main sources of free radicals and ROS. The plasma membrane-associated flavocytochrome b558 (composed of gp91^{phox} and p22^{phox} proteins) catalyzes the electron transfer from NADPH to O₂, resulting in the superoxide anion (O₂⁻). Both subunits are transcriptionally regulated by pro-inflammatory cytokines (TNF α and IL-1) and by the ROS themselves, although other cytosolic subunits are also essential for a complete enzyme function (Almeida et al., 2005; van der Vliet, 2008). The upregulation of NADPH oxidase subunits and increase in NADPH oxidase activity contribute to the elevated O₂⁻ radical and ROS generation during inflammation by activated mononuclear cells.

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Heme oxygenase-1 (HMOX-1) plays an important role in the cytoprotection against oxidative stress by participating in the anti-inflammatory, anti-proliferative and anti-apoptotic processes. As HMOX-1 is activated in the early phase of oxidative stress, it is a frequently used marker of the antioxidant system via which the adaptation mechanisms follow. Different HMOX-1 levels have been observed in various pathological conditions and inflammatory diseases. Environmental and pro-inflammatory stimuli, such as oxidative stress, hypoxia and certain cytokines (TNF α and IL-1 α), may regulate the HMOX-1 transcriptionally (Slebos et al., 2003; Lee et al., 2009; Taillé et al., 2004). It has been already known that inflammation and oxidative stress in the airways modify the expression of HMOX-1 (Slebos et al., 2003; Farkas et al., 2008), but there are few data about the changes of the enzyme level from blood leukocytes and about the events in HMOX-1 regulation in persistent stable asthma.

In certain chronic diseases, including asthma, the pathological events and symptoms originate from primarily affected organs; however, the systemic effects are also considerable. The aim of the present study was therefore to compare the systemic oxidative damage and antioxidant status in the blood of asthmatic children and age-matched healthy controls. Blood is suitable for the study of the systemic effects of inflammation, and is readily obtained in pediatric practice. We measured the levels of both enzymatic and non-enzymatic components of the antioxidant system such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), reduced and oxidized glutathione (GSH vs. GSSG); and the concentrations of some damaged biomolecules indicating the extent of the direct molecular damage caused by free radicals. We were also interested in the expressions of a free radical source, the gp91^{phox} subunit of NADPH oxidase (the NOX2 isoform, which is highly expressed in leukocytes), and the expression of the antioxidant HMOX-1 isoenzyme (both are important in the bronchial system) in the leukocytes, and investigated whether these correlate with the biochemical parameters in the blood. Since all asthmatic patients were treated medically, the effects of the medication were compared with the biochemical parameters and the gene expression profiles.

Patients and methods

The study groups consisted of children with a confirmed diagnosis of bronchial asthma ($n=58$), and healthy children or young adults as controls ($n=30$). All of the asthmatic patients were admitted to inpatient and outpatient wards of the Department of Pediatric and Health Care Center Faculty of Medicine at University of Szeged in Hungary. Healthy controls were enrolled from ward of surgery waiting for elective intervention and from the outpatient department of our institution recalled for control medical check-up after recovery. No controls had acute disease one month prior to sample collection. No controls had positive atopic status, respiratory disease and chronic disease in their anamnesis, which could have influenced their oxidative status. There were 36 boys (mean age: 14.55 years) and 22 girls (mean age 16.29 years) among the patients, and 16 boys (mean age: 14.94 years) and 14 girls (mean age: 16.03 years) among the healthy controls. The level of asthma control was assessed via the GINA (Global Initiative for Asthma) references, and the medication was modulated according to the complaints and the parameters reported in the protocol. The asthmatic patients were classified in 4 subgroups: those who did not require inhaled corticosteroid (ICS) ($n=22$), those who received a low daily dose of ICS (below 200 $\mu\text{g/day}$) ($n=20$), those who received a medium daily dose of ICS (200–400 $\mu\text{g/day}$) ($n=6$), and those who received a medium or high dose ICS (≥ 200 –400 $\mu\text{g/day}$ ICS) combined with a long-acting β_2 -receptor agonist (LABA) ($n=10$). The medication had been applied for at least six months before sample collection. For all these patients, the possibility was given for the use of short-acting β_2 -agonists (SABA) as rescue therapy. In some cases, histamine-2 receptor antagonists and leukotriene receptor antagonist

were also administered as maintenance therapy when required. The study was approved in advance by The Scientific Committee and Ethical Council of the University of Szeged, and written informed parental consent was obtained in all cases before the study.

Sample collection

Six milliliters of blood was taken from a peripheral vein in both groups. For the gene expression experiments, blood was stabilized (RNA/DNA Stabilization Reagent for Blood/Bone Marrow, Roche), and EDTA-coated tubes were used for the collection and analyses of oxidant and antioxidant parameters.

Gene expression analysis

The gp91^{phox} (CYBB) and HMOX-1 gene expression was determined by using a real-time polymerase chain reaction (RT-PCR) technique. Briefly, after the extraction of mRNA from whole blood as prescribed by the manufacturer (mRNA isolation Kit for Blood/Bone Marrow, Roche), reverse transcription was performed by using a cDNA Synthesis Kit (Fermentas). The RT-PCR process was carried out with a LightCycler Carousel-based System 1.5 (Roche) and LightCycler Software Version 3.5, with a FastStart HybProbe Kit (Roche). The following primers and hybridization probes were designed by the LightCycler Probe Design Software 2.0: *forward primer-CYBB*: 5'-AACACCCTAATACCAGA-3', *reverse primer-CYBB*: 5'-CATGGAAGAGACAAGT-3', *P1-CYBB*: 5'-CTCTGTGACCTGAAGCC-Fluo3', *P2-CYBB*: 5'-LcRed705-GGCTGAAACCTGACTAAC-Pho3', *forward primer-HMOX-1*: 5'-GTTCTGCTCAACATCCA-3', *reverse primer-HMOX-1*: 5'-GCTTCCCTCTGGGAGTCT-3', *P1-HMOX-1*: 5'-GCTGACCCATGACACC-Fluo3', *P2-HMOX-1*: 5'-LcRed705-GGACCAGAGCCCCTCA-Pho3'. The annealing temperature was set at 55 °C. Human porphobilinogen deaminase (h-PBGD, HPBGD Gene Set, Roche) was used as housekeeping gene, marked by different dyes, in the same capillary, to normalize the differences between the sample's mRNA concentrations. Relative expressions of candidate mRNAs were determined by using the threshold cycle number (Ct) data obtained by real-time PCRs, with each sample normalized to its own h-PBGD housekeeping gene (ΔCt). The ΔCt data for the overall group and the individual subgroups were compared with those for the healthy controls ($\Delta\Delta\text{Ct}$).

Biochemical analysis

We measured the levels of GSH vs. GSSG, thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) and protein carbonylation (an oxidized protein end-product), and the activities of SOD, CAT, GPx and GR enzymes in the red blood cells. The plasma cortisol level was also examined. GSH and GSSG were measured in whole blood. All other parameters were determined on red blood cells after a period of washing with phosphate-buffered saline (pH 7.4) three times. For all analyses, 10- and 100-fold diluted hemolyzates were used.

GSH concentrations were determined in specific reactions with GR. To determine GSSG concentrations, thiol scavenging was carried out with N-ethylmaleimide (Németh and Boda, 1994). The total quantity of proteins was determined with the Folin–Ciocalteu phenol reagent, using bovine serum albumin as standard. Results are expressed in mg/ml (Lowry et al., 1951). After removal of the hemoglobin from hemolyzed samples, SOD activity was measured in the supernatant via the inhibition of the spontaneous epinephrine–adrenochrome transformation (McCord and Fridovich, 1969; Misra and Fridovich, 1972; Matkovic et al., 1982). CAT activity was measured spectrophotometrically, following the decomposition of H₂O₂, at 240 nm (Beers and Sizer, 1952). To determine GPx activity, we measured the GSH consumption in each sample and its own control, with cumene hydroperoxide and GSH as substrates. Determination was performed spectrophotometrically with measurement of the residual GSH with 5,5'-dithiobis-2-nitrobenzoic acid (Novák et al., 1990; Chiu et al., 1976; Pinto and Bartley, 1969). In

the determination of GR activity, GSSG was applied as substrate and NADPH as cofactor (Novák et al., 1990; Pinto and Bartley, 1969). Enzyme activities are expressed in U/mg protein, or in the case of CAT in Bergmeyer units (BU)/mg protein (Beers and Sizer, 1952). The concentration of TBARS derived from the red blood cells was measured with thiobarbituric acid (TBA), which gives the level of total TBA-reactive substances. Results are reported in nmol/mg protein (Placer et al., 1966). Carbonylated proteins are oxidatively modified proteins. The level of protein damage was examined with 2,4-dinitrophenylhydrazine (2,4-DNPH). All samples had their own controls without 2,4-DNPH. Results are expressed in nmol/mg protein (Levine et al., 1996).

Statistical analysis

Comparisons were performed by using one-way ANOVA, followed by Tukey's post-test; or for TBARS, the Kruskal–Wallis test. All values have had normal distribution, and means $\pm$ SD were calculated for all groups except for MDA, where median values are shown with the 25th and 75th percentiles. In latter case the variances were different and non-parametric test was applied. The p values <0.05 were considered indicative of a significant difference.

Results

NADPH oxidase gp91phox subunit mRNA in blood

We analyzed the relative mRNA level in each subgroup and determined the difference in expression between the subgroups treated or not treated with ICS ($\Delta\Delta Ct = 0.819$ vs. $\Delta\Delta Ct = -0.363$, respectively; $p < 0.03$; ΔCt values referred to the healthy controls). This result suggested a difference due to medical treatment. The post test after ANOVA analysis revealed a significant difference between the subgroup not treated with ICS and that treated with a low daily dose of ICS ($\Delta\Delta Ct = 1.445$, $p < 0.05$) indicating an approximately two-fold difference. These ΔCt values in each subgroup were compared with non steroid treated asthmatics in Table 1. Negative $\Delta\Delta Ct$ values mean upregulation of the gene expression according to the comparative ΔCt method, contrarily to the positive values which indicate downregulation. No significant difference was observed between the three other ICS-treated subgroups, where the taken higher ICS doses did not influence the CYBB gene expression significantly as compared with the subgroup treated with a low daily dose of ICS. For a better presentation in Fig. 1, the relative expression profile of the NADPH oxidase subunit coding CYBB gene was shown relative to healthy controls (Fig. 1).

HMOX-1 gene expression in blood

There was no significant difference in HMOX-1 gene expression between the asthmatic patients and the healthy controls, and the ICS therapy did not influence the expression of this gene either (Fig. 2).

Biochemical analyses in blood

The TBARS and carbonylated protein concentrations in the red blood cells were significantly higher in the asthmatic children than

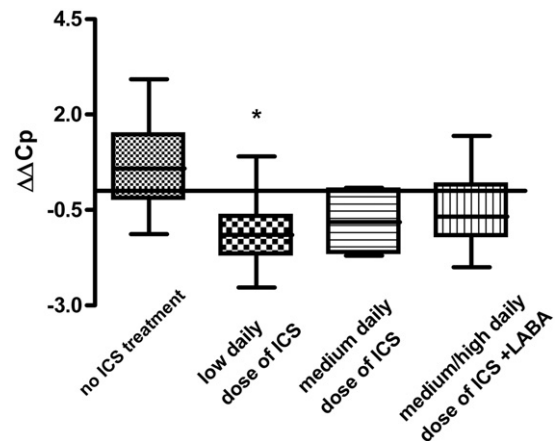


Fig. 1. Relative expressions of CYBB in blood samples from the three subgroups of asthmatic patients. Relative mRNA levels of gp91^{phox} coding CYBB gene were determined by using the threshold (Ct) values of the CYBB gene by real-time PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were compared ($\Delta\Delta Ct$). Values are referred to healthy controls for the better presentation. *Significantly lower mRNA levels $p < 0.05$ were measured in the subgroup treated with a low daily dose of ICS than in those who were not treated with ICS. The blocking effect was lower (higher mRNA levels) in the patients who received medium/high daily dose of ICS alone or in combination with LABA, but this difference did not reach the level of significance. Statistical analysis was performed by one-way ANOVA test.

in the healthy controls ($p < 0.05$ and $p < 0.03$, respectively). Both parameters were somewhat higher in the subgroup which did not require steroids than in those who received ICS medication (Table 2). The TBARS level was also significantly higher in all the ICS-treated subgroups as compared the asthmatic children not treated with ICS. The highest carbonylated protein level was measured in the subgroup that did not require ICS, but the difference was significant only relative to those who received a low daily dose of ICS. Neither parameter was influenced by sex or asthma control (data not shown).

The GSH and GSSG levels and the ratio GSSG/2GSH did not differ significantly in any of the subgroups from those in the healthy controls. Nor were the GPx or GR activities in the asthmatic children significantly different from those in the healthy children. The ICS-treated asthmatic subgroup displayed higher GPx activities than those who did not receive ICS treatment, but the difference did not reach the

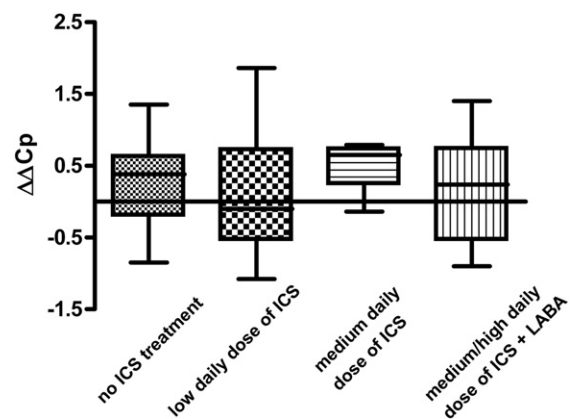


Fig. 2. Relative expressions of HMOX-1 in blood samples from the three subgroups of asthmatic patients. Relative mRNA levels of the HMOX-1 gene were determined by using the threshold (Ct) values of the HMOX-1 gene by real-time PCR. Each sample was normalized to its own h-PBGD housekeeping gene (ΔCt). ΔCt data on groups and subgroups were determined ($\Delta\Delta Ct$). Values are referred to the healthy controls. No significant difference was observed between the individual subgroups. Statistical analysis was performed by one-way ANOVA test.

Table 1

$\Delta\Delta Ct$ data derived from asthmatic patients treated with different ICS doses compared with non ICS treated ones.

Treatment	$\Delta\Delta Ct$
Low daily dose of ICS	1.445*
Medium daily dose of ICS	1.168
Medium/high daily dose of ICS + LABA	1.034

Statistical analysis was performed by one-way ANOVA test.

* $p < 0.05$: compared with non steroid treated patients.

Table 2

Biochemical oxidative stress and antioxidant parameters in blood of asthmatic children and healthy controls.

Parameters	Healthy	Steroid treated	Steroid non treated
TBARS (nmol/mg prot.)	0.56 (0.44–0.65)	0.77 (0.66–0.89)*	0.78 (0.66–1.05)*
Carbonylated protein ($\times 10^{-5}$ μ mol/mg prot.)	6.77 $\pm$ 1.12	7.22 $\pm$ 1.25	8.10 $\pm$ 1.63*
GSH (μ mol/g Hb)	8.69 $\pm$ 2.31	9.31 $\pm$ 1.51	9.19 $\pm$ 1.36
GSSG (μ mol/g Hb)	18.50 $\pm$ 4.35	19.63 $\pm$ 5.28	18.95 $\pm$ 3.51
GSSG/2GSH	0.22 $\pm$ 0.06	0.21 $\pm$ 0.04	0.21 $\pm$ 0.03
SOD (U/mg prot.)	2.54 $\pm$ 1.07	2.49 $\pm$ 0.86	3.13 $\pm$ 0.97*+
CAT ($\times 10^{-5}$ BU/mg prot.)	0.89 $\pm$ 0.22	0.86 $\pm$ 0.29	1.04 $\pm$ 0.36
GPx ($\times 10^{-3}$ U/mg prot.)	2.41 $\pm$ 0.45	3.18 $\pm$ 1.57	2.63 $\pm$ 0.45
GR ($\times 10^{-4}$ U/mg prot.)	7.68 $\pm$ 1.84	7.44 $\pm$ 2.28	8.28 $\pm$ 2.78

Data are reported as mean $\pm$ SD and in case of TBARS median (25th–75th percentiles). One-way ANOVA and Kruskal–Wallis tests were performed to compare the groups.

* $p < 0.05$ significant difference relative to healthy controls.

*+ $p < 0.05$ significant difference relative to healthy controls and to steroid treated patients.

level of significance. These data too were not influenced by sex or asthma control (*data not shown*).

The SOD activity was significantly higher in the asthmatic patients who did not receive ICS medication than in the healthy children or in those who required ICS therapy (Table 1).

The CAT activity exhibited a small, but not significant elevation in the asthmatic subgroup not treated with ICS, but there was no noteworthy difference in enzyme level as a function of sex or asthma control.

Discussion

This study focused on the possible use of peripheral blood, which is the most performable medium for examination of the systemic oxidative damage in children. We measured the mRNA levels of the NADPH oxidase enzyme complex gp91^{phox} subunit (CYBB), one of the main free radical sources, and the antioxidant HMOX-1 protein. Besides the transcriptional activities of the above genes, we determined the level of a number of antioxidant enzymes and the blood levels of lipid peroxidation and protein damage. All the results were considered in the context of the medical treatment too.

The major role of the NADPH oxidase complex in free radical generation and the importance of the NF- κ B transcriptional factor and pro-inflammatory cytokines in the transcriptional regulation of the gp91^{phox}, p67^{phox} and p47^{phox} subunits were demonstrated earlier in the MonoMac1 cell line in vitro, but the mRNA level of CYBB has previously not been measured in blood leukocytes of childhood asthmatic patients (Bedard and Krause, 2007; Gauss et al., 2007; Yao et al., 2007). The CYBB mRNA level proved to be significantly lower in the patients treated with a low daily dose of ICS than in those who did not receive ICS therapy. There was also a tendency to a lower expression in the patients treated with a medium/high daily dose of ICS or with an ICS–LABA combination, relative to asthmatics without ICS treatment, but these differences did not reach the level of significance. Although it is widely accepted that inhaled corticosteroids in normal therapeutic doses exhibit effects only locally in the airways (Chung et al., 2009), our results clearly demonstrated that the CYBB expression was altered in the blood. This systemic effect may be due to the activated inflammatory cells which appear in the circulation, and the alterations in CYBB expression may be a consequence of the inhibitory effects of ICS on NF- κ B-regulated genes as in the case of the NADPH oxidase gp91^{phox} subunit (Almeida et al., 2005; Gauss et al., 2007; Yao et al., 2007; Condino-Neto et al., 1998). The present findings in the subgroups that received a higher dose of steroid or ICS combined with LABA, which are known to improve the symptoms (Hew et al., 2006), may be linked with relative steroid resistance, which demands further examinations. Despite the administration of higher steroid doses, a lower level of CYBB gene transcription was found in steroid

treated asthmatic children than in healthy children, even when the serum cortisol concentration was similar to that in the healthy controls. The activity of the NADPH oxidase complex is also determined by several posttranscriptional modifications (van der Vliet, 2008; Bedard and Krause, 2007). The lack of transcriptional difference in the CYBB gene in the blood from asthmatics and healthy children does not exclude a possible role of the NADPH oxidase complex in free radical generation at the protein level, as demonstrated in other studies.

The HMOX-1 gene, transcriptionally activated in the early phase of the oxidative stress, is used as an acute marker in studies of the anti-oxidant system (Slebos et al., 2003; Jin and Choi, 2005). Our results did not indicate any change in the HMOX-1 mRNA levels in the analyzed groups, in accord with the chronic oxidative stress previously supposed in bronchial asthma. The difference in expression profile between CYBB and HMOX-1 genes in our samples may originate from the different signaling pathways: the CYBB gene is mainly regulated by NF- κ B, while HMOX-1 is regulated by Nrf2 during airway inflammation (Yao et al., 2007; Lacy et al., 2003; Lee et al., 2000).

Elevated TBARS and carbonylated protein levels were detected in our asthmatic patients relative to healthy children, confirming earlier data (Schock et al., 2003; Shanmugasundaram et al., 2001; Perišić et al., 2007; Sugiura and Ichinose, 2008; Novák et al., 1991). The level of TBARS is indicative of lipid damage, which is an early marker of the ROS-mediated injury, whereas the carbonylated protein elevation due to the protein damage is a later marker of oxidative stress. The TBARS and carbonylated protein levels in the subgroup that did not participate in corticosteroid treatment were higher than in those treated with inhalative steroid. Higher SOD activity was also measured in the subgroup that did not require steroid, indirectly indicating the high O₂^{•−} generation. The enzymatic antioxidant CAT, GR and GPX concentrations in the red blood cells were not changed significantly.

The clinical status and therapy requirement of patients with asthma are influenced by various factors, including genetic, environmental and nutritional ones (Wong et al., 2001; Matsumoto et al., 2004). Recently, there have been many reports suggesting the presence of vitamin D insufficiency in immune-regulated inflammatory diseases such as rheumatoid arthritis or in bronchial asthma in which the balance between the Th1 and Th2 immune response is disturbed (Cutolo, 2009; Sandhu and Casale, 2010). It was also reported that vitamin D supplementation may be favorable on immune response of cells obtained from patients who required ICS–LABA combination therapy or oral steroid treatment in vitro (Sandhu and Casale, 2010; Searing et al., 2010). The immunomodulatory role, the possible effect of vitamin D on the corticosteroid pathway and results in adult asthmatics suggest that vitamin D may have positive effects in asthmatic children too. This hypothesis, as well as the possible relationship of vitamin D levels and oxidative stress requires further examinations.

To the best of our knowledge, there have been no reports of monocytic/myeloid cell lines expressing the gp91^{phox} subunit coding gene in the blood of asthmatic patients, and nor of the modifying effects of ICS on this pro-oxidant gene from blood leukocytes. Our results were measured on native leukocytes from asthmatic children: cells after isolation were not treated in vitro. Besides the local inflammation in the airways, we could detect the systemic effects of chronic oxidative stress in childhood asthma.

Conclusions

In conclusion, we confirmed the presence of oxidative stress in the circulation of pediatric asthmatic patients: higher damaged lipid and carbonylated protein concentrations reflected a chronic oxidative overload. We also detected the effects of inhaled corticosteroid medication on the gene expression also in the blood, and examined for the first time the transcriptional activity of the main, electron transfer-catalyzing gp91^{phox} subunit of the NADPH–oxidase complex on native leukocytes gained from asthmatic children. The examination of

blood samples proved suitable for the following oxidative processes which mainly occur locally in the lungs, but further investigations are needed to identify the best blood parameters and methods in pediatric practice and asthma management. It is also essential to determine reference values for oxidative stress in different pathological conditions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

Special thanks for all participating patients and their parents, for Noémi Nagy and for the members of the pulmonology ward.

References

- Almeida AC, Rehder J, Severino SD, Martins-Filho J, Newburger PE, Condino-Neto A. The effect of IFN- γ and TNF- α on the NADPH oxidase system of human colostrum macrophages, blood monocytes, and THP-1 cells. *J Interferon Cytokine Res* 2005;25:540–6.
- Baraldi E, Carraro S. Exhaled NO and breath condensate. *Paediatr Respir Rev* 2006;7(Suppl. 1):S20–2.
- Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007;87:245–313.
- Beers Jr RF, Sizer IW. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 1952;195:133–40.
- Busse WW, Wanner A, Adams K, Reynolds HY, Castro M, Chowdhury B, et al. Investigative bronchoprovocation and bronchoscopy in airway diseases. *Am J Respir Crit Care Med* 2005;172:807–16.
- Chiu DT, Stults FH, Tappel AL. Purification and properties of rat lung soluble glutathione peroxidase. *Biochim Biophys Acta* 1976;445:558–66.
- Chung KF, Caramori G, Adcock IM. Inhaled corticosteroids as combination therapy with beta-adrenergic agonists in airways disease: present and future. *Eur J Clin Pharmacol* 2009;65:853–71.
- Comhair SAA, Ricci KS, Arroliga M, Lara AR, Dwelk RA, Song W, et al. Correlation of superoxide dismutase deficiency to airflow obstruction in asthma. *Am J Respir Crit Care Med* 2005;172:306–13.
- Condino-Neto A, Whitney C, Newburger PE. Dexamethasone but not indomethacin inhibits the human phagocyte nicotinamide adenine dinucleotide phosphate oxidase activity by downregulating expression of genes encoding oxidase components. *J Immunol* 1998;161:4960–7.
- Cutolo M. Vitamin D, and autoimmune rheumatic diseases. *Rheumatology* 2009;48:210–2.
- Dworsky R. Oxidant stress in asthma. *Thorax* 2000;55:S51–3.
- Farkas I, Maróti Z, Katona M, Endreffy E, Monostori P, Máder K, et al. Increased heme-oxygenase-1 expression in premature infants with respiratory distress syndrome. *Eur J Pediatr* 2008;167:1379–83.
- Gauss KA, Nelson-Overton LK, Siemsen DW, Gao Y, DeLeo FR, Quinn MT. Role of NF- κ B in transcriptional regulation of the phagocyte NADPH-oxidase by tumor necrosis factor- α . *J Leukoc Biol* 2007;82:729–41.
- Grob NM, Aytekin M, Dweik RA. Biomarkers in exhaled breath condensate: a review of collection, processing and analysis. *J Breath Res* 2008;2:037004.
- Hew M, Bhavsar P, Torrego A, Meah S, Khorasani N, Barnes PJ, et al. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. *Am J Respir Crit Care Med* 2006;174:134–41.
- Jin Y, Choi AMK. Cytoprotection of heme oxygenase-1/carbon monoxide in lung injury. *Proc Am Thorac Soc* 2005;2:232–5.
- Lacy P, Latif DA, Steward M, Musat-Macru S, Paulman SF, Moqbel R. Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects. *J Immunol* 2003;170:2670–9.
- Lee PJ, Camhi SL, Chin BY, Alam J, Choi AMK. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. *Am J Physiol Lung Cell Mol Physiol* 2000;279:175–82.
- Lee IT, Luo SF, Lee CW, Wang SW, Lin CC, Chang CC, et al. Overexpression of HO-1 protects against TNF- α mediated airway inflammation by down-regulation of TNFR1-dependent oxidative stress. *Am J Pathol* 2009;175:519–32.
- Levine RL, Garland D, Oliver CN. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1996;186:464–78.
- Lowry OH, Rosebrough EA, Farr AL. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- Mak JCW, Chan-Yeung MMW. Reactive oxidant species in asthma. *Curr Opin Pulm Med* 2006;12:7–11.
- Matkovic B, László A, Szabó L. A comparative study of superoxide dismutase, catalase and lipid peroxidation in red blood cells from muscular dystrophy patients and normal controls. *Clin Chim Acta* 1982;118:289–92.
- Matsumoto K, Inoue H, Fukuyama S, Tsuda M, Ikegami T, Kibe A, et al. Decrease of interleukin-10-producing T cells in the peripheral blood of severe unstable atopic asthmatics. *Int Arch Allergy Immunol* 2004;134:295–302.
- McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein). *J Biol Chem* 1969;244:6049–55.
- Misra HP, Fridovich I. A role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972;247:3170–5.
- Nadeem A, Chhabra SK, Masood A, Raj HG. Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol* 2003;111:72–8.
- Németh I, Boda D. Blood glutathione redox ratio as a parameter of oxidative stress in premature infants with IRDS. *Free Radic Biol Med* 1994;16:347–53.
- Novák Z, Kovács L, Pál A, Pataki L, Isz Varga, Matkovic B. Comparative study of antioxidant enzymes and lipid peroxidation in cord maternal red blood cells. *Acta Paediatr Hung* 1990;30:391–7.
- Novák Z, Németh I, Gyurkovits K, Varga IS, Matkovic B. Examination of the role of oxygen free radicals in bronchial asthma in childhood. *Clin Chim Acta* 1991;201:247–51.
- Novak Z, Petak F, Banfi A, Toth-Szuzi V, Barati L, Kosa L, et al. An improved technique for repeated bronchoalveolar lavage and lung mechanics measurements in individual rats. *Respir Physiol Neurobiol* 2006;154:467–77.
- Ozars R, Tahan V, Turkmen S, Talay F, Besirli K, Aydin S, et al. Changes in malondialdehyde levels in bronchoalveolar fluid and serum by the treatment of asthma with inhaled steroid and beta2-agonist. *Respir* 2000;3:289–92.
- Park CS, Kim TB, Lee KY, Moon KA, Bae YJ, Jang MK, et al. Increased oxidative stress in the airway and development of allergic inflammation in a mouse model of asthma. *Ann Allergy Asthma Immunol* 2009;103:238–47.
- Perišić T, Srečković M, Matić G. An imbalance in antioxidant enzymes and stress proteins in childhood asthma. *Clin Biochem* 2007;40:1168–71.
- Pinto RE, Bartley W. The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochem J* 1969;112:109–15.
- Placer ZA, Cushman L, Johnson SC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem* 1966;16:359–64.
- Rahman I, Biswas SK, Kode A. Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* 2006;533:222–39.
- Rochelle LG, Fisher BM, Adler KB. Concurrent production of reactive oxygen and nitrogen species by airway epithelial cells in vitro. *Free Radic Biol Med* 1998;24:863–8.
- Sandhu MS, Casale TB. The role of vitamin D in asthma. *Ann Allergy Asthma Immunol* 2010;105:191–9.
- Schock BC, Young IS, Brown V, Fitch PS, Shields MD, Ennis M. Antioxidants and oxidative stress in BAL fluid of atopic asthmatic children. *Pediatr Res* 2003;53:375–81.
- Searing DA, Zhang Y, Murphy JR, Hauk PJ, Goleva E, Leung DYM. Decreased serum vitamin D levels in children with asthma are associated with increased corticosteroid use. *J Allergy Clin Immunol* 2010;125:995–1000.
- Shanmugasundaram KR, Kumar SS, Rajasee S. Excessive free radical generation in the blood of children suffering from asthma. *Clin Chim Acta* 2001;305:107–14.
- Slebos DJ, Rytter SW, Choi AMK. Heme oxygenase-1 and carbon monoxide in pulmonary medicine. *Rev Respir Res* 2003;4:7.
- Sugiura H, Ichinose M. Oxidative and nitrate stress in bronchial asthma. *Antioxid Redox Signal* 2008;10:785–97.
- Taillé C, El-Benna J, Lanone S, Dang MC, Ogier-Denis E, Aubier M, et al. Induction of heme-oxygenase-1 inhibits NADPH-oxidase activity by downregulating cytochrome b558 expression via the reduction of heme availability. *J Biol Chem* 2004;279:28681–8.
- van der Vliet A. NADPH oxidases in lung biology and pathology. Host defence enzymes and more. *Free Radic Biol Med* 2008;44:938–55.
- Vural H, Aksoy N, Ceylan E, Gencer M, Ozguner F. Leukocyte oxidant and antioxidant status in asthmatic patients. *Arch Med Res* 2005;36:502–6.
- Wong CK, Ho CY, Ko FWS, Chan CHS, Ho ASS, Hui DSC, et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN- γ , IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 2001;125:177–83.
- Yao H, Yang SR, Kode A, Rajendrasozhan S, Caito S, Adenuga D, et al. Redox regulation of lung inflammation: role of NADPH oxidase and NF- κ B signaling. *Biochem Soc Trans* 2007;35:1151–5.