Superoxide anion production by monocytes of corticosteroid-treated asthmatic patients

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Superoxide anion production by monocytes of corticosteroid-treated asthmatic patients. M. Majori, I. Vachier, P. Godard, M. Farce, J. Bousquet, P. Chanez. ©ERS Journals Ltd 1998.
ABSTRACT: Reactive oxygen species play an important role in promoting inflammation. Blood monocytes have been described to release higher amounts of superoxide anion in uncontrolled and untreated asthmatics. Corticosteroids are widely used in asthma, but little is known about their molecular mechanism of action. The aim of our study was to analyse the *ex vivo* effect of corticosteroid treatments on superoxide anion release by blood monocytes.

Superoxide anion release was measured by a spectrophotometric method based on the superoxide dismutase (SOD) -inhibitable reduction of ferricytochrome C by blood monocytes from untreated patients and asthmatics treated with *i.v.*, inhaled and oral corticosteroids.

Monocytes from uncontrolled and untreated asthmatics, released high amounts of superoxide anions. After short-term treatment with *i.v.*, corticosteroids, this release was found to decrease significantly (1.410 *versus* 0.340 nM, p<0.05). Cells from asthmatics who had undergone long-term treatment with inhaled or oral steroids presented low amounts of superoxide anion production, with a significant difference as compared to untreated asthmatics (0.375 nM p<0.01 and 0.620 nM p<0.02 respectively).

In general, patients with controlled asthma (treated with short-term oral steroids, or with long-term inhaled steroids) released lesser amounts of superoxide anion than uncontrolled and untreated asthmatics. In the case of steroid-dependent asthmatics there was no difference between patients with a controlled or uncontrolled disease.
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Bronchial asthma is a chronic inflammatory disease of the airways characterized by an increased recruitment of inflammatory cells, including mononuclear phagocytic cells such as monocytes [1]. The ability of these cells to generate highly reactive oxygen species (ROS), including super-oxide anion, following activation of a membrane-associated reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important part of the host defence system, and has also been implicated in inflammation in a variety of pulmonary diseases [2, 3], including asthma [4, 5]. Oxygen metabolites cause tissue injury, smooth muscle contraction, increased airway vascular permeability, and may increase airway responsiveness and release of var-ious mediators [4].

In previous studies, it has been shown that alveolar macrophages [6], blood monocytes [5] and eosinophils [7] from asthmatic patients were able to produce high amounts of ROS. Alveolar macrophage activation was correlated with the severity of asthma [6]. This activation occurred immediately after antigen challenge, and the appearance of high-density macrophages which release higher amounts of superoxide anion occurred during the late airway response [8]. Several investigators have also suggested that polymorphonuclear leucocytes from asthmatic patients generated more ROS than control subjects [9, 10]. This activation was correlated with the level of bronchial hyperreactivity [11].

Corticosteroids are the most potent anti-inflammatory agents available for the treatment of asthma [12], but little is known about their molecular mechanism of action in improving symptoms or the way they reduce hyperresponsiveness. It has been proposed that corticosteroids inhibit the inflammatory process by redirection of lymphocyte traffic, inhibition of cytokine gene expression [13] and inhibition of the expression of adhesion molecules [14].

The effect of anti-inflammatory corticosteroids on oxygen radical production by phagocytes has been less extensively studied, and the results are contradictory. *In vitro* studies have demonstrated an inhibitory effect of high corticosteroid concentrations on oxygen radical production in human granulocytes [15]. In contrast, dexamethasone treatment has been reported to have no effect on ROS secretion in human blood-derived macrophages [16], but have a stimulatory effect on human monocytes [17]. It has been reported that *in vivo* corticosterone had no effect on murine peritoneal inflammatory cells [18]. *Ex vivo* inhaled steroid treatment had no effect on superoxide anion production in human alveolar macrophages [19, 20]. In contrast, a recent study showed that *in vivo* administration of dexamethasone or methylprednisolone led to a dose-dependent inhibition of rat peritoneal leucocyte chemiluminescence [21]. Moreover, hydrocortisone was found to inhibit superoxide anion production during an asthma attack [22].

The present study was carried out to clarify the *ex vivo* effects of corticosteroids on superoxide anion production by blood monocytes from asthmatic patients. We compared superoxide anion production in blood monocytes from untreated asthmatic patients and three groups of asthmatic patients treated with corticosteroids. In the first group, superoxide anion release was assessed before and after short-term therapy with high-dose *i.v.* corticosteroids in order to test the acute effects induced by corticosteroids. The second group included patients receiving long-term therapy with inhaled corticosteroids, and the third group included patients receiving long-term therapy with inhaled and oral corticosteroids (for at least 1 yr) called steroid-dependent asthmatic patients.

Materials and methods

Patients

Twenty seven nonsmoking asthmatic patients, ranging 22–72 yrs of age (mean±SD 48.68±15.13 yrs), were included in this study. Asthma was defined according to the American Thoracic Society [23] criteria, and all patients had a reversible airway disease. Informed consent was obtained from all patients in accordance with the Ethics Committee of the University of Montpellier. Patients who did not present any nocturnal asthma symptoms and who did not use any β_2-agonist were classified as patients with controlled disease.

Nine asthmatic patients had not undergone any treatment (group 1), and demonstrated a 12% increase in their forced expiratory volume in one second (FEV1) after inhalation of 200 μg salbutamol. All these patients were symptomatic, with no anti-inflammatory treatment when entering the study. These patients were treated with a short course of *i.v.* methylprednisolone given over 10 days (80 mg·day^{-1}), and demonstrated at least a 15% improvement in their morning prebronchodilator FEV1. Six patients were seen again after the treatment for evaluation but three were lost to follow-up.

Six asthmatic patients (moderate persistent asthma) included in this study had been treated with 1.5 mg of inhaled beclomethasone dipropionate (BDP) for at least 6 months, along with 100 μg salmeterol (group 2). This dose of inhaled steroid was the minimal dose required to control their symptoms.

Twelve patients with severe steroid-dependent asthma (severe persistent asthma) were included in this study (group 3). They had already been monitored in our clinic for at least 1 yr prior to the start of the present study. They were described as steroid-dependent, since we failed to wean them from corticosteroids in the previous year and they had presented with recurrent threatening episodes of acute asthma during the past 2 yrs. They were treated on a regular basis with prednisone from 10–60 mg·day^{-1} (mean dose: 34.3 mg) and 1,600 μg of inhaled budesonide, 100 μg salmeterol and 10 mg·kg^{-1} of long-acting theophylline, according to recently published guidelines [24].

Preparation of human blood monocytes

Blood monocytes were prepared as previously described [5]. Peripheral blood was recovered by venipuncture using heparin (25 U·mL^{-1}). Cells were isolated by centrifugation of blood samples over isotonic Percoll solutions at densities of 1.097 (Percoll 72%) and 1.086 (Percoll 63%). A 5 mL volume of a 63% Percoll solution was layered over 5 mL of 72% solution in a 15 mL conical tube. Whole blood (5 mL) was layered over the Percoll gradient and then centrifuged at 1,200 × g for 20 min at room temperature, resulting in the formation of one band of mononuclear cells and bands of polymorphonuclear leucocytes (PMN) above the 72% layer.

The mononuclear cell suspension in Percoll was removed and washed once with an equal volume of RPMI 1640 medium. Contaminating erythrocytes were lysed by incubation for 10 min in a lysing buffer solution of 130 mM NH_4Cl, 10 mM Tris buffer, and 16 mM K_2CO_3, pH 7.4, and recovered by centrifugation. Cells were then washed in RPMI 1640 medium and counted in the initial suspension. A total of 10^5 blood monocytes were then incubated in 96-multiwell dishes with 100 μL RPMI 1640, containing antibiotics (L-glutamine, streptomycin and penicillin) and 20% foetal calf serum (FCS), for 2 h at 37°C in a humid atmosphere of 95% air and 5% CO_2 to separate adherent monocytes from suspended lymphocytes. At the end of this incubation period nonadherent cells were then removed.

Superoxide anion generation assay

The superoxide anion concentration produced by adherent blood monocytes was quantified using a spectrophotometric method based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C, adapted for the 96-well microtitre assay, according to JONHSTON *et al.* [5]. Ferricytochrome C, SOD, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Company (St Louis, MO, USA). Each well contained 10^5 cells, and 80 μmol of ferricytochrome C in phosphate-buffered saline (PBS) (with Ca^{2+}/Mg^{2+}). Superoxide anion production was measured under two conditions, spontaneously and after PMA stimulation for 90 min. PMA was freshly prepared by diluting a stock solution in PBS to a final concentration of 100 ng·mL^{-1}. Paired wells were established with and without SOD (30 μg·mL^{-1} final concentration) to assess the specificity of cytochrome C reduction as control. Optical density was then determined with absorbance at 550 nm for 90 min, with the results expressed in nmol. Each reaction was run in quadruplicate and measurements performed at 10, 15, 30, 45, 60 and 90 min. The stimulatability coefficient was defined as the ratio of PMA-stimulated/nonstimulated superoxide anion production at 60 min after stimulation [9].

Statistical analysis

Untreated asthmatics and three groups of treated patients were compared using the analysis of variance (ANOVA), and the Mann-Whitney U-test was used for unpaired data. The Wilcoxon W-test was used to compare superoxide anion levels in patients before and after short-term methyl prednisolone treatment. The kinetic study results are ex-

Table 1. – Characteristics of asthmatic patients and stimulatability coefficient in the superoxide anion release at 60 min

Asthmatics	Sex M/F	Age yrs	FEV1 predicted	Treatment	CS dose mg·day^{-1}	Stimulatability coefficient mean (25–75% percentiles)
Group 1: Untreated	3/6	51.3±16.4	68.1±10.4	β$_2$ as required	-	60 (3.5–8.5)
after short-term treatment	3/3	50.0±17.8	90.8±7.8[+]	i.v. CS	80	3.5 (1.0–4.9)
Group 2: Inhaled-steroids	2/4	56.7±14.1	83.3±13.4[‡]	Inh CS	1.5	3.0 (1.0–4.7)
Group 3: Steroid-dependent	7/5	45.6±16.7	51.4±22.1[†]	Inh + oral CS	34.3±13.1	6.9 (1.9–17.7)

Values are expressed as mean±SD. M: male; F: female; FEV1: forced expiratory volume in one second. CS: corticosteroid; Inh: inhaled. [+]: p=0.03 significant difference between FEV1 before and after treatment (Wilcoxon W-test); [‡]: p=0.003 significant different in FEV1 between steroid-dependent and inhaled treatment (Mann-Whitney test); [†]: p<0.02 significant difference in FEV1 between untreated and inhaled treatment (Mann-Whitney U-test).

pressed as mean, and the time point analysis results expressed as median with a 25–75th percentile.

Results

Patients

The demographic characteristics of the groups of asthmatic patients are summarized in table 1.

In group 1, untreated asthmatic patients (sex matched), with a mean±SD age of 51.3±16.4 yrs and with an FEV1± SD of 68.1±10.4 % of pred value were classified as uncontrolled asthmatics requiring a course of i.v. corticosteroids. After 10 days of administration of 80 mg methylprednisolone, symptoms had improved and FEV1 increased significantly in all patients, being 90.8±7.8 % pred (p<0.05 (Wilcoxon W-test)).

In group 2, patients with a mean±SD age of 56.7±14.1 yrs were classified with moderate persistent asthma (sex matched), requiring long-term treatment with inhaled steroids. They were in a steady state with a mean±SD FEV1 of 83.3±13.4 % pred, and significantly different from untreated patients (p<0.02 (Mann-Whitney U-test)).

Group 3 included steroid-dependent asthmatics with severe persistent asthma (sex matched), with a mean±SD of 45.6±16.7 yrs, requiring long-term therapy with oral steroids. Their mean±SD FEV1 51.4±22.1 % pred was significantly different (p=0.003) from patients undergoing long-term treatment with inhaled steroids. The oral corticosteroid dose was the minimal dose required to improve their symp-toms, i.e. a mean of 34.3±13.1 mg per day.

Forty per cent of the patients enrolled in this study were allergic, as assessed by at least three skin-prick tests positive to common allergens.

Superoxide anion production analysis

Time-kinetic studies. Time-kinetic studies were performed for up to 90 min, and superoxide anion accumulation was measured on unstimulated and PMA-stimulated blood monocytes.

For unstimulated cells, the time-kinetic curves for superoxide anion production by blood monocytes from untreated asthmatics and the three groups of treated asthmatic patients did not show any significant differences (fig. 1). However, a nonsignificant decrease in spontaneous superoxide anion production in blood monocytes was
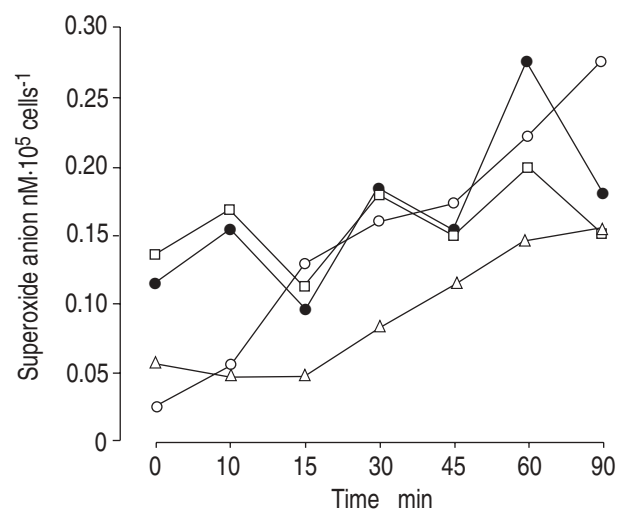

Fig. 1. – Kinetic curves for spontaneous superoxide anion production by blood monocytes. ● : untreated; ○ : short-term treatment; ▢ : steroid-dependent asthmatics; △ : patients treated with inhaled steroids. Each symbols represents the mean of four reactions performed simultaneously.

observed in asthmatic patients treated with inhaled steroids.

For PMA-stimulated cells, the time-kinetic curve for blood monocytes of untreated asthmatic patients was higher than for the three groups of treated asthmatic patients, with significant differences in superoxide anion production from 10–90 min for steroid-dependent asthmatic patients, from 15–90 min for inhaled treatment asthmatic patients, and from 30–90 min for short-term-treated asthmatic patients (fig. 2). No differences were observed in the shape of the four curves, but the slopes were very different between the treated groups (slope coefficients: 0.005 for steroid-dependent, 0.006 for inhalation treated, 0.007 for short-term-treated patients and 0.013 for the untreated group).

Time point analysis. For untreated asthmatic patients, maximum release was obtained at 60 min. This time was chosen to study individual data for all four populations of asthmatic patients. The ratio of PMA-stimulated/spontaneous superoxide anion production from blood monocytes was calculated for each individual result at 60 min and represents the stimulatability coefficient (table 1).

No significant difference was observed for spontaneous production, despite a lower release for all three popula-
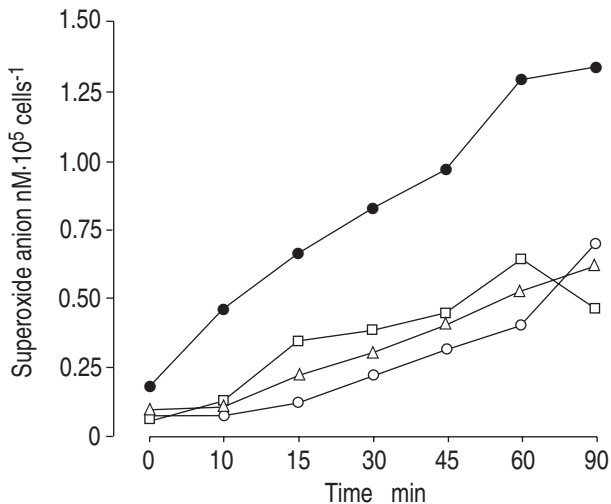
Fig. 2. – Kinetic curves for phorbol 12-myristate 13-acetate (PMA)-stimulated superoxide anion production by blood monocytes. ● : untreated; ○ : short-term treatment; □ : steroid-dependent asthmatics; △ : patients treated with inhaled steroids. Each symbol represents the mean of four reactions performed simultaneously.

Fig. 3. – Phorbol 12-myristate 13-acetate-stimulated superoxide anion production by blood monocytes in untreated, short-term treatment, steroid-dependent asthmatics and patients treated with inhaled steroids after: a) 60 min stimulation; and b) before and after treatment. Each symbols represents superoxide anion production in an individual subject, and the measurements are the means of quadruplicate determinations. U: untreated; STT: short-term treatment; IS: inhaled steroids; SD: steroid dependent; *: $p < 0.05$; **: $p < 0.01$.

tions of treated asthmatic patients as compared to untreated patients.

Concerning PMA-stimulated production, the three populations of treated asthmatic patients showed a significantly lower superoxide anion production by blood monocytes as compared to untreated asthmatic patients (fig. 3). Significant differences were observed using the Mann-Whitney U-test between untreated asthmatics and those treated with inhaled steroid ($p < 0.01$) and those who were steroid-dependent ($p < 0.02$) and using the Wilcoxon W-test between asthmatic patients before and after treatment ($p < 0.05$).

Except for steroid-dependent asthmatic patients, a significant difference in superoxide anion release by blood monocytes was observed using the Mann-Whitney U-test between untreated (uncontrolled) and short-term treated asthmatic patients and those treated with inhaled steroids (controlled) ($p < 0.02$) (fig. 4).

The stimulatability coefficient was identical for untreated asthmatic patients and patients treated with inhaled
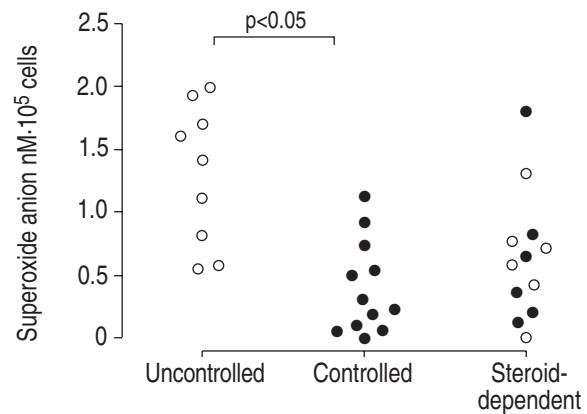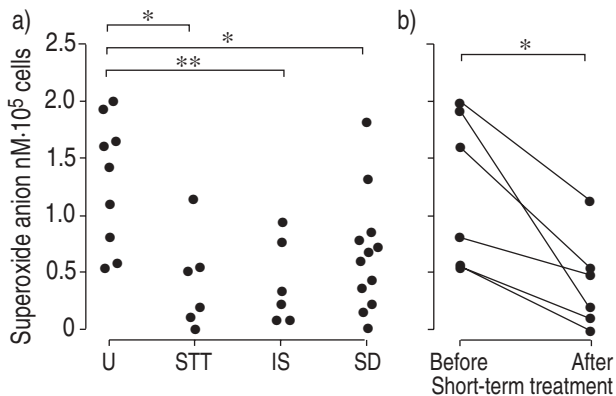

Fig. 4. – Phorbol 12-myristate 13-acetate-stimulated superoxide anion production by blood monocytes from untreated (uncontrolled), short-term treatment and patients treated with inhaled steroids (controlled), and steroid-dependent asthmatics after 60 min stimulation. Each symbol represents superoxide anion production in an individual subject, and the measurements are the means of quadruplicate determinations.

steroids, with no significant difference between long-term oral and short-term *i.v.* treatment of asthmatic patients.

Discussion

This study demonstrated that long-term treatment with oral or inhaled corticosteroids and short-term treatment with high doses of corticosteroids showed reduced PMA-stimulated superoxide anion production by blood monocytes from asthmatic patients as compared to untreated asthmatic patients. Spontaneous superoxide anion production was identical in untreated and treated asthmatics and was not significantly decreased in blood monocytes from inhalation treated asthmatic patients. The shapes of the kinetic curves for PMA-stimulated blood monocytes were all similar, with increasing amounts of superoxide anion production until 60 min. A plateau was then reached for untreated asthmatic patients. Consequently, a period of 60 min was chosen to analyse individual time point levels of superoxide anion production in all groups of asthmatic patients. This analysis showed a significant decrease in superoxide anion production by blood monocytes from all treated asthmatics as compared to untreated patients. It has been demonstrated that the PMA concentration used (100 ng·mL^{-1}) remained the optimal concentration in steroid-dependent asthmatic patients, without a shift in the dose-response curve (data not shown).

In previous work, it was observed that cells from controlled asthmatic patients produced significantly lower amounts of superoxide anion than uncontrolled ones [9]. In the present study, all untreated asthmatic patients were included on the basis of their instability, characterized by nocturnal asthma symptoms and high β_2-agonist usage. These patients were subjected to short-term treatment with a high dose of corticosteroids to rapidly improve their asthma symptoms, with a minimum 15% increase in their FEV1 after 10 days of therapy. After this treatment, all patients had improved symptoms, and all had a significantly decreased superoxide anion production by blood monocytes. These results could be correlated with other studies investigating the effects of corticosteroids on bronchial responsiveness [25].

The anti-inflammatory effects of corticosteroids on ROS inhibition in various cell types are controversial [21]. *In vitro* studies have suggested that the anti-inflammatory action of corticosteroids depends at least partially on an interference with the formation of reactive oxygen species by phagocytes [26].

SZEFLER *et al.* [27] showed that when human monocytes from normal subjects were treated with hydrocortisone *in vitro*, there was a significant inhibition of superoxide anion release over 32 h. Moreover, prednisone and progesterone which are non-active corticosteroids, had no effect on this production. To our knowledge, no studies have been performed to analyse superoxide anion production from steroid-dependent asthmatic patients treated with the minimum dose of corticosteroids required in corticosteroid therapy. Our results show that superoxide anion release by blood monocytes, from steroid-dependent asthmatics or patients undergoing long-term treatment with inhaled steroids after PMA-stimulation, are quite similar to those obtained after short-term treatment with a high dose of methylprednisolone. From patients who had inhaled steroids, there was a decrease in superoxide anion production by blood cells, suggesting that they have a systemic effect or that they activate a molecule which affects monocytes progenitors in the bone marrow. However, we did not observe the same inhibitory effects of corticosteroids in patients who had been clinically well controlled with their treatment (short-term *i.v.* corticosteroids, or long-term inhaled corticosteroids) and in steroid-dependent asthmatic patients who were clinically different. Indeed, we did not observe a correlation between FEV_1 and the release of superoxide anion by blood monocytes in steroid-dependent asthmatic patients (data not shown). These patients, who had been monitored in the department for at least 1 yr, presented with a different clinical pattern [28], and respond differently to their treatment on a long-term basis.

Interestingly, the coefficient of stimulatability for asthmatic patients with inhaled steroid treatment was the same as that obtained for untreated patients, due to the fact that the spontaneous release of superoxide anion was very low. In a previous study, we observed that in control subjects blood monocytes were not able to release any detectable amounts of superoxide anion [5]. The weak release of superoxide anion by blood monocytes from patients treated with inhaled steroids, and the fact that they were very well controlled by their treatment, suggests that superoxide anion release may reflect good control of the disease.

It is well known that anti-inflammatory effects of corticosteroids are partly mediated by the inhibition of arachidonic acid, induced by the synthesis and release of phospholipase-A2 inhibitors such as lipocortin-1 [29]. However, it is not yet clear whether corticosteroids suppress superoxide anion production by a phospholipase-dependent [30] or independent [31] mechanism. The corticosteroid effects are due to a specific interaction with the glucocorticoid receptor, and the complex hormone-receptor is able to modulate the transcription of corticosteroid-responsive genes. Activation of respiratory burst involves several steps and can be induced by a variety of stimuli. The phorbol ester PMA binds and activates protein kinase C which is then translocated to the membrane [32]. The responses to the common three stimulators used to stimulate superoxide anion production (PMA, N-formyl-methionyl leucyl phenylalanine (fMLP) and opsonized zymosan) were affected similarly by corticosteroids [21]. This suggests that corticosteroids commonly affect components in the activation pathway, possibly protein kinase C or NADPH oxidase. A direct effect of dexamethasone on phagocytes has been demonstrated *in vitro*, leading to a dose-dependent reduction of messenger ribonucleic acid coding for the NADPH oxidase component [33]. Furthermore, corticosteroids may influence oxygen-free radicals by scavenging reactive molecules directly as superoxide dismutase, catalase or gluthation peroxidase. Corticosteroids, in addition to the suppression of reactive oxygen species production, may thus also raise intracellular levels of their scavengers [34].

In conclusion, it is interesting to note that corticosteroids administered in three different ways inhibits superoxide anion release by blood monocytes in asthmatic patients. Inhaled steroids are able to inhibit superoxide anion release by blood monocytes, suggesting an action of these inhaled steroids outside of the lungs.

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