Effects of an inhaled corticosteroid, budesonide, on alveolar macrophage function in smokers

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Abstract
Selected functions of alveolar macrophages obtained by bronchoalveolar lavage of 12 healthy smokers were examined before and after eight weeks' treatment with an inhaled glucocorticosteroid, budesonide (400 µg twice daily). After budesonide treatment spontaneous as well as opsonised zymosan triggered prostaglandin E₂ (PGE₂) secretion from harvested cells was reduced; no such reduction in opsonised zymosan triggered leukotriene B₄ (LTB₄) production was observed. Neither the capacity to phagocytose opsonised yeast particles nor the superoxide radical generation triggered by the calcium ionophore A23187, 4α-phorbol 12-myristate 13-acetate (PMA), or opsonised zymosan ex vivo were more than marginally affected by the glucocorticosteroid treatment in vivo. Lavage fluid concentrations of angiotensin converting enzyme (ACE), however, after treatment were twice those before treatment and concentrations of fibronectin were reduced to half. Albumin concentrations in lavage fluid were not affected by the glucocorticosteroid treatment. In separate experiments treatment of alveolar macrophages with 10⁻⁷ or 10⁻⁸ M budesonide overnight in vitro did not affect their superoxide radical or PGE₂ generation but significantly blocked LTB₄ release. These data indicate that inhaled glucocorticosteroid treatment may affect synthesis or release (or both) of ACE and fibronectin by alveolar macrophages from healthy smokers whereas other functions of these cells, such as the generation of reactive oxygen derived products ex vivo, are only marginally affected.

Studies using the technique of bronchoalveolar lavage have shown smoking to be associated with a substantial increase in alveolar macrophages in the lower respiratory tract. Although these cells serve as a first line of defence, they are also capable of contributing to the tissue injury induced by chronic smoking. They can release several potentially harmful proteolytic enzymes and various reactive oxidants, and smoking primes them for increased oxygen radical generation after stimulation in vitro. On the other hand, smoking is reported to reduce the phagocytic activity of alveolar macrophages as well as the stimulus triggered production of arachidonic acid metabolites, including prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄).

The mechanism or mechanisms for the anti-inflammatory activity of glucocorticosteroids remains elusive; the induction of the synthesis and secretion of putative phospholipase A₂ inhibitor proteins such as lipocortin is one possible mechanism of action. The aim of the present study was to examine whether treatment in vivo with inhaled budesonide, a potent glucocorticosteroid suitable for topical treatment, could influence alveolar macrophage function. The latter was assessed ex vivo by evaluating the capacity of these cells to generate oxygen derived reactive species, to secrete LTB₄ and PGE₂, and to phagocytose particles. In addition, angiotensin converting enzyme (ACE) and fibronectin, putative markers of alveolar macrophage activity, as well as albumin were analysed in lavage fluid harvested before and after treatment with budesonide in an attempt to monitor alveolar macrophage function in situ. We also examined whether overnight culture in vitro with budesonide could influence the capacity of alveolar macrophages obtained by bronchoalveolar lavage from other subjects, to generate superoxide radicals or arachidonic acid metabolites after appropriate triggering.

Methods
SUBJECTS AND DRUG TREATMENT
Bronchoalveolar lavage' was performed on 12 healthy smokers (three male) with a mean age of 28 (SEM 2) years and a mean cigarette consumption of 12 (2) pack years. All 12 smokers had a normal chest radiograph.

Six individuals had a productive cough in the morning; the remaining subjects had no respiratory symptoms. Forced expiratory volume in one second (FEV₁) and forced vital capacity (FVC) were measured by wedge spirometer (Vitalograph) and the best of three values was used. Lung function was measured before (baseline) and 15 minutes after inhalation of 2.5 mg terbutaline sulphate from a metered dose inhaler connected to a pear shaped plastic tube (Nebulator, Draco, Stockholm).

After the first lavage the patients received inhaled budesonide (Pulmicort, Draco), 400 µg twice daily, for eight weeks. One and a half hours after the final dose a second lavage was performed.

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Lavage cells were also collected for in vitro studies from 24 different smokers and four non-smoking individuals undergoing bronchoalveolar lavage for other purposes. The smokers included healthy subjects and patients with allergic alveolitis, asthma, chronic bronchitis, and sarcoidosis; one of the non-smoking subjects had sarcoidosis. All subjects gave their informed consent and the study had the approval of the local ethical committee.

BRONCHOALVEOLAR LAVAGE AND CHARACTERISATION OF LAVAGE FLUID CELLS

Lavage fluid was obtained during fibreoptic bronchoscopy. In short, lavage cells were seeded on multispot microscope slides (10^4 cells/slide) and allowed to adhere for 60 minutes. Adherent cells were incubated for 5–60 minutes with yeast particles labelled with fluorescein isothiocyanate (Sigma). Trypan blue was used to quench the fluorescence of non-phagocytosed yeast particles. The percentage of phagocytosing cells (cells containing more than one yeast particle per cell) was assessed from 400 counted cells and the mean number of ingested particles per phagocytosing cell was determined.

ALVEOLAR MACROPHAGE SUPEROXIDE RADICAL GENERATION

Superoxide radical generation was recorded as superoxide dismutase inhibitable ferricytochrome C reduction according to the principles of Pick and Mizel, as described in detail previously. In brief, after 20–24 hours in the cold (during transport) the lavage cells were cultured for 20–24 hours in Earle’s balanced salt solution without phenol red but buffered with sodium bicarbonate to pH 7.4 and supplemented with 5% fetal calf serum (Flow) and 50 μg/ml gentamicin. Cultures were performed in 96 well microtitre plates prepared for cell culture (Nunc) in a humidified incubator containing 95% air and 5% carbon dioxide. Non-adherent cells were removed by three washes with medium and the adherent cells were cultured for 30 minutes in Earle’s medium supplemented with ferricytochrome C with or without superoxide dismutase. Stimuli or vehicles (final concentrations given below) were added, the optical density at 550 nm was determined with a Flow Titertek Multiscan at selected time intervals up to two hours after triggering, and the amount (nmol) of superoxide radicals produced per 10^6 plated cells at each examined time point was calculated. On the basis of the time course curve defined by these measurements, the amount of superoxide radicals produced was also expressed in arbitrary units of the area under the curve (AUC). The following were used as stimuli: 4β-phorbol 12-myristate 13-acetate (PMA), 160 nmol/l; serum treated zymosan, 1 mg/ml; and the calcium ionophore A23187, 0.5 μg/ml (for sources and preparations see Bergstrand et al.).

The actual number of cells remaining in the wells after culture and washes was estimated by measuring in replicate the protein content of wells before and after the treatment with the aid of the bichinchoninic acid protein assay (Pierce). A standard curve was obtained for each assay with bovine serum albumin.

EFFECTS OF IN VITRO TREATMENT WITH Budesonide ON ALVEOLAR MACROPHAGE SUPEROXIDE RADICAL GENERATION

Bronchoalveolar lavage cells were obtained from 19 different subjects not receiving glucocorticosteroids. Thirteen of these were smokers without overt airflow obstruction and three had obstruction, one asthma, one sarcoidosis, and one extrinsic alveolitis. The cells were cultured overnight with or without 10^-7 M budesonide. In the continued presence or absence of the drug they were triggered with vehicle, PMA, or serum treated zymosan and examined for spontaneous and stimulus induced superoxide radical generation.

SECRETION OF LTB4 AND PGE2 BY ALVEOLAR MACROPHAGES

The lavage cells were resuspended at 0.5–5 x 10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum and a 2 ml cell suspension was placed in each well of a 24 well tissue culture plate (Nunc). Incubation was performed for one hour at 37°C in a 5% carbon dioxide atmosphere and non-adherent cells were removed by washing. The adherent cells were cultured overnight and then triggered with vehicle or with opsonised zymosan (prepared as described), having a final concentration in the cultures of 1 mg/ml. The cells were treated with the stimulus at 37°C for 90 minutes, and supernatants and cells were harvested and stored at –70°C until they were analysed.

Concentrations of LTB4 and PGE2 in supernatants from alveolar macrophage cultures were analysed with radioimmunoassay kits (NEN) and were related to the content of DNA, determined as described by Labarca and Paigen.

EFFECTS OF IN VITRO TREATMENT WITH Budesonide ON LTB4 AND PGE2

Alveolar macrophages were obtained from different subjects not being treated with glucocorticosteroids. The cells were cultured overnight at 37°C with or without 10^-7 M budesonide and then triggered with opsonised zymosan as described above. Three of these subjects were
immunoelectrophoresis with commercial antiserum (rabbit anti-human albumin, Dakopatts) and human serum albumin (Kabi Vitrum) as standard; concentrations were expressed in mg/l. Intra-assay and interassay coefficients of variation were 3% and 7%.

Fibronectin was analysed by a double sandwich ELISA developed in our laboratory. The aliquots of lavage fluid were thawed at 37°C. Briefly, microtitre plates (Nunc) were coated with rabbit anti-human fibronectin antibodies (Dakopatts) in phosphate-saline buffer, pH 7.2. After addition of unconcentrated lavage fluid samples, the plates were incubated at room temperature for two hours. Horseradish peroxidase labelled anti-human fibronectin was added as a second antibody and the plates were incubated for one hour. The amount of bound peroxidase, which is proportional to the amount of fibronectin in the sample, was measured by analysing its enzymatic activity on 100 μl orthophenylenediamine. Serum fibronectin of nephelometric quality from Behring-Hoechst was used as standard. Concentrations of fibronectin were expressed in µg/l. The detection limit was 10 µg/l and intra-assay and interassay coefficients of variation were 3.7% and 6.4%.

**Determination in Lavage Fluid of Angiotensin Converting Enzyme, Albumin and Fibronectin**

ACE activity was assayed in the concentrated lavage fluid according to the method of Lieberman as described previously; concentrations were expressed in U/l, corresponding to nmol hippuric acid/min/l. Intra-assay and interassay coefficients of variation (CV) were 3.5% and 3.7%.

Albumin was determined by rocket immunoelectrophoresis with commercial antiserum (rabbit anti-human albumin, Dakopatts) and human serum albumin (Kabi Vitrum) as standard; concentrations were expressed in mg/l. Intra-assay and interassay coefficients of variation were 3% and 7%.

**Results**

**Treatment with Inhaled Budesonide**

**Lung Function**

Baseline (% predicted) FEV1 was 91 (SEM 3), FVC 100 (2), and FEV1/FVC 93 (4). All smokers had an FEV1, and an FEV1/FVC of over 70% predicted value. The increase in FEV1 after inhalation of terbutaline was 4%.

**Statistics**

Results are presented as means with standard errors in parentheses. The significance of the effects of treatment was assessed with Wilcoxon's signed rank test on paired observations.
obtained from healthy smokers before (open bars) and after (hatched bars) eight weeks' treatment with inhaled budesonide: mean (SEM) superoxide produced, expressed in area under the curve (AUC) units calculated from kinetic measurements (during two hours) of nmoles of superoxide radicals produced per 10^6 plated cells. PMA—4β-phorbol 12-myristate 13-acetate; STZ—serum treated zymosan; A23187 is a calcium ionophore.

(2%) and did not exceed 20% in any subject. Thus according to these indices lung function was within normal limits in all participating subjects.

Cell counts
Absolute and differential cell counts in lavage fluid did not differ before and after glucocorticosteroid treatment, except that counts of epithelial cells were significantly lower after treatment than before (mean (SEM) 0.47% (0.12) and 0.10 (0.05); p < 0.01). A comparison of absolute and differential cell counts in lavage fluid from smokers and non-smokers has been presented elsewhere.7

Secretion of LTB₄ and PGE₂
Treatment with inhaled budesonide led to a reduction in spontaneous as well as opsonised zymosan triggered secretion of PGE₂ from alveolar macrophage cultures. In contrast, LTB₄ secretion in these cultures was not reduced after the glucocorticosteroid treatment period (fig 1).

Phagocytosis
The percentage of phagocytosing cells remained unchanged after the steroid treatment (fig 2). The mean number of yeast particles ingested by the alveolar macrophages after 60 minutes' incubation was slightly increased after treatment with inhaled budesonide.

Generation of superoxide radicals
The spontaneous release of superoxide radicals from the cultured adherent lavage fluid cells was low throughout and there was no difference in this variable after treatment with inhaled budesonide (results not shown).

The net stimulus triggered superoxide radical generation (corrected for spontaneous release) was not significantly affected by budesonide treatment when serum treated zymosan was used as a stimulus. When the alveolar macrophages were challenged with A23187 or with PMA, superoxide radical generation in some individuals tended to be slightly less in cells obtained after inhalation of glucocorticosteroid than in cells obtained before treatment. In neither of these cases, however, were significant reductions of the mean net stimulus triggered superoxide radical generation recorded. This was the case whether these figures were based on kinetics expressed in nmoles of superoxide radicals produced per 10^6 cells added to the wells or per μg cellular protein remaining in the wells. The protein content did not differ for cells harvested before and after inhaled budesonide treatment (8-9
EFFECTS OF IN VITRO TREATMENT WITH Budesonide ON SELECTED ALVEOLAR MACROPHAGE FUNCTIONS

Secretion of LTB4 and PGE2

Cells cultured with $10^{-5}$ M budesonide overnight showed a significant reduction in LTB4, but not PGE2 secretion. The effect of budesonide on alveolar macrophages was similar in healthy non-smokers and smokers, so the data from all eight subjects examined are presented in figure 5.

Superoxide radical generation

Overnight treatment with $10^{-7}$ M budesonide caused a slight but significant reduction in PMA induced production of superoxide radicals by the alveolar macrophages, but that induced by serum treated zymosan was unaffected. The findings were similar whether the cells from the 13 smokers without overt airflow obstruction were taken alone or cells from all 19 individuals were considered. The latter are shown in figure 6. The alveolar macrophages obtained from the patients with allergic alveolitis (n = 4), asthma (n = 1), and sarcoidosis (n = 1) did not differ in their response to budesonide from the cells of smokers.

Discussion

This report examines the effects of glucocorticosteroid treatment on selected functions of human alveolar macrophages from cigarette smokers. The effects of in vitro treatment with budesonide on selected functions of alveolar macrophages were also assessed.

Bronchoalveolar lavage fluid levels of two putative products of alveolar macrophages, angiotensin converting enzyme and fibronectin, were found to be changed by treatment with budesonide, whereas albumin concentration was not affected. Although ACE is found in endothelial and epithelial cells of various origins, glucocorticosteroid treatment has previously been shown to enhance ACE activity only in cultured alveolar macrophages and in lung tissue.3-8 ACE levels in bronchoalveolar lavage fluid are considered to reflect alveolar macrophage activity.33-34 The increased levels of ACE we record in lavage fluid harvested after budesonide treatment is thus fully compatible with previous findings. As ACE is considered important in bradykinin inactivation in the lung,35-36 the physiological implication of the present finding is interesting.

Activated alveolar macrophages are considered to be major producers of the fibronectin present in lavage fluid.17-20 Our finding of reduced fibronectin concentrations in the presence of changed concentrations of albumin in lavage fluid after glucocorticosteroid treatment also suggests that glucocorticosteroid is affecting the macrophage rather than having a general effect on protein extravasation in the lung. Taken together, these data on soluble lavage fluid constituents indicate that inhaled glucocorticosteroid has reached alveolar macrophages and influenced at least some functions of the cells in vivo.
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In view of these findings the lack of a substantial effect of glucocorticosteroids on alveolar macrophage functions when examined ex vivo is somewhat surprising. Cells from treated patients showed significantly reduced production of PGE₂, in vitro, however, a finding in keeping with an indirect action of glucocorticosteroids through reduction of phospholipase A₂ activity. Thus the capacity, already reduced, of macrophages from smokers to produce arachidonic acid metabolites after suitable triggering¹²-¹⁴ is reduced further by glucocorticosteroid treatment in vivo and not increased. The reason why there is no simultaneous reduction in LTB₄ generation from cells from treated patients is not clear. Nor can we provide a plausible explanation for the fact that treatment of alveolar macrophages in vitro, in contrast to treatment in vivo, leads to reduced LTB₄ but unaffected PGE₂ production.

In vitro treatment of murine peritoneal macrophages with glucocorticosteroid suppresses their capacity to ingest yeast.³⁷-³⁸ We found that treatment with inhaled budesonide did not influence alveolar macrophage phagocytic capacity ex vivo. Physiological differences between murine peritoneal and human alveolar macrophages, use of different steroids, and different ways of exposing cells to the drug may account for the apparent discrepancy in these data.

As discussed above, there was no difference in the protein content of wells containing alveolar macrophages after treatment with budesonide. These data imply that the treatment with inhaled budesonide does not affect adherence properties of the lavage cells.

We found that budesonide treatment had only a small effect on the capacity of the alveolar macrophage to mount an oxidative burst. This is in agreement with most previous reports. Wallaert et al.⁴⁹ reported that the respiratory burst activity of alveolar macrophages, both unstimulated and triggered by PMA and serum treated zymosan, from patients with systemic lupus erythematosus was unaffected by corticosteroid treatment; similar findings were obtained for cells from patients with sarcoidosis by Calhoun et al.⁵⁰ Schaffner and Rollstab⁵¹ showed that dexamethasone impairs microbicidal activity of human blood monocytes against Listeria monocytogenes without affecting their respiratory burst. In contrast, however, Clement et al.⁵² reported that three patients with interstitial lung disease showed substantially reduced oxygen radical generation after two months' treatment with prednisone.

We also failed to influence more than marginally the generation of superoxide radicals by culturing the macrophages with budesonide overnight in vitro. A similar inability of a glucocorticosteroid, examined at reasonable concentrations, to influence generation of hydrogen peroxide in vitro has previously been reported for human monocytes.⁵³ It is worth noting that in our hands analogous treatment overnight in vitro of human leucocytes leads to a substantially reduced capacity for triggered histamine release,⁴⁸ showing that clearcut effects are obtained with a glucocorticosteroid in certain cases.

Monocytes and macrophages need to be primed to respond with a respiratory burst after appropriate stimulation. A necessary precursor for inducing such priming is γ interferon.⁴⁵-⁴⁶ When comparing the oxidative microbicidal activity of human blood monocytes and alveolar macrophages, Kemmerich et al.⁴⁷ found that the former but not the latter could be enhanced substantially by interferon. As prolonged in vivo treatment with budesonide failed to influence superoxide radical generation triggered by defined stimuli, we conclude that budesonide treatment does not influence the degree of priming of the cells to any extent, at least when the cells are obtained from smokers.

Our results therefore imply that budesonide treatment of smokers modulates angiotensin converting enzyme and fibronectin production by alveolar macrophages in vivo and possibly also certain pathways of arachidonic acid metabolism but does not substantially affect the oxidative burst produced by these cells. These findings conform to the general idea that many macrophage functions are sensitive to glucocorticosteroid treatment. If the lack of effect of glucocorticosteroid treatment on the respiratory burst, however, is true for mononuclear phagocytes, it may have an impact on ideas for developing new antiinflammatory drugs.

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