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Influence of *in vivo* prednisolone on increased *in vitro* O_2^- generation by neutrophils in emphysema

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Influence of in vivo *prednisolone on increased* in vitro *O_2^- generation by neutrophils in emphysema. T.E.J. Renkema, D.S. Postma, J.A. Noordhoek, H.J. Sluiter, H.F. Kauffman.*

ABSTRACT: Evidence is accumulating that neutrophil-derived oxidants substantially contribute to the development of emphysema, especially in smoking individuals. It is not clear, however, why not all smokers develop emphysema.

We tested the hypothesis that an abnormality in the oxidative metabolism of polymorphonuclear leucocytes (PMNs) might contribute to the development of emphysema. We investigated *in vitro* O_2^- production by peripheral PMNs in patients with stable emphysema and in healthy controls. In addition, we investigated whether *in vivo* prednisolone may modulate *in vitro* O_2^- production by PMNs in patients with emphysema during a stable phase of the disease.

Spontaneous O_2^- production by PMNs was not significantly different in patients and controls. After stimulation with submaximal concentrations of calcium ionophore A23187 and phorbol myristate acetate, however, PMNs from patients with stable emphysema produced more O_2^- than those from healthy controls, especially in smoking subjects. Moreover, *in vitro* O_2^- generation by PMNs significantly decreased after *in vivo* prednisolone treatment in patients with emphysema.

We suggest that our findings reflect an abnormality of PMNs, acting as one of the factors that contribute to the development of emphysema. This abnormality may, at least partially, be dampened by *in vivo* prednisolone treatment. These findings may provide new insights into the pathogenesis and treatment of pulmonary emphysema. Further studies on pulmonary PMNs are necessary to extend our findings.

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It is generally accepted that pulmonary emphysema results from a long-term imbalance between proteases, especially neutrophil elastase, and antiproteases in lung tissue [1]. In patients with normal serum α_1-antitrypsin (α_1-AT) levels, the precise mechanisms underlying this imbalance are unknown.

There is no doubt that emphysema is related to cigarette smoking. Cigarette smoke is rich in highly reactive oxidizing radicals. Smokers' lungs contain increased numbers of macrophages and neutrophils, suggesting an increased burden of proteolytic and oxidative activity [2, 3]. Evidence is accumulating that oxidant-mediated injury substantially contributes to the development of emphysema. Oxidants are thought to disturb the protease-antiprotease balance through oxidative inactivation of α_1-AT [1] and antileucoprotease (ALP) [4], and possibly through activation of latent proteolytic enzymes [5, 6]. In addition, they may cause direct injury to lung tissue components [7–10]. Despite these findings, it is still unclear why not all smokers develop emphysema.

In an earlier study, we were unable to demonstrate an increased release of elastase by polymorphonuclear leucocytes (PMNs) in patients with emphysema [11]. In this study, we put forward the hypothesis that an abnormality in the oxidative metabolism of PMNs may contribute to the development of emphysema. Therefore, we investigated superoxide anion production *in vitro* by PMNs from patients with emphysema and healthy controls.

Systemic corticosteroids are widely used in the treatment of patients with obstructive airway disease. A beneficial long-term effect of corticosteroids on lung function has been suggested by two studies in patients with chronic airflow obstruction and clinical signs of emphysema [12, 13]. The mechanisms underlying this favourable influence of corticosteroids are unknown. *In vitro* studies have suggested that the anti-inflammatory action of corticosteroids depends, at least partially, on interference with formation of reactive oxygen species by phagocytes [14, 15]. Therefore, we also investigated whether

oral prednisolone may modulate superoxide anion production by PMNs in patients with stable emphysema.

Patients and methods

Patients and healthy controls

Fifty seven male patients with emphysema (25 smokers and 32 ex-smokers) participated in the study. Selection criteria were:

1. Clinical diagnosis of pulmonary emphysema according to history (persistent dyspnoea, mainly on exertion, without sudden attacks of dyspnoea), physical examination (hyperresonant percussion of the chest with decreased intensity of breath sounds) and chest radiography (flat and/or low diaphragm, increased retrosternal space, vessel narrowing or loss) [16].
2. Forced expiratory volume in one second (FEV_1) <80% of predicted value.
3. Residual volume (RV) >100% of the predicted value.
4. Specific compliance expressed as a percentage of the predicted value (Csp % pred) greater than 100% after bronchodilation; when, however, air trapping (calculated as thoracic gas volume measured by body plethysmography minus functional residual capacity measured by an indicator gas) [17] was greater than 1.5 *l*, Csp was allowed to be smaller than 100% of predicted.
5. No signs of allergy (negative skin tests, total immunoglobulin E (IgE) <200 IU, eosinophils in peripheral blood <250 cells·mm^{-3}).
6. Stable phase of the disease, *i.e.* no exacerbation for at least 3 months.

All patients used anticholinergics, beta-mimetics, theophylline, or a combination of these drugs as maintenance therapy, without oral or inhaled corticosteroids for at least 3 months before entry into the study. All patients had serum α_1-AT levels within the normal range. Smoking history was expressed as pack years (average number of packs of 25 cigarettes smoked per day × number of years of smoking). All ex-smokers had stopped smoking at least one year before entering the study, except for one patient who had stopped 4 months previously. A group of healthy volunteers, matched for age, sex, and smoking habits, was taken as a control group. No subject had signs or symptoms of cough, sputum production or dyspnoea. None had airflow obstruction as measured by spirometry. All had a normal chest radiograph. All participants gave informed consent. The study protocol was approved by the hospital Medical Ethics Committee.

Isolation procedure

PMNs were isolated from peripheral venous blood, essentially according to the method of BÖYUM [18]. Details of the procedure have been described previously [11]. Briefly, 50 ml of heparinized venous blood were diluted 1:1 with saline solution and layered on Ficoll-Paque (Pharmacia Fine Chemicals). After centrifugation (20 min, 2,600 rpm) the mononuclear cell layer was removed. Erythrocytes were removed from the PMN containing fraction by dextran (Pharmacia Fine Chemicals) sedimentation and Percoll (Pharmacia Fine Chemicals) centrifugation (20 min, 2,600 rpm). Finally, the cells were resuspended in Hank's Balanced Salt Solution (HBSS) + 0.1% glucose. Cytocentrifuge smears were made and differential counts were performed after staining with May-Grünwald-Giemsa. Contamination with lymphocytes and monocytes by this method was always less than 5%. Viability as tested by trypan blue exclusion was always higher than 95%. All reagents used for PMN isolation were free of endotoxin. The isolation procedure was always performed over roughly the same time period.

Superoxide generation

Superoxide anion production was measured as the superoxide dismutase (SOD) inhibitable reduction of cytochrome C (cyt C, 3). Details of the procedure have been described previously [19]. Briefly, 0.4 × 10^6 PMN were incubated with cyt C solution (with or without SOD, respectively) and a soluble stimulus in polystyrene test tubes. The following stimuli were used: 1) phorbol myristate acetate (PMA) (Sigma), at concentrations of 5, 10, and 20 ng·ml^{-1}, 2) calcium ionophore A23187 (Sigma), at concentrations of 1 and 5 μM; 3) N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Sigma), at concentrations of 0.2 and 2 μM.

Spontaneous reduction by PMNs was measured by including test tubes without stimulus. Total reduction was checked using a saturated sodium hydrosulphite solution (Sigma). After incubation in a water bath (37°C), the mixtures were centrifuged to stop the reaction. The optical density (OD) of the supernatants was measured spectrophotometrically (Model 25, Beckman Instruments Inc.) at 550 nm. SOD inhibitable reduction of cyt C was calculated using an extinction coefficient of 29.5 × 10^3 M^{-1} cm^{-1} (Biochemicals, Organic Compounds for Research, and Diagnostic Reagents, Sigma Chemical Co., 1990). The results are expressed as nmol cyt C reduced in 15 min per 0.4 × 10^6 PMN.

Study protocol

Superoxide anion generation was measured in healthy subjects, in patients with emphysema before prednisolone treatment, and in the same patients after prednisolone treatment. Prednisolone was given orally, 40 mg *q.d.*, for 8 days in a double-blind, randomized, placebo-controlled, cross-over design. Both placebo

and prednisolone periods were always preceded by a 3 month period without any corticosteroids.

Statistics

Data analysis was performed on a personal computer utilizing SPSS/PC+ programs. Differences between patient and control groups were analysed using two-way analysis of variance (ANOVA), with superoxide anion production as dependent variable and pulmonary condition ("healthy" or "emphysema") and smoking habits ("smoker" or "ex-smoker") as independent variables. Differences before and after prednisolone treatment in the patient groups were analysed with the Student's t-test for paired observations. Significance levels were set at 5%. Values are presented as mean±SEM.

Results

Characteristics of the patients and healthy controls

Clinical characteristics of the patients with emphysema and the healthy controls are shown in table 1. The group of healthy ex-smokers had fewer pack years of smoking than the healthy smokers and the patients with emphysema. White blood cell count (WBC) was significantly higher in smokers than in ex-smokers, in patients as well as in controls. Spirometric values of the controls were in the normal range and significantly higher than the corresponding values in the patient group ($p<0.05$). Prednisolone treatment induced a significant increase in WBC, both in smoking and in ex-smoking patients (values after treatment being 9.28±0.67 and 7.51±0.44 $\times 10^9$ cells·l^{-1} in smokers and ex-smokers, respectively).

Superoxide anion production

Superoxide anion production by PMNs in healthy subjects and patients with emphysema before and after prednisolone treatment is shown in figure 1. Comparing the results in the control group with those obtained in the patient group before prednisolone treatment, spontaneous O_2^- production ("vehicle" in figure 1) was not significantly different in healthy subjects and patients with emphysema. After stimulation with A23187 and PMA, however, O_2^- generation tended to be higher in patients with emphysema than in healthy controls. Differences reached statistical significance after stimulation with A23187 1 μM (values being 3.9±0.5 and 2.7±0.3 nmol cyt C in healthy ex-smokers and healthy smokers, and 4.9±0.4 and 5.4±0.5 nmol cyt C in emphysematous ex-smokers and emphysematous smokers, respectively, $p<0.01$), PMA 5 ng·ml^{-1} (8.4±1.0 and 7.2±1.7, and 12.3±1.3 and 12.4±1.7 nmol cyt C, $p<0.05$), and PMA 10 ng·ml^{-1} (12.4±1.2 and 8.5±1.5, and 18.6±1.5 and 16.9±1.6 nmol cyt C, $p<0.001$). On stimulation with FMLP, no significant differences in O_2^- generation were found between both groups (data not shown). The observed differences were more pronouced in smokers than in ex-smokers. Within both groups, however, no significant differences in (spontaneous or stimulated) O_2^- production existed between smoking and ex-smoking individuals.

Table 1. – Characteristics of the emphysematous patients and the healthy controls

	Emphysematous patients		Healthy controls	
	Ex-smokers	Smokers	Ex-smokers	Smokers
n	32	25	12	9
Age yrs	56±2	55±2	55±2	53±2
Smoking history pack yrs	24.6±3.7	32.2±3.2	12.7±2.9†	23.8±4.3
WBC $\times 10^9$ cells·l^{-1}	5.92±0.26	7.66±0.43*	5.48±0.49	7.66±0.84*
FEV_1 l	2.03±0.11	1.92±0.11	3.58±0.20	3.62±0.21
FEV_1 % pred	63±3	62±4	115±5	112±5
FIV_1 % pred	98±2	99±3	117±7	126±13
FEV_1 % VC %	45±2	44±2	72±2	75±2
TLC % pred	110±3	115±3	-	-
RV % pred	149±7	168±7	-	-
Csp % pred	110±7	116±8	-	-
Air trapping l	1.46±0.15	1.40±0.27	-	-
PC_{20} histamine mg·ml^{-1}	5.75	4.68	-	-

All values are given as mean±SEM, except PC_{20} histamine which is geometric mean. *: significantly higher ($p<0.05$) in smokers than in ex-smokers; †: significantly smaller ($p<0.05$) as compared with emphysematous smokers. FEV_1: forced expiratory volume in one second; % pred: expressed as a percentage of the predicted value; FIV_1: forced inspiratory volume in one second; FEV_1%VC: FEV_1 expressed as a percentage of slow inspiratory vital capacity; WBC: white blood cell count; TLC: total lung capacity; RV: residual volume; Csp: specific compliance (after bronchodilation); PC_{20} histamine: provocative concentration of histamine causing a 20% fall of FEV_1 from baseline after inhalation during 30 s.

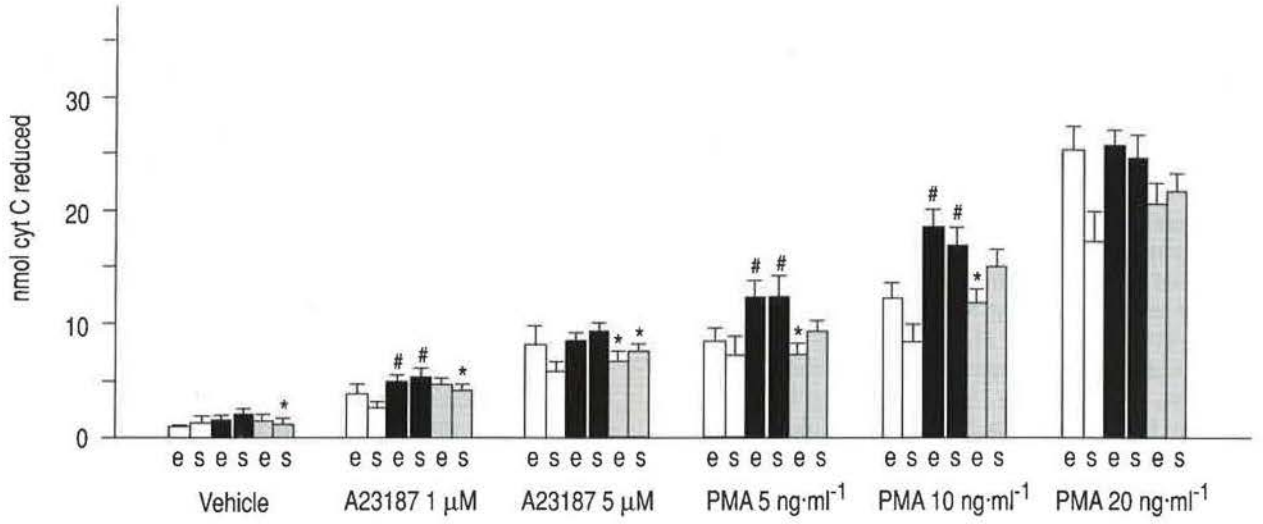

Fig. 1. – Mean (±SEM) superoxide anion production (nmol cytochrome C reduced in 15 min per 0.4 × 10^6 cells) by PMNs in healthy subjects (□), in patients with emphysema before prednisolone treatment (■), and in patients with emphysema after prednisolone treatment (▨). In all three groups, "s" are smokers and "e" are ex-smokers. #: significantly different ($p<0.05$) in patients with emphysema before prednisolone treatment as compared with healthy subjects; *: significantly different ($p<0.05$) in patients with emphysema after prednisolone treatment as compared with the same subgroup of patients before treatment. PMNs: polymorphonuclear leucocytes; PMA: phorbol myristate acetate.

Superoxide anion production by unstimulated as well as stimulated PMNs was not significantly correlated with pack years of smoking in patients with emphysema and healthy subjects, or in current smokers, with actual daily tobacco consumption. In the patient group, no significant correlation was found between superoxide anion production and degree or severity of emphysema, as measured by FEV_1 % pred, FEV_1 expressed as percentage of slow inspiratory vital capacity (FEV_1 % VC), total lung capacity (TLC) % pred, residual volume (RV) % pred, Csp % pred, or air trapping.

Comparing the results in emphysematous patients before prednisolone treatment with those obtained after prednisolone treatment, spontaneous O_2^- production was not significantly different before and after treatment in ex-smoking patients; in smoking patients, however, spontaneous neutrophil O_2^- generation after prednisolone treatment (1.2±0.3 nmol cyt C) was significantly lower than spontaneous O_2^- generation by PMNs in the same patients before treatment (1.9±0.4 nmol cyt C, $p<0.05$). O_2^- production by PMNs stimulated with A23187 and PMA tended to be lower after prednisolone treatment as compared with the corresponding values obtained before treatment, both in smoking and ex-smoking patients. For ex-smoking patients, differences reached statistical significance after stimulation with A23187 5 μM (values being 8.4±0.5 and 6.6±0.8 nmol cyt C before and after prednisolone treatment, respectively, $p<0.05$), PMA 5 ng·ml^{-1} (12.3±1.3 and 7.5±0.6 nmol cyt C, $p<0.05$), and PMA 10 ng·ml^{-1} (18.6±1.5 and 11.8±1.3 nmol cyt C, $p<0.05$), and for smoking patients after stimulation with A23187 1 μM (5.4±0.5 and 4.1±0.4 nmol cyt C before and after treatment, respectively, $p<0.05$) and A23187 5 μM (9.2±0.7 and 7.5±0.7 nmol cyt C, $p<0.01$). FMLP-induced O_2^- generation was not significantly different before and after prednisolone treatment, either in smoking or in ex-smoking patients (data not shown). The observed differences in O_2^- production between smoking and ex-smoking patients after prednisolone treatment were, just as before treatment, not significantly different.

Discussion

In this study, we investigated *in vitro* O_2^- generation as a measure of oxygen radical production by PMNs in patients with emphysema and healthy controls. Our results demonstrate an increased superoxide anion generation after submaximal stimulation of PMNs in patients with emphysema as compared to healthy controls. Moreover, *in vitro* O_2^- generation by PMNs from patients with emphysema significantly decreased after prednisolone treatment *in vivo*.

Several investigators have shown an enhanced O_2^- production by stimulated peripheral PMNs in patients with asthma as compared to normal control subjects [20, 21]. In this study, we have demonstrated an increased O_2^- generation by peripheral PMNs in patients with emphysema. Increased neutrophil oxidative metabolism suggests an increased oxidant burden in the lungs of these patients. It has to be noted, that we studied an *in vitro* model using peripheral blood PMNs. These cells may behave differently from PMNs in lung tissue, due to differences in environmental factors. KELLY *et al.* [22], however, observed an increased release of reactive oxygen species by pulmonary PMNs obtained by bronchoalveolar lavage in patients with asthma, suggesting similar behaviour of peripheral PMNs and pulmonary PMNs in

these patients. It is not inconceivable that the same phenomenon exists in patients with emphysema. Further studies with pulmonary PMNs in patients with emphysema are necessary to investigate this hypothesis.

The observed differences in neutrophil O_2^- generation between healthy controls and patients with emphysema were more pronounced in smokers than in ex-smokers. It is important to note, however, that we did not find significant differences in O_2^- generation by PMNs in smoking and ex-smoking individuals. Similar findings are reported by KELLY *et al.* [22]. We believe this indicates that the differences that we observed are not related to, or caused by, actual smoking habits. Emphysema is undoubtedly related to smoking, but the fact that only a relatively small percentage of smokers develop disabling airflow obstruction [2] suggests that cigarette smoke alone does not cause emphysema but acts as a co-determinant, allowing or amplifying emphysema caused by other factors. We suggest that the increased O_2^- generation by PMNs in patients with emphysema may be one of these factors. Although the observed differences in O_2^- production are small, they may, nevertheless, be relevant, as emphysema is a slowly progressive disease in which small but ongoing disturbance of the normal balance between proteases and antiproteases and/or oxidants and antioxidants may be important.

From our results, it is not clear where the proposed abnormality in PMNs from patients with emphysema is located. Investigation of the signal-transduction systems involved is needed to clarify this aspect. The observed differences in O_2^- generation between patients and normals were most pronounced upon stimulation with submaximal concentrations of PMA and A23187. These results, in accordance with findings in asthmatic patients [21], suggest that the maximal capacity of PMNs for generation of oxygen metabolites is not abnormal in patients with emphysema, but that PMNs of these patients have an increased responsiveness to low levels of stimulation. The absence of differences in O_2^- generation upon stimulation with FMLP is probably due to the fact that the FMLP concentrations that we used were not submaximal, as they both induced O_2^- production in the same range as the highest PMA concentration that we used.

Healthy controls were matched for age and smoking habits. A factor that could not be controlled, however, was drug therapy. All patients used anticholinergics, beta-mimetics, theophylline, or a combination of these drugs as maintenance therapy. Evidence from the literature [23, 24] suggests that if there is an influence of these drugs on neutrophil function, it is a suppressive one, thus, this only strengthens our findings.

Decreased O_2^- generation by PMNs after incubation with corticosteroids *in vitro* has been reported by several investigators [8, 9]. To our knowledge, we are the first to describe decreased O_2^- generation *in vitro* by PMNs after oral prednisolone treatment in patients with stable emphysema. Treatment with corticosteroids induces an elevation of the number of neutrophils in the circulation. This phenomenon is partly due to an increased production of neutrophils by the bone marrow, which is reflected by an increase in the number of immature PMNs in the circulation. It has been shown that oxygen radical production is a late manifestation of neutrophil functional maturation [25]. Although morphological differentiation of the isolated PMN suspensions in our experiments showed no increase in the number of immature cells after prednisolone treatment, we cannot, with certainty, exclude the possibility that a PMN population shift contributes to the observed favourable influence of prednisolone on O_2^- production.

In earlier studies [12, 13] in patients with chronic airflow obstruction and clinical signs of emphysema, we observed that a daily dosage of at least 10 mg prednisolone delayed the decline in FEV_1. In the light of these observations, and the potential importance of neutrophil-derived oxidants in the development of emphysema, our present findings suggest a beneficial influence of corticosteroid treatment in patients with emphysema, which may depend, at least partially, on a reduction of O_2^- generation by PMNs. We treated our patients with a daily dose of 40 mg prednisolone [26]. Because of the current knowledge of the side-effects of prednisolone, it is not a daily practice to treat patients with chronic airflow obstruction using high doses of corticosteroids. A long-term prospective study in progress in our department should reveal whether dose reduction or the use of inhaled corticosteroids will have a similar beneficial effect.

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