Biochemistry (Moscow), Vol. 69, No. 4, 2004, pp. 462-467. Translated from Biokhimiya, Vol. 69, No. 4, 2004, pp. 568-574.

Originally published in Biochemistry (Moscow) On-Line, Papers in Press, as Manuscript BM03-184, February 29, 2004.

Iron, Nitric Oxide, and Myeloperoxidase in Asthmatic Patients

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Received August 21, 2003
Revision received August 29, 2003

Abstract—Plasma nitric oxide (NO), myeloperoxidase (MPO), and iron (Fe) levels were determined in bronchial asthma. The relations among these parameters in different stages of asthma were interpreted. Their association with airway inflammation observed in patients with bronchial asthma as well as the roles and the contributions to the pathological processes were evaluated. A total of 62 individuals, 32 asthmatics and 30 controls, were included into the scope of this study. Plasma nitric oxide metabolites (NOx) and MPO and Fe levels were determined by the Griess reaction, ELISA, and the automated TPTZ (2,4,6-tri[2-pyridyl]-5-triazine) method, respectively. In the asthmatic individuals, plasma NOx, MPO, and Fe concentrations were 133 ± 13 μM, 95 ± 20 ng/ml, and 159 ± 20 μg/dl, respectively; in the control group these values were 82 ± 11 μM, 62 ± 11 ng/ml, and 96 ± 9 μg/dl. Increased values were detected for plasma MPO ($p > 0.05$), NOx ($p < 0.01$), and Fe ($p < 0.01$) concentrations in asthmatic individuals. Considering the facts that NO modulates the catalytic activity of MPO and induces the expression of heme oxygenase as important contributors to the mechanisms causing free Fe release, it is concluded that elevated NOx, MPO, and Fe levels observed in the asthmatic group act in a concerted manner and appear to be involved in the pathogenesis of asthma.

Key words: asthma, nitric oxide, myeloperoxidase, nitrate, nitrite

Asthma is a chronic inflammatory disease of the airways, and reactive oxygen and nitrogen species (ROS/RNS) are suggested to contribute to its pathology. Data on several ROS/RNS profiles as well as the presence of iron (Fe) and iron binding proteins in different lung compartments and the amount of Fe and its distribution in different pulmonary diseases have already been reported [1-3]. However, more studies on the matter are needed in view of the increasing incidence of asthma and because many questions about Fe metabolism and its relation with the metabolisms of other related parameters in the lung remained unanswered. For example, the relationship between nitric oxide (NO) and Fe as well as the participation of myeloperoxidase (MPO) into this association and the role of Fe in the defense against infectious agents in the lung are not fully understood.

Nitric oxide plays important bioregulatory roles in a number of physiological processes. Recent reports on the interaction of NO and Fe metabolism have shown that NO plays a role in the regulation of Fe metabolism [4].

Despite the fact that MPO functions in biological environments replete with NO, the potential interaction between NO and the heme moiety of MPO has not yet been explored.

The aim of this study was to evaluate plasma levels of NO products (NOx), MPO (which has bactericidal effect as an indicator of neutrophil activation), and Fe (a parameter that is closely associated with the metabolisms of the other two parameters). Also, the correlation between conventional measures of asthma monitoring (symptoms, spirometry) and these parameters (known to be closely associated with each other), their association with airway inflammation in patients with mild to moderate to severe persistent bronchial asthma, as well as their roles and contributions to pathological processes were investigated.

For this purpose, plasma NOx (nitrite NO_2^-, nitrate NO_3^-), MPO, and Fe values both in patients with asthma and healthy individuals were determined, and the rela-

Abbreviations: NOx) nitric oxide metabolites (NO_3^- and NO_2^-); MPO) myeloperoxidase; ROS/RNS) reactive oxygen and nitrogen species; NOS) nitric oxide synthase; TPTZ) 2,4,6-tri[2-pyridyl]-5-triazine; FEV_1) measurement of forced expiratory volume in 1 sec; FVC) forced vital capacity.

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tions among these parameters in different stages of asthma have been interpreted.

METHODS OF INVESTIGATION

A total of 62 individuals, 32 asthmatics and 30 controls, were included into the scope of this study. Thirty-two asthmatic subjects who fulfilled the diagnostic criteria of asthma from the American Thoracic Society were recruited for the study. They are divided into four groups based on Classification of Asthma Severity by Clinical Features before Treatment: Steps 1, 2, 3 and 4 were described as intermittent, mild persistent, moderate persistent, and severe persistent, respectively [5].

Twenty-one were atopic and 2 had atopy in the individuals within their families, defined by positive skin prick tests to common allergens. Four, 12, 10, and 6 of the patients had Stage 1, Stage 2, Stage 3, and Stage 4 asthma, respectively. Patients with intermittent and mild persistent asthma were not previously or currently being treated with inhaled corticosteroids and stable but symptomatic patients were receiving treatment with inhaled steroids. All patients took inhaled salbutamol for intermittent relief of wheeze. None of the subjects studied had received oral corticosteroids during the preceding 12 months. Ex-smokers of more than one pack years were excluded.

Mean age ± S.E.M for patient and control groups was 36 ± 1 years. Body mass indexes for patients and control group were calculated as 25 ± 0.5 and 24 ± 0.7 kg/m^2, respectively.

Pulmonary function tests were performed. Measurement of forced expiratory volume in 1 sec (FEV_1) and forced vital capacity (FVC) are determined during a forced expiratory maneuver using a spirometer. Baseline spirometric parameters—FVC (ml), FVC (%), FEV_1 (ml), FEV_1 (%), FEV_1/FVC (%), FEF_{25-75} (liter/sec), and FEF_{25-75} (%)—were recorded.

Plasma NOx concentrations, MPO levels, and Fe concentrations were determined in both groups. Plasma NOx concentrations were determined by a method based on the principle using nitrate reductase for the conversion of nitrate to nitrite and using Griess reagent for color production [6, 7]. ELISA assay [8] and an automated method [9] using TPTZ (2,4,6-tri[2-pyridyl]-5-triazine) were used to determine plasma MPO and Fe levels, respectively.

This study was approved by the Istanbul University, Cerrahpaşa Medical Faculty Hospital Ethics Committee, and all patients gave their informed consent.

Statistical treatment of the data. Data were expressed as mean ± S.E.M. as well as medians as appropriate. Data parametrically distributed were presented as a mean ± S.E.M. Data nonparametrically distributed were presented as median with lower and upper quartiles. Normality of the distribution of the data was determined based on the Chi-square test.

Statistical analyses of comparisons between groups were performed using Student's *t*-test for parametric data (NOx, NO_3^-, Fe, pulmonary function tests) and the Mann–Whitney U-test for nonparametric data (MPO and NO_2^-).

Comparisons of parameters between all the groups (4 patient subgroups) were first made using the Kruskal–Wallis test. In case of significant difference, this was followed by the Dunn's multiple comparisons test between groups.

Correlations between different parameters were assessed by Pearson's or Spearman's rank correlation tests as appropriate, using data from both healthy individuals and asthmatics.

Statistical analyses were done by the statistical program STATA 5.0.

Differences associated with values $p < 0.05$ were accepted as significant.

RESULTS AND DISCUSSION

Physical parameters of the subjects with persistent asthma and healthy individuals are shown in Table 1.

Table 1. Physical parameters of the subjects with persistent asthma and healthy individuals

Parameter (mean ± SEM) / Group	Control	Patients	Step 1	Step 2	Step 3	Step 4
Age, years	36 ± 1	36 ± 1	33 ± 6	36 ± 3	36 ± 3	37 ± 3
Height, cm	164 ± 20	162 ± 1	165 ± 3	160 ± 2	163 ± 2	164 ± 3
Weight, kg	65 ± 2	65 ± 1	60 ± 2	63 ± 3	68 ± 3	68 ± 3
Body mass index, kg/m^2	24 ± 0.7	25 ± 0.5	22 ± 0.2	25 ± 1	26 ± 1	26 ± 1

Table 2. Parameters related to pulmonary function tests performed on control and patients groups

Parameter (mean ± SEM) / Group	Control (C)	Patients (P)	Step 1 (S1)	Step 2 (S2)	Step 3 (S3)	Step 4 (S4)
FVC, ml[a,c,e,g]	3943 ± 145	3050 ± 164	3935 ± 347	3060 ± 164	2906 ± 338	2682 ± 509
FVC, %[a,e,g]	104 ± 2	93 ± 3	113 ± 7	100 ± 3	85 ± 5	78 ± 8
FEV_1, ml[a,b,d,g]	3294 ± 123	2226 ± 133	2970 ± 394	2379 ± 125	2036 ± 233	1738 ± 374
FEV_1, %[a,d,f]	102 ± 2	78 ± 3	98 ± 4	90 ± 3	69 ± 4	55 ± 7
FEV_1/FVC, %[a,c]	84 ± 1	72 ± 2	75 ± 4	78 ± 1	70 ± 3	62 ± 4
FEF_{25-75}, liter/sec[a,b,d,f]	3.6 ± 0.2	1.7 ± 0.1	2.6 ± 0.5	2 ± 0.2	1.5 ± 0.2	1.1 ± 0.3
FEF_{25-75}, %[a,b,d,f]	89 ± 4	46 ± 3	58 ± 11	57 ± 3	37 ± 3	29 ± 6

[a] $p < 0.01$ (C vs P).
[b] $p < 0.01$ (C vs S2).
[c] $p < 0.05$ (C vs S2).
[d] $p < 0.001$ (C vs S3).
[e] $p < 0.01$ (C vs S3).
[f] $p < 0.001$ (C vs S4).
[g] $p < 0.01$ (C vs S4).

Table 2 shows parameters related to pulmonary function tests performed on both control and patient groups.

Increased values were detected for plasma MPO ($p > 0.05$), NOx ($p < 0.05$), and Fe ($p < 0.01$) concentrations. In asthmatic individuals, plasma NOx, MPO, and Fe concentrations were 133 ± 13 μM, 95 ± 20 ng/ml, and 159 ± 20 μg/dl, respectively; in the control group these values were 82 ± 11 μM, 62 ± 11 ng/ml, and 96 ± 9 μg/dl. The values for these parameters for intermittent, mild, moderate, and severe persistent asthmatics as well as for the healthy individuals are listed in Table 3.

Oxidative stress is known to participate in the pathogenesis of chronic inflammatory diseases, e.g., atherosclerosis, carcinogenesis, and asthma. Mediators including NO released during chronic inflammation were shown to increase heme oxygenase expression in asthmatic patients. Heme oxygenase reaction, with its products bilirubin, carbon monoxide, and free Fe, is interpreted as an antioxidative defense mechanism due to release of bilirubin [10]. However, free Fe, a catalyst during ROS production, is another product of this reaction and therefore may possess some inflammatory effects.

The usual source of Fe in the lung is serum Fe, which is derived from catabolized erythrocytes and absorbed Fe. Iron metabolism is of crucial importance in the biology and pathophysiology of the lower respiratory tract. As with many other factors involved in inflammation, it is very important that an appropriate Fe balance is maintained. Excessive accumulation of Fe exerts toxic effects through its ability to catalyze formation of highly reactive hydroxyl radicals.

Studies have reported that nitric oxide synthase (NOS) can bind transition metals; however, only Fe increased the rate of the reaction, whereas the others, e.g., nickel and cobalt, inhibited the reaction or were without effect like manganese. On the other hand, NO was reported to cause Fe release from erythrocytes [11-13].

Nitric oxide synthase and MPO are colocalized in primary granules of neutrophils and concomitantly secreted into the extracellular space and phagolysosomal compartments. It is therefore likely that at sites of inflammation, MPO will experience environments where high levels of NO are produced. NO modulates the catalytic activity of MPO. NO was shown to bind to both forms of Fe in MPO and modulates peroxidase catalytic activity. The high affinity of NO for MPO-Fe(III) suggests that the leukocyte peroxidase may serve as a target of NO within biological systems. The influence of NO on MPO catalytic activity may have broad implications for the regulation of local inflammatory and infectious events *in vivo* [14-16].

Studies have already been performed on trace elements in patients with bronchial asthma. So far, lower zinc and higher copper levels in serum have been well confirmed [7-21]. However, reports on serum Fe levels in bronchial asthma are scarce [20, 22]. Due to the contradictory findings on the matter, the possible relations between Fe and the other parameters in bronchial asthma are not clear yet. Serum Fe levels were reported to have only limited importance for diagnosis and prognosis of bronchitis and bronchial asthma and the estimation of this parameter was recommended in special cases only

Table 3. Plasma MPO, nitrite (NO_2^-), nitrate (NO_3^-), NOx, and Fe levels in patients with asthma and control group

Parameter (mean ± SEM) / Group	Control (C)	Patients (P)	Step 1 (S1)	Step 2 (S2)	Step 3 (S3)	Step 4 (S4)
MPO, ng/ml	62 ± 11	95 ± 20	90 ± 49	139 ± 37	48 ± 18	89 ± 57
Median	32	43	64	114	30	43
Min-Max	7–203	5–447	10–223	5–447	7–206	5–370
NOx, μM (NO_2^-/NO_3^-)	82 ± 11	133 ± 13[a]	113 ± 38	107 ± 20	160 ± 23[c]	160 ± 34
Median	70	130	140	92	140	162
Min-Max	14–196	28–280	40–160	28–280	48–236	52–236
Nitrites, μM	9 ± 2	6 ± 1	4 ± 1	4 ± 1	8 ± 3	7 ± 5
Median	75 ± 53	3	4	3	4	2
Min-Max	1–42	1–32	2–7	1–13	1–28	1–32
Nitrates, μM	75 ± 11	127 ± 13[a]	109 ± 31	103 ± 20	152 ± 22	152 ± 31
Median	64	126	133	88	135	161
Min-Max	14–192	24–273	38–155	24–273	39–233	51–223
Fe, μg/dl	96 ± 9	159 ± 20[b]	198 ± 83	175 ± 31	125 ± 28	155 ± 59
Median	89	118	194	170	89	121
Min-Max	22–206	24–430	36–368	30–327	24–313	29–430

[a] $p < 0.05$ (C vs P).
[b] $p < 0.01$ (C vs P).
[c] $p < 0.01$ (C vs S3).

[22]. In some reports, no change was detected in serum Fe levels in patients with bronchial asthma compared to controls [20]. However, significantly increased plasma Fe levels in patients were found ($p < 0.01$) in our study. No difference was noted in plasma Fe concentrations between various stages of the disease. This finding has showed that Fe initially increased significantly and continued its increasing trend to the last stages of the disease. Values above the higher limit of the normal range were detected only for 3 individuals in the control group. On the other hand, 38% of the asthmatic patients had Fe concentrations above this value.

The mean MPO concentration in plasma obtained from controls was 62 ± 11 ng/ml. This was consistent with the findings previously reported on 8 healthy individuals [23].

Decreased MPO levels were reported in iron deficiency anemia and anemia of chronic disorders [16]. This confirms our findings. In our study, significant increases were detected both in plasma MPO levels and Fe concentrations in asthmatics in comparison with those of healthy individuals.

In patients of the mild persistent asthma group, a positive correlation between plasma Fe and MPO levels was found ($r = 0.66$, $p < 0.05$). This finding may be due to the relationship between smoking and increased plasma Fe levels in this group, with the concomitant rise in plasma MPO levels. It is known that high Fe concentrations are observed in smokers. It was reported that tobacco has a high iron load (440-1150 μg/g of tobacco and 420 μg/g paper). If one assumes that each cigarette has 0.7 mg of tobacco and that each inhalation supplies 0.1%, a smoker of one pack per day would inhale 1.12 μg of Fe. In this context, exogenous Fe intake in smokers will be significantly higher than that of nonsmokers [24]. Healthy individuals as well as moderate and severe persistent asthmatic patients in this study were nonsmokers. However, 3 individuals in the mild persistent asthmatic group were current smokers (2-10 cigarettes a day).

Nitric oxide (NO), a biological mediator as well as a simple free radical, elicits a wide range of physiological and pathophysiological effects. It is generally measured as NO product (nitrite plus nitrate (NOx)) levels in physiological fluids.

Recently, the finding of no significant difference was reported in serum NOx levels of patients with non-acute asthma and controls. It is suggested that asthma patients in this "steady-state" appear to have "normal" serum NOx levels [25]. However, in our study increased values were detected for plasma NOx and nitrate concentrations in asthmatic patients ($p < 0.05$). Nitrite levels did not show any difference ($p > 0.05$).

Ranges obtained for NOx values exhibit great differences from one study to another. The values obtained in this study were consistent with some findings reported previously [26-30].

Exhaled NO levels increase in asthma, but there is not much evidence on the relationship between this parameter and airway inflammation in asthmatic patients treated with inhaled steroids. It was reported that since there is no correlation between exhaled NO and conventional measures of asthma, measurement of exhaled NO levels may not be suitable for the assessment of asthma control or airway inflammation [31]. However, we have found a weak and inverse correlation ($r = -0.38$; $p < 0.05$) between plasma NOx levels and FEV_1/FVC (%), an important pulmonary function test, in asthmatics.

Because diseases other than those causing airflow limitation may result in reduced FEV_1, a useful assessment of airflow limitation can be obtained as the ratio of FEV_1 to FVC. As known, in the normal lung, flow limitation on forced expiration results in FEV_1/FVC ratios of greater than 80%. Any values less than this is suggestive of airflow limitation [5].

In the moderate persistent asthmatic group, we found a very strong negative correlation between NOx and MPO levels, which indicates that as NOx levels increased MPO levels decreased ($r = -0.90$, $p < 0.01$). In this group, significantly decreased MPO levels were detected in comparison to mild or severe persistent asthmatic groups. The durations of disease were 4.4, 2.5, and 4.8 years in mild, moderate, and severe persistent asthmatics, respectively. Significantly decreased MPO levels as NOx levels increased, in the moderate persistent asthma group, may be due to the short duration of disease, which greatly differs from the other groups.

In the severe persistent asthmatic group, there was a moderately strong negative correlation ($r = -0.89$; $p < 0.05$) between FEF_{25-75} (liter/sec) and MPO levels. In this group comprising severe asthmatic patients FEF_{25-75} values (in control group 3.6 ± 0.3 liter/sec) decreased by 70% to 1.1 ± 0.3 liter/sec. This strong correlation caused by the increased MPO levels reflects the important association between FEF_{25-75} (liter/sec), a pulmonary function test, and MPO levels during the terminal stage of the disease. In a similar manner, a weak correlation was found between the ratio of FEV_1 to FVC (%) and plasma NOx values when all the patients as a whole were taken into consideration ($r = -0.38$; $p < 0.05$).

Finally, considering the facts that NO modulates the catalytic activity of MPO and induces the expression of heme oxygenase as an important contributor to the mechanism causing free Fe release, it is concluded that elevated NOx, MPO, and Fe levels observed in the asthmatic group act in a concerted manner and appear to be involved in the pathogenesis of asthma.

This work was supported by the Research Fund of the University of Istanbul (project number T-1135/18062001).

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