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Activation of cannabinoid receptors prevents antigen-induced asthma-like reaction in guinea pigs

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Abstract

In this study we evaluated the effects of the CB_1/CB_2 cannabinoid receptor agonist CP55, 940 (CP) on antigen-induced asthma-like reaction in sensitized guinea pigs and we tested the ability of the specific CB2 receptor antagonist SR144528 (SR) and CB1 receptor antagonist AM251 (AM) to interfere with the effects of CP. Ovalbumin-sensitized guinea pigs placed in a respiratory chamber were challenged with the antigen given by aerosol. CP (0.4 mg/kg b.wt.) was given i.p. 3 hrs before ovalbumin challenge. Sixty minutes before CP administration, some animals were treated i.p. with either AM, or SR, or both (0.1 mg/kg b.wt.). Respiratory parameters were recorded and quantified. Lung tissue specimens were then taken for histopathological and morphometric analyses and for eosinophilic major basic protein immunohistochemistry. Moreover, myeloperoxidase activity, 8-hydroxy-2-deoxyguanosine, cyclic adenosine monophosphate (cAMP) and guanosine monophosphate (cGMP) levels, and CB_1 and CB_2 receptor protein expression by Western blotting were evaluated in lung tissue extracts. In the bronchoalveolar lavage fluid, the levels of prostaglandin D_2 and tumour necrosis factor-α TNF-α were measured. Ovalbumin challenge caused marked abnormalities in the respiratory, morphological and biochemical parameters assayed. Treatment with CP significantly reduced these abnormalities. Pre-treatment with SR, AM or both reverted the protective effects of CP, indicating that both CB_1 and CB_2 receptors are involved in lung protection. The noted treatments did not change the expression of cannabinoid receptor proteins, as shown by Western blotting. These findings suggest that targeting cannabinoid receptors could be a novel preventative therapeutic strategy in asthmatic patients.

Keywords: cannabinoid receptors • CB_1 • CB_2 • asthma-like reaction • PGD_2 • TNF-α • eMBP

Introduction

Asthma is a chronic inflammatory disease of the airways characterized by eosinophilia, increased vascular permeability in the bronchial mucosa, mucus hypersecretion and airway hyperresponsiveness. The prevalence of asthma has increased dramatically over the past decades and currently affects about 10% of the population in developed countries [1]. Although there is a general consensus about the use of corticosteroids and bronchodilators as main therapeutic measures for the prevention and management of asthma, the identification and development of promising new substances with anti-asthmatic effects that can flank and co-operate with the above drugs is a fertile field for basic and clinical research because of its primary medical interest.

Recently, claims have been made for the beneficial effects of cannabis and cannabinoids, the active components of *Cannabis sativa*. This plant has a long history as a drug source. Over the centuries, it has been used for many purposes, including the treatment of asthma [2]. Early studies have indicated that cannabinoids have bronchodilatory effects in asthmatic patients when administered either orally or by aerosol [3, 4]. Moreover, cannabinoids also have anti-inflammatory effects [5, 6] and have been recently used as novel therapeutic tools in immune-mediated diseases, such as multiple sclerosis [7], rheumatoid arthritis [8] and diabetes [9].

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In murine models of ovalbumin-induced allergic airway response, cannabinoids have shown inhibitory effects on T cell cytokine expression, serum IgE levels and mucus overproduction [10].

Cannabinoids bind to specific G-protein-coupled receptors [11, 12]. To date, two cannabinoid receptors, termed CB_1 and CB_2, have been isolated and cloned. CB_1 is predominantly expressed in the central nervous system and has been also detected in the testis, spleen cells and leukocytes [13, 14]. CB_2, mainly expressed by cells of the immune system, is also present on microglial cells [12–14].

A recent report has suggested that the endocannabinoid system could play a role in lung function. In fact, anandamide, an endocannabinoid agonist, is produced in lung tissue and CB_1 receptors are expressed on axon terminals of nerve fibres in rat bronchiolar smooth muscle [15]. In a rodent model of bronchial hyper-responsiveness, anandamide exerted opposite effects: it strongly inhibited bronchospasm and cough evoked by capsaicin, whereas it caused bronchospasm when the constricting tone exerted by the vagus nerve was removed [15]. Therefore, the activation of CB_1 receptors by locally released anandamide could participate in the control of bronchial contractility. The existence of an intrinsic endocannabinoid-mediated control of airway function may open new perspectives for the development of new anti-tussive and anti-asthmatic agents.

Recently, selective agonists and antagonists for the CB_1 and CB_2 receptors have been made available, thus offering a suitable pharmacological tool to better understand the role of the cannabinoid receptors in lung physiopathology. The aim of this study was to investigate the role of CB_1 and CB_2 receptors on allergic asthma-like reaction in a well-established *in vivo* model of ovalbumin (OV)-sensitized guinea pigs [16, 17]. We used the cannabinoid receptor agonist CP55, 940 (CP) [18], and we tested the ability of the specific CB2 receptor antagonist SR144528 (SR) [19] and CB_1 receptor antagonist AM251 (AM) [20] to interfere with the effects of CP.

Materials and methods

Animals

Male adult albino guinea pigs were quarantined for 7 days at 22–24°C on a 12 hrs light, 12 hrs dark cycle before use. The experimental protocol was basically the same used previously for similar purposes [16, 17]. It complied with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and was approved by the animal care committee of the University of Florence (Florence, Italy). At the end of the treatments, the animals weighed 350–400 g.

Treatments

Group 1. Eight guinea pigs were injected with saline (5 ml/kg i.p., plus 5 ml/kg s.c.). Two weeks later, they were treated with an aerosol of ovalbumin (OV; Fluka, Buchs, Switzerland) suspended in PBS (5 mg/ml). They are referred to as naive controls.

Other guinea pigs were sensitized with OV (100 mg/kg i.p., plus 100 mg/kg s.c.), dissolved in water to a concentration of 20 mg/ml. Two weeks later, they were challenged with an OV aerosol (5 mg/ml saline) to verify that sensitization had occurred. The animals were withdrawn from antigen exposure at the first sign of respiratory abnormality. The animals that developed a clear-cut immediate asthma-like reaction to the inhaled antigen are referred to as sensitized animals. After 4–8 days, they were randomly divided in four further groups, eight animals each, and treated as indicated below.

Group 2. Treatment with an i.p. injection of 1 ml Phosphate buffered Saline (PBS). Three hours later, the animals underwent challenge with OV aerosol, as described below. They are referred to as OV-challenged animals.

Group 3. Treatment with an i.p. injection of CP55,940 (CP; Tocris Cookson, Bristol, UK; 0.4 mg/kg b.wt.), dissolved in 1 ml PBS. Three hours later, the animals underwent challenge with OV aerosol, as described below. They are referred to as CP-treated animals.

Group 4. Treatment with an i.p. injection of the CB_2 antagonist SR144528 (SR; kindly provided by Sanofi Recherche, Montpellier, France; 10 mg/kg b.wt.), dissolved in 1 ml PBS. One hour later the animals were treated with an i.p. injection of 1 ml CP (0.4 mg/kg b.wt.), and after further 3 hours, underwent challenge with OV aerosol. They are referred to as SR+CP-treated animals

Group 5. Treatment with an i.p. injection of the CB_1 antagonist AM251 (AM; Tocris Cookson, Bristol, UK; 10 mg/kg b.wt.), dissolved in 1 ml PBS. One hour later the animals were treated with an i.p. injection of 1 ml CP (0.4 mg/kg b.wt.), and after further 3 hrs, underwent challenge with OV aerosol. They are referred to as AM+CP-treated animals

Group 6. Treatment with an i.p. injection of SR (10 mg/kg b.wt.) plus AM (10 mg/kg b.wt.), dissolved in 1 ml PBS. One hour later the animals were treated with CP (0.4 mg/kg b.wt.), and after further 3 hrs, underwent challenge with OV aerosol. They are referred to as SR+AM+CP-treated animals.

The above dose of CP was selected basing on preliminary observations that it did not cause substantial hypomotility of the treated animals. The doses of AM and SR were chosen by comparison with that of CP, based on their receptor affinity [19, 20].

Evaluation of respiratory activity

The guinea pigs of all the groups were placed in a whole body respiratory chamber, as described previously [16, 17]. The changes in inner pressure induced by breathing were monitored with a high sensitivity pressure transducer connected with a polygraph (Battaglia-Rangoni, Comerio, Italy). Upon stabilization of the breath pattern, the guinea pigs were challenged with an OV aerosol (5 mg/ml in water) for 10 sec. Changes in the respiratory activity of the animals subjected to the different treatments were recorded for 10 min. after OV aerosolization. Evaluation of the following parameters was achieved: (*i*) latency time (sec.) for the appearance of respiratory abnormalities after the onset of aerosolization; (*ii*) cough severity score, assessed as the product of cough frequency (cough strokes/min.) and mean cough amplitude (in mmHg); (*iii*) latency time (sec.) for the appearance of dyspnea, recognized in breath recordings as a series of irregular breaths of abnormally elevated or reduced amplitude compared with the basal breath. Any motion- and sneezing-related changes in the inner pressure of the body chamber were visually detected and discarded.

At the end of the test, the guinea pigs were killed by lethal i.p. injections of sodium thiopenthal (Abbott, Latina, Italy). Bronchoalveolar lavage (BAL) was carried out by cannulation of the trachea and instillation of 3 ml of PBS, pH 7.4. BAL fluid was then centrifuged at 1,100 g for 30 min. The cell-free supernatant was collected, its volume measured and frozen at −70°C until needed. The thorax was then opened allowing for the gross appearance of lungs to be examined. Tissue specimens from the right lung were excised and processed for further analyses, as described below.

Histological and morphometric analyses

Tissue samples, two from each animal (one from the middle lobe and one from the lower lobe), were fixed by immersion in Mota fluid and embedded in paraffin. This fixative solution allows a rapid infiltration of the tissue, with only minimal artifactual shrinking, thus providing a tissue morphology which is representative of the lung features *in vivo*. For each animal, sections 5 μm thick cut from the two different lung samples were stained with haematoxylin and eosin or with Astra blue (Fluka, Buchs, Switzerland) to reveal mast cell granules.

A first series of determinations was carried out on haematoxylin and eosin-stained sections to evaluate the surface area of alveolar aerial spaces, examined with a ×10 objective. Five randomly chosen microscopical fields per animal were analysed. At the chosen magnification, each field corresponded to a tissue area of 570,224 μm^2. The same tissue sections were used to evaluate the surface area of bronchial lumina, selected by: (*i*) histological appearance of small-sized, muscular bronchi; (*ii*) transverse or slightly oblique cross-section. In each guinea pig, measurements were carried out on 4–6 randomly chosen bronchial profiles, examined with a ×20 objective. For both alveolar and bronchial luminal areas, digital images of the microscopical fields were taken. On these images, surface area measurements were carried out using the Scion Image β 4.0.2 image analysis program (Scion Corp., Frederick, MD) upon appropriate thresholding to include only blank, tissue-free aerial spaces. The mean values (± SEM) of alveolar and bronchial luminal areas were then calculated for each experimental group.

A second series of determinations was carried out on Astra blue-stained sections to evaluate the optical density of lung mast cells, which is related to their secretion granule content, as described previously [16, 17]. Digital images of mast cells, taken with a ×100 oil immersion objective, were used for measurement of optical density, carried out on selected mast cell profiles, using the Scion Image β 4.0.2 image analysis program. In each animal, 30 different mast cells, 15 from each lung sample, were analysed and the mean optical density value (± SEM) was then calculated for the entire experimental group.

Immunohistochemisty for eosinophilic major basic protein (eMBP)

Lung tissue sections were treated with 0.3% (v/v) H_2O_2 in 60% (v/v) methanol to quench endogenous peroxidase, permeabilized with 0.1 % (w/v) Triton X 100 in PBS for 20 min. and incubated overnight with human monoclonal anti-eMBP (clone BMK13, Chemicon, Temecula, CA; working dilution: 1:50 in PBS). Immune reaction was revealed by indirect immunoperoxidase method (Vectastain Elite kit, Vector, Burlingame, CA, USA), using 3, 3´-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used. In each guinea pig, the number of eMBP-positive eosinophils was counted in 10 randomly chosen microscopical fields at a ×200 final magnification (test area: 72,346 μm^2). Values obtained from two different observers were averaged.

Evaluation of myeloperoxidase activity

Myeloperoxidase activity, a marker for leukocyte accumulation in tissues, was evaluated according to Bradley *et al.* [21] Briefly; frozen lung tissue fragments weighing approximately 100 mg were thawed and homogenized in 1.5 ml of 50 mmol/l potassium phosphate buffer, pH 6. One ml of the homogenate was centrifuged at 10,000 ×*g* for 10 min. and the pellet suspended in 1 ml of potassium phosphate buffer (50 mmol/l), pH 6, containing 0.5% hexadecyltrimethylammonium bromide (Sigma, Milan, Italy) to negate the pseudoperoxidase activity of haemoglobin and to solubilize membrane-bound myeloperoxidase. The suspensions were sonicated on ice and centrifuged at 12,000 ×*g* for 10 min. Myeloperoxidase activity was determined in 0.1 ml of the supernatant, mixed with 2.9 ml of potassium phosphate buffer (50 mmol/l), pH 6, containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005 % H_2O_2 as a substrate for myeloperoxidase. The absorbance of oxidised *o*-dianisidine was determined spectrophotometrically over 2 min. at 460 nm wave length The values of tissue myeloperoxidase activity were obtained by comparison with standard concentrations of *o*-dianisidine and excess H_2O_2 and expressed as mU/mg of proteins, these latter determined with the Bradford method [22].

Determination of 8-hydroxy-2´-deoxyguanosine (8-OHdG)

8-OHdG was measured as a marker of free radical-induced DNA damage, according to Lodovici *et al.* [23]. DNA was isolated from frozen tissue samples, which were thawed, homogenized in 1 ml of 10 mM phosphate buffered saline, pH 7.4, sonicated on ice for 1 min, added with 1 ml of 10 mM Tris-HCl buffer, pH 8, containing 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, 0.5 % SDS, and incubated for 1 hr at 37°C with 20 μg/ml RNAse (Sigma). Then, the samples were incubated overnight at 37°C under argon, in the presence of 100 μg/ml proteinase K (Sigma). The mixture was then extracted with chloroform/isoamyl alcohol (10:2 v/v) and DNA precipitated from the aqueous phase with 10 M ammonium acetate, dissolved in 200 μl of 20 mM acetate buffer, pH 5.3 and denatured at 90°C for 3 min. The extract was then incubated for 1 hr at 37°C with 10 IU of P1 nuclease and 5 IU of alkaline phosphatase in 0.4 M phosphate buffer, pH 8.8. The mixture was filtered by an Amicon Micropure-EZ filter (Amicon, MA, USA) and 50 μl of each sample were used for 8-hydroxy-2´-deoxyguanosine (8-OHdG) determination using a Bioxytech EIA kit (Oxis, Portland, OR, USA), following the instructions provided by the manufacturer. The protein concentration was determined as described above and the values are expressed as ng of 8-OHdG/mg of protein.

Tumour necrosis factor-α (TNFα) and prostaglandin D_2 (PGD_2) determination in BAL fluid

The release of TNFα and PGD_2 into the BAL fluid were measured using commercial enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions. Protein content of the BAL samples taken from animals belonging to the different experimental groups, measured by the Bradford method, was substantially similar. Thus, the results have been expressed as ng/ml of BAL fluid.

Determination of cyclic nucleotides

Lung tissue samples were homogenized in the presence of 3-isobutyl-1-methylxanthine (IBMX, 50 μm) to inhibit phosphodiesterase activity. The levels of cGMP and cAMP were measured in the aqueous phase of the tissue homogenates, extracted in 10% trichloroacetic acid added with

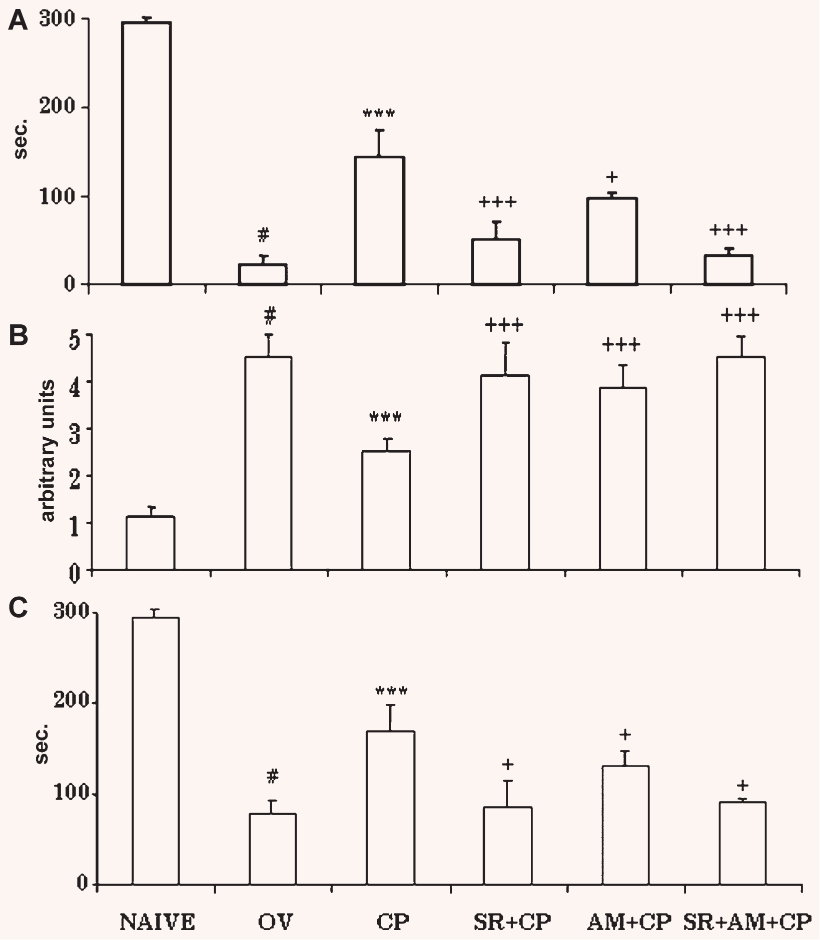

Fig. 1 Occurrence of respiratory abnormalities in guinea pigs of the different experimental groups. (**A**) latency time (sec.) for the onset of cough; (**B**) cough severity; (**C**) latency time (sec.) for the appearance of dyspnea. Compared with the sensitized, ovalbumin (OV)-challenged animals (group 2), a 3-hrs treatment with the CB_1/CB_2 receptor agonist CP of the sensitized guinea pigs before OV challenge (group 3) resulted in a statistically significant reduction of the respiratory abnormalities. Pretreatment of the sensitized guinea pigs with the CB_1 receptor antagonist SR (SR+CP, group 4) or the CB_2 receptor antagonist AM (AM+CP, group 5) or both (SR+AM+CP, group 6) before CP administration markedly reverted the protection afforded by CP. Significance of differences (one-way ANOVA; each group: $n = 8$): # $P < 0.001$ *versus* naive; *** $P < 0.001$ *versus* OV; + $P < 0.05$ and +++ $P < 0.001$ *versus* CP.

0.5 mM tri-n-octylamine in 1,1,2-trichlorotrifluoroethane, using commercial radioimmunoassay kits (Amersham, Bucks, UK). Determinations were performed in duplicate. Values are expressed as fmol cGMP and nmol cAMP per min. per mg of proteins, the latter determined as described above.

Determination of cannabinoid receptors by Western blotting

Lung tissue samples were homogenized in cold lysis buffer (20 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 2.5 mM leupeptin, 2.5 mM aprotinin). Homogenates were centrifugated at 10,000 × *g* for 20 min. at 4°C and the supernatant was collected. Proteins were quantified using Bio-Rad Protein Assay reagent (Hercules, CA, USA). Then, samples containing 75 μg of proteins each were loaded on a 10% SDS-PAGE gel, resolved by electrophoresis and blotted onto a polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After treatment with blocking buffer (5% dry milk and 0.05% Tween 20 in PBS: PBS-T) for 1 hr at room temperature, the membranes were incubated overnight with primary antibodies against CB_1 and CB_2 receptors (1:250; Alexis Biochemicals, San Diego, CA, USA) in PBS-T. Then, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce, Rockford, Il, USA). Immunoreactivity was detected by an enhanced-chemiluminescence assay (Supersignal, Pierce, Rockford, Il, USA). As controls, membranes incubated with antibodies against α-tubulin as invariant protein (1:10,000 in PBS-T) were used.

Statistical analysis

Statistical comparison of differences between the experimental groups was carried out using one-way ANOVA test followed by Student–Newman–Keuls multiple comparison test. $P < 0.05$ was considered significant. Calculations were done using a GraphPad Prism 4.0 statistical program (GraphPad Software, San Diego, CA, USA).

Results

CP reduces OV-induced respiratory abnormalities

The values of the respiratory parameters assayed are reported in Fig. 1A–C. There were no substantial abnormalities in the naive

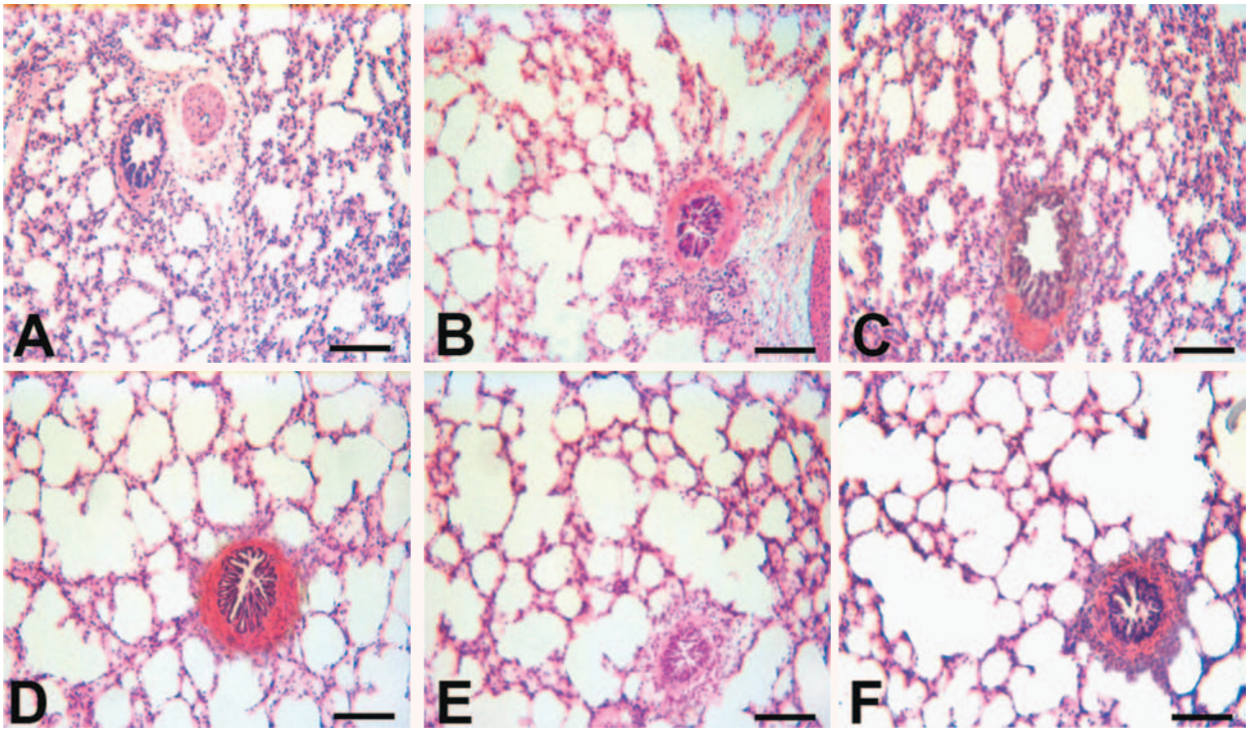

Fig. 2 Representative light micrographs of lung tissue from naive guinea pigs given OV aerosol (**A**; group 1), sensitized guinea pigs challenged with OV aerosol (**B**; group 2), sensitized guinea pigs treated with the CB_1/CB_2 receptor agonist CP before OV challenge (**C**; group 3), sensitized guinea pigs pre-treated with CP in combination with the CB_1 receptor antagonist SR (**D**; group 4), the CB_2 receptor antagonist AM (**E**; group 5) or both (**F**; group 6). Compared with the animals of group 1, those of group 2 show reduction of bronchiolar lumen and dilation of the respiratory air spaces. These alterations are not evident in the CP-treated guinea pigs of group 3, but persist in the animals given SR, AM or both 3 hours before CP (groups 4, 5 and 6, respectively). Haematoxylin and eosin. Bars = 100 μm.

control guinea pigs after inhalation of OV aerosol (group 1), apart from sporadic cough strokes arising about 2 min. after the onset of the aerosol. Challenge of PBS-pre-treated, sensitized guinea pigs with the OV aerosol (group 2) resulted in striking abnormalities of the respiratory pattern, consisting of a significant reduction of the latency time for the onset of cough and dyspnea and a significant increase in the severity of cough. Conversely, a 3-hr pre-treatment with CP of the sensitized guinea pigs before OV challenge (group 3) resulted in a marked, statistically significant reduction of the respiratory abnormalities compared with the OV-challenged animals of group 2. In particular, the latency for cough and dyspnea increased and the cough severity decreased. Pre-treatment of the sensitized guinea pigs with the CB_2 antagonist SR (group 4) and the CB_1 antagonist AM (group 5) before CP administration reverted the protection afforded by CP; in this context, SR was more effective than AM. The respiratory parameters of the animals treated with both the antagonists (group 6) were similar to those of the animals treated with SR (group 4).

CP reduces OV-induced lung histopathological changes

Compared with the naive controls (group 1), macroscopic examination of the lungs showed prominent changes in the OV-challenged guinea pigs (group 2). The pulmonary lobes were swollen because of air entrapment and focal subpleural hemorrhagic foci were observed. Sectioning of trachea or of main bronchi did not cause lung deflation, thus indicating that peripheral airway obstruction had occurred. Lung inflation and subpleural haemorrhage were not found in the CP-treated guinea pigs (group 3), whereas they were present in most of the animals given SR, AM or both before CP (group 4, 5 and 6). By light microscopy (Fig. 2), the lung parenchyma of naive guinea pigs (group 1) had a normal appearance: intrapulmonary bronchi showed open lumina and respiratory air spaces were mostly small-sized (Fig. 2A). Conversely, the lungs from the OV-challenged guinea pigs (group 2) mostly showed a reduction of the lumen of intrapulmonary bronchi, with long mucosal folds

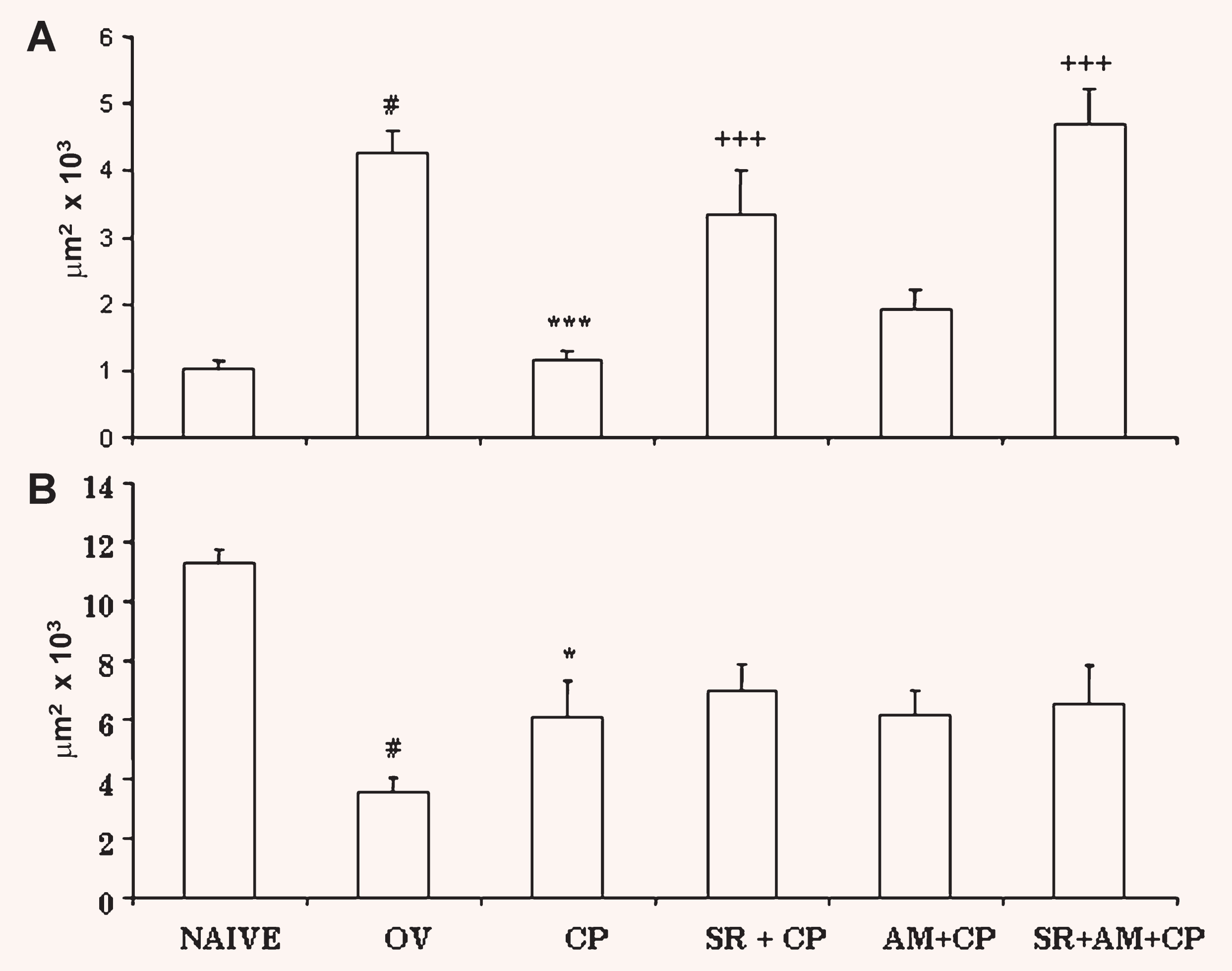

Fig. 3 Surface area of alveolar air spaces (**A**) and small-sized bronchial lumina (**B**) in the lungs of guinea pigs from the different experimental groups. Compared to the OV-challenged, untreated animals (group 2) in the guinea pigs treated with the CB_1/CB_2 receptor agonist CP (group 3) the mean surface area of alveolar air spaces nearly returned to the control values and the mean surface area of bronchiolar lumina was significantly increased. With respect to the CP-treated group, pre-treatment of the sensitized guinea pigs with the CB_1 receptor antagonist SR (SR+CP, group 4) or the CB_2 receptor antagonist AM (AM+CP, group 5) or both (SR+AM+CP, group 6) before CP administration significantly prevented the reduction of the mean surface area of alveolar air spaces but not the increase of the mean surface area of bronchiolar lumina. Significance of differences (one-way ANOVA): # $P < 0.001$ *versus* naive; *** $P < 0.001$ and * $P < 0.05$ *versus* OV; +++ $P < 0.001$ *versus* CP.

expanding into the lumen and markedly dilated respiratory air spaces (Fig. 2B). In the CP-treated guinea pigs (group 3), the histological lung abnormalities were nearly abrogated. In fact, the intrapulmonary bronchi usually showed no appreciable signs of constriction, and most respiratory air spaces were not dilated (Fig. 2C). In the guinea pigs pre-treated with SR, AM or both the antagonists before CP (groups 4, 5 and 6), the histological features were quite similar to those of the OV-challenged animals (Fig. 2D–F).

The visual observations were objectified by morphometric analysis (Fig. 3A and B). Compared with the naive guinea pigs of group 1, the OV-challenged guinea pigs (group 2) showed a significant increase in the mean surface area of alveolar air spaces and a significant decrease in the mean surface area of bronchiolar lumina. In the CP-treated guinea pigs (group 3) the mean surface area of alveolar air spaces nearly returned to the control values and the mean surface area of bronchiolar lumina was significantly increased compared to the OV-challenged animals. With respect to the CP-treated group, pre-treatment with SR, or AM, or both the antagonists significantly prevented the CP-induced reduction of the mean surface area of alveolar air spaces but had little effects on the mean surface area of bronchiolar lumina.

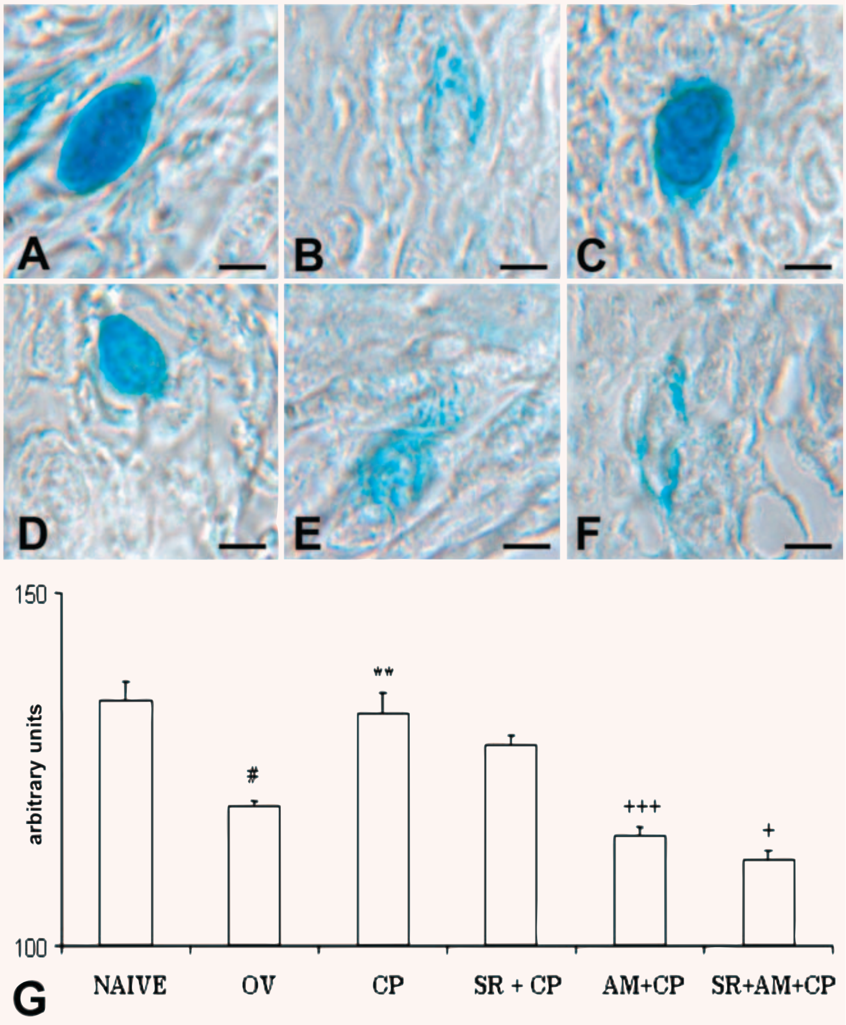

Fig. 4 Representative images of Astra Blue-stained mast cells from naive guinea pigs given OV aerosol (**A**, group 1), sensitized guinea pigs challenged with OV aerosol (**B**, group 2), sensitized guinea pigs treated with the CB$_1$/CB$_2$ receptor agonist CP before OV challenge (**C**, group 3), sensitized guinea pigs pre-treated with CP in combination with the CB$_1$ receptor antagonist SR (**D**, group 4), the CB$_2$ receptor antagonist AM (**E**, group 5) or both (**F**, group 6). Compared with the animals of group 1, those of group 2 show a clear-cut reduction of mast cell staining intensity. These alterations are not evident in the CP-treated guinea pigs of group 3, but persist in the animals given SR, AM or both 3 hrs before CP (groups 4, 5 and 6, respectively). Astra blue staining. Bars = 10 μm. The visual observations are confirmed and objectified by computer-aided densitometry on selected mast cell profiles (**G**). Significance of differences (one-way ANOVA): # $P < 0.001$ *versus* naive; ** $P < 0.01$ *versus* OV; + $P < 0.05$ and +++ $P < 0.001$ *versus* CP.

CP reduces OV-induced mast cell granule release

Lung mast cells from the OV-challenged guinea pigs (group 2) underwent a marked decrease in Astra blue staining intensity, which depends on secretion granule content, as compared with those from the naive controls (group 1), thus indicating that granule discharge has occurred (Fig. 4A and B). In the CP-treated guinea pigs (group 3), the mast cell staining intensity was markedly increased in respect to the OV-challenged animals (group 2), attaining values similar to those of the controls (Fig. 4C). As compared with the animals of group 3, mast cell staining intensity was slightly decreased in the guinea pigs pre-treated with SR (group 4), or AM (group 5), or both the antagonists (group 6) (Fig. 4D–F). Computer-aided densitometry confirmed the visual observations (Fig. 4G). Compared with the naive controls (group 1), the optical density of Astra blue-stained mast cells was significantly reduced in OV-challenged guinea pigs (group 2), but not in the CP-treated guinea pigs (group 3). As compared with the latter animals, mast cell optical density was decreased, but not significantly, in the guinea pigs pre-treated with SR (group 4), whereas the decrease reached statistical significance in the guinea pigs pre-treated with AM (group 5) and in those pre-treated with both the antagonists (group 6).

CP reduces OV-induced lung leukocyte infiltration

Myeloperoxidase activity (Fig. 5), a marker of leukocyte infiltration into inflamed tissues, as well as eMBP-positive eosinophils (Fig. 6) were assayed in the different experimental groups. Both parameters markedly and significantly increased in the OV-challenged guinea pigs (group 2) compared with the controls (group 1). In the CP-treated guinea pigs (group 3), myeloperoxidase activity and the number of eMBP-positive eosinophils were significantly

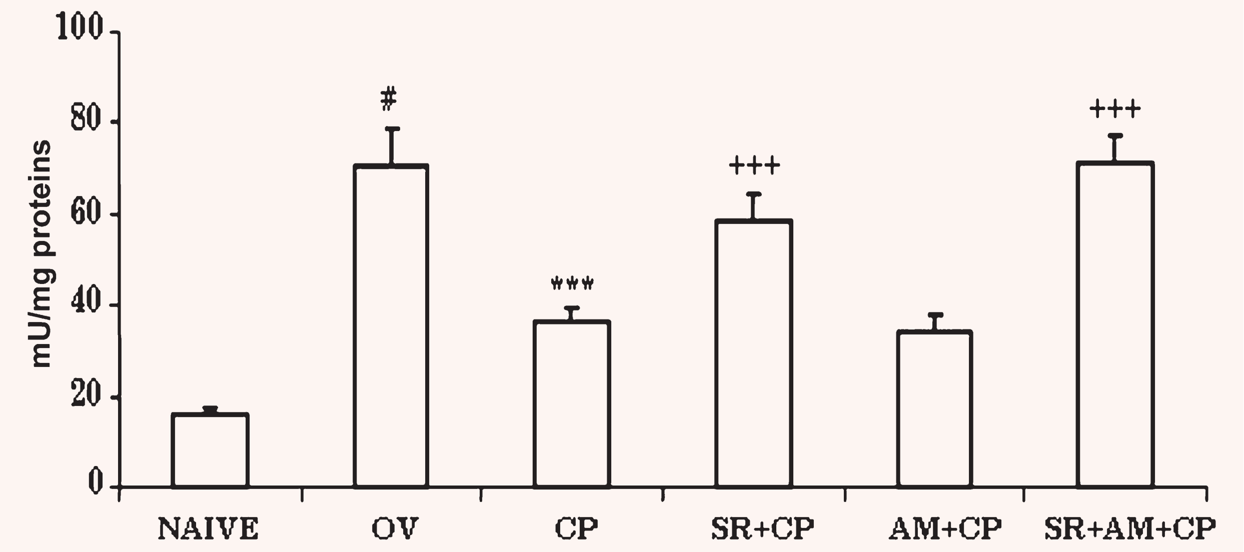

Fig. 5 Lung tissue myeloperoxidase (MPO) activity in the guinea pigs from the different experimental groups. In the guinea pigs treated with the CB_1/CB_2 receptor agonist CP (group 3) MPO activity is significantly decreased compared with the OV-challenged untreated guinea pigs (group 2). Pre-treatment with the CB_1 receptor antagonist SR (SR+CP, group 4) or SR and the CB_2 receptor antagonist AM (SR+AM+CP, group 6) before CP administration prevented the reduction of MPO activity, while pre-treatment with AM alone (AM+CP, group 5) had no effects. Significance of differences (one-way ANOVA): # $P < 0.001$ *versus* naive; *** $P < 0.001$ *versus* OV; +++ $P < 0.001$ *versus* CP.

decreased compared with the animals of group 2. Pre-treatment with SR significantly prevented the reduction of myeloperoxidase activity and the number of eMBP-positive eosinophils due to CP, while pre-treatment with AM only prevented the reduction of the number of eMBP-positive eosinophils. Myeloperoxidase activity and the number of eMBP-positive eosinophils evaluated in the animals pre-treated with both the antagonists (group 6) were similar to those of animals pre-treated with SR alone (group 4).

CP reduces OV-induced 8-OHdG production

Tissue levels of 8-OHdG (Fig. 7), a marker of free radical-induced DNA damage, significantly increased in the OV-challenged guinea pigs (group 2) compared with the controls (group 1). In the CP-treated guinea pigs (group 3), the levels of 8-OHdG were significantly decreased compared with the OV-challenged animals of group 2. Pre-treatment with SR, but not with AM, significantly prevented the reduction of 8-OHdG. The levels of 8-OHdG evaluated in the animals treated with both the antagonists (group 6) were slightly increased as compared with the animals pre-treated with SR alone (group 4).

CP reduces OV-induced release of TNFα and PGD_2 in BAL fluid

The inflammatory cytokine TNFα (Fig. 8A) and PGD_2 (Fig. 8B) were markedly increased in the BAL fluid from OV-challenged guinea pigs (group 2) compared with the controls (group 1). In the CP-treated guinea pigs, the values of TNFα and PGD_2 in BAL fluid were significantly lower than in the animals of group 2. Pre-treatment with SR or AM prevented the CP-induced decrease of TNFα and PGD_2 levels in BAL fluid, with SR being the most effective. Pre-treatment with both the antagonists (group 6) caused similar effects as SR alone.

CP reduces OV-induced increase of cAMP levels

As CB_1 and CB_2 receptors are G protein-associated, cyclic nucleotide-operating receptors, we evaluated the levels of cAMP and cGMP in lung tissue extracts. Cyclic AMP levels (Fig. 9A) in lung tissue were similar in the samples from the OV-challenged animals (group 2) and the controls (group 1). In the CP-treated guinea pigs (group 3), the values of cAMP were significantly lower than in the previous groups, while in the animals pre-treated with the antagonists, given alone or together (groups 4–6), the cAMP values were similar to those of the control (group 1) and OV-challenged animals (group 2). On the other hand, cGMP levels did not change among the experimental groups (Fig. 9B).

CP treatment does not modify the expression of cannabinoid receptor proteins

Western blot analysis showed that both CB_1 and CB_2 receptors are expressed in guinea pig lung tissue and that neither the treatment

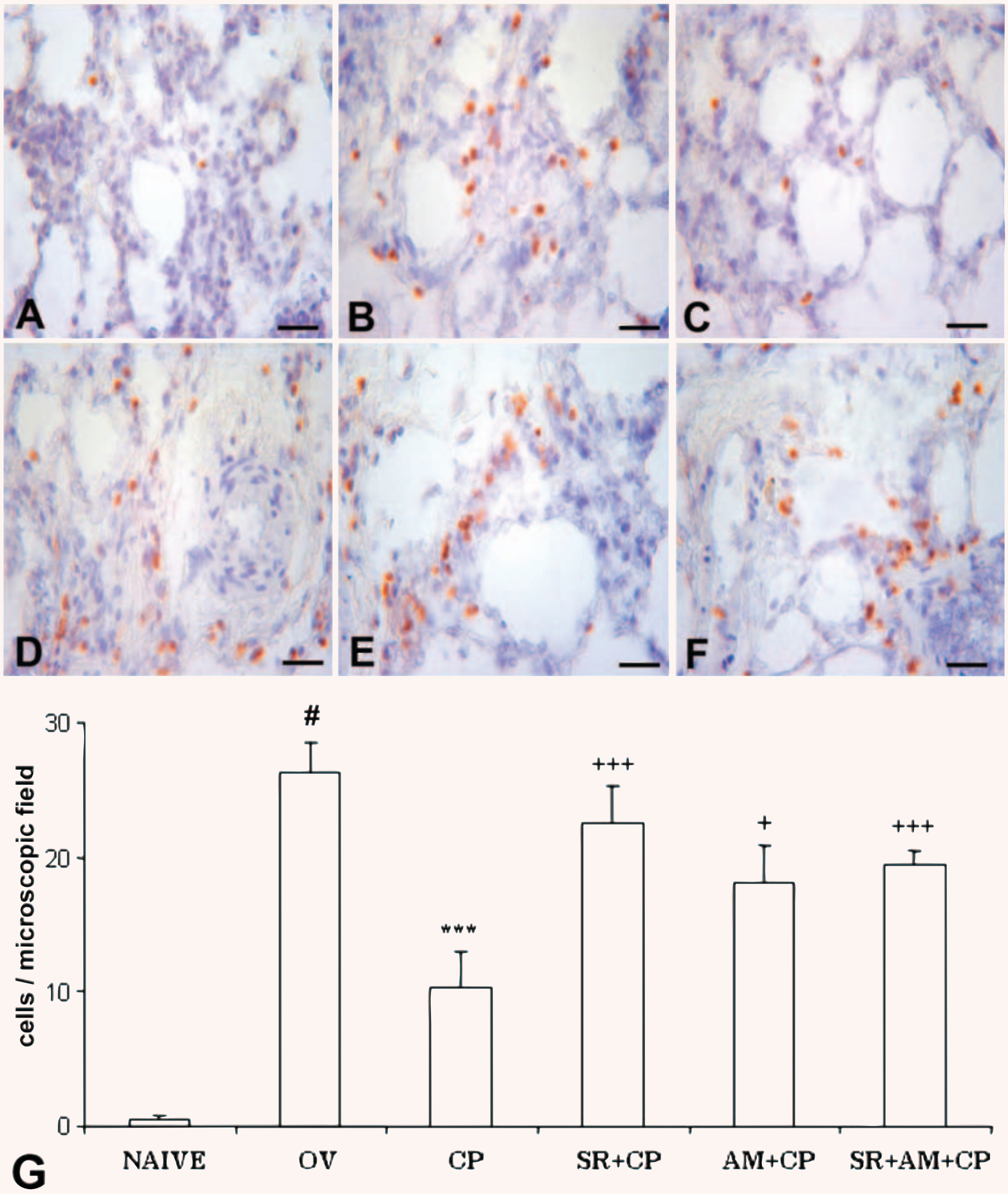

Fig. 6 Eosinophils positive for eMBP in the lung tissue from naive guinea pigs given OV aerosol (**A**, group 1), sensitized guinea pigs challenged with OV aerosol (**B**, group 2), sensitized guinea pigs treated with the CB$_1$/CB$_2$ receptor agonist CP before OV challenge (**C**, group 3), sensitized guinea pigs pre-treated with CP in combination with the CB$_1$ receptor antagonist SR (**D**, group 4), the CB2 receptor antagonist AM (**E**, group 5) or both (**F**, group 6). Pre-treatment with CP (group 3) reduces the amount of eMBP-positive eosinophils compared with the untreated, OV-challenged guinea pigs (group 2). Pre-treatment with the CB$_1$ receptor antagonist SR (SR+CP, group 4), the CB2 receptor antagonist (AM+CP, group 5) or both (SR+AM+CP, group 6) before CP administration prevents the reduction eMBP-positive eosinophils. Morphometrical analysis (**G**) confirmed the visual observations. Significance of differences (one-way ANOVA): # $P < 0.001$ *versus* naive; *** $P < 0.001$ *versus* OV; + $P < 0.05$ and +++ $P < 0.001$ *versus* CP.

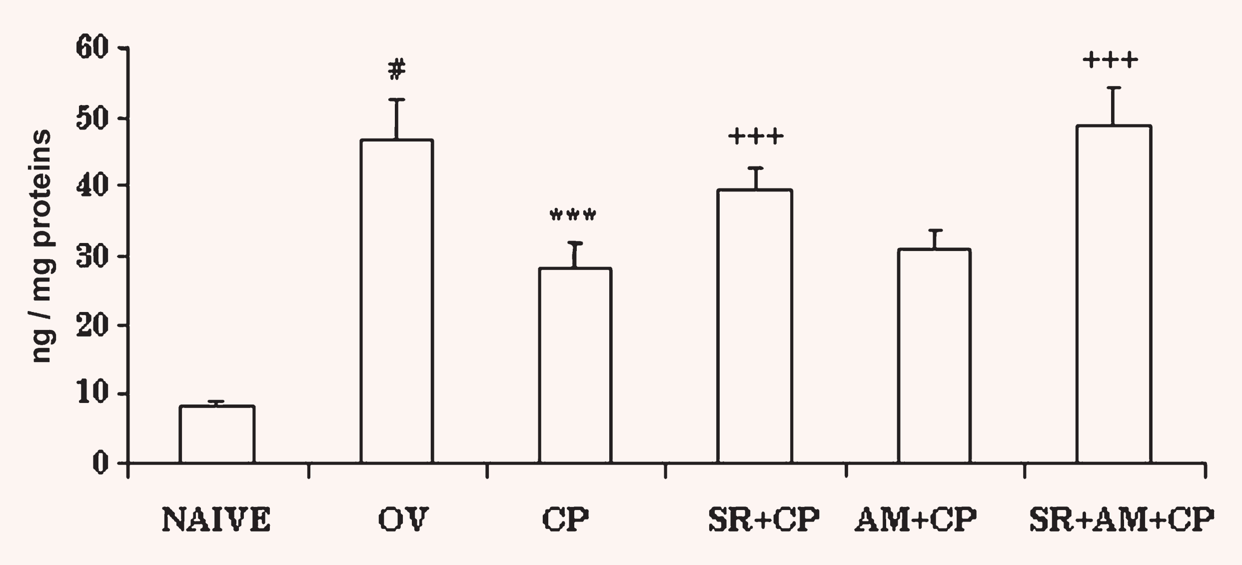

Fig. 7 Lung tissue levels of 8-hydroxy-2´-deoxyguanosine (8-OHdG) in the guinea pigs from the different experimental groups. In the guinea pigs treated with the CB$_1$/CB$_2$ receptor agonist CP (group 3), the levels of 8-OHdG were significantly decreased compared with the untreated, OV-challenged animals (group 2). Pre-treatment with the CB$_1$ receptor antagonist SR (SR+CP, group 4) or SR and the CB$_2$ receptor antagonist AM (SR+AM+CP, group 6) before CP administration prevented the reduction of 8-OHdG. Significance of differences (one-way ANOVA): # $P < 0.001$ *versus* naive; ***$P < 0.001$ *versus* OV; +++ $P < 0.001$ *versus* CP.

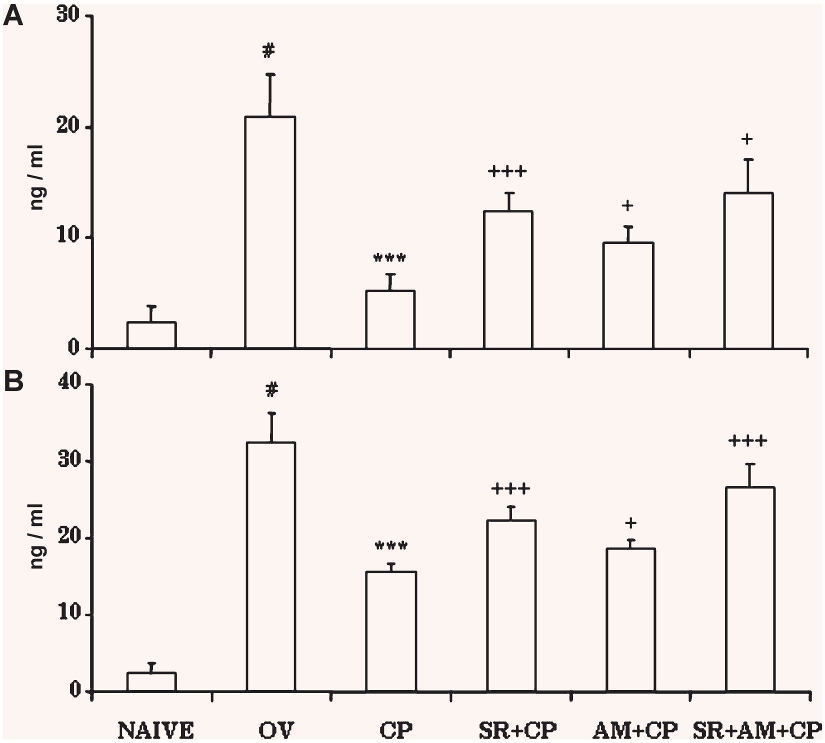

Fig. 8 Levels of tumour necrosis factor-α TNF-α (**A**) and PGD$_2$ (**B**) in BAL fluid of guinea pigs from the different experimental groups. In the guinea pigs treated with the CB$_1$/CB$_2$ receptor agonist CP (group 3), the values of TNFα and PGD2 were significantly lower than in the untreated, OV-challenged animals (group 2). Pre-treatment with the CB$_1$ receptor antagonist SR (SR+CP, group 4) or the CB2 receptor antagonist AM (AM+CP, group 5) or both (SR+AM+CP, group 6) before CP administration prevented the CP-induced decrease of TNFα and PGD2 levels. Significance of differences (one-way ANOVA): # $P < 0.001$ versus naive; ***$P < 0.001$ *versus* OV; + $P < 0.05$ and +++ $P < 0.001$ *versus* CP.

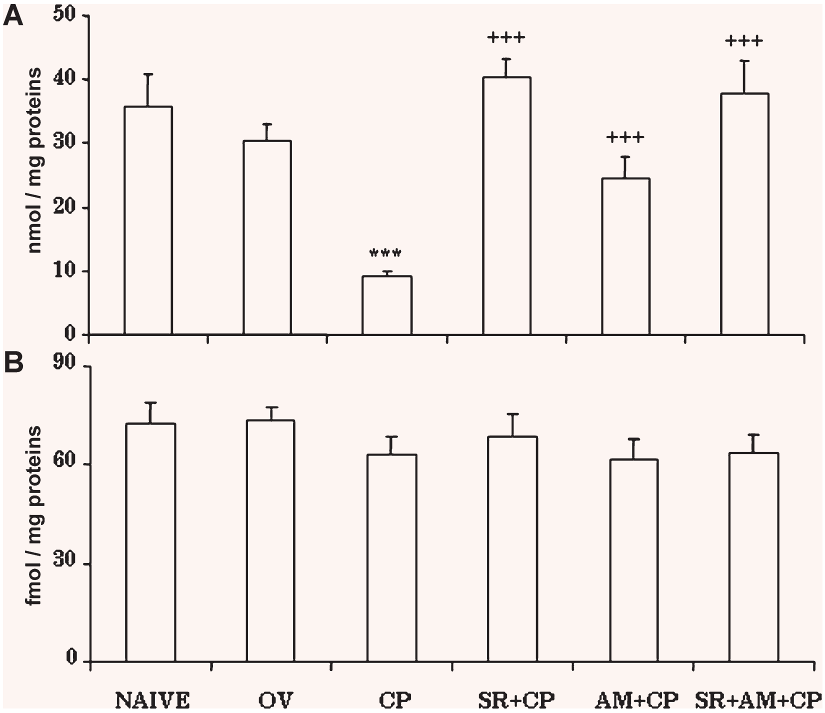

Fig. 9 Lung tissue levels of cAMP (**A**) and cGMP (**B**) in the guinea pigs from the different experimental groups. In the guinea pigs treated with the CB$_1$/CB$_2$ receptor agonist CP (group 3), the values of cAMP were significantly lower than in the untreated, OV-challenged animals (group 2) and in the naive animals (group 1). Pre-treatment with the CB$_1$ receptor antagonist SR (SR+CP, group 4) or the CB$_2$ receptor antagonist AM (AM+CP, group 5) or both (SR+AM+CP, group 6) before CP administration prevented this decrease. The levels of cGMP were not different among the experimental groups. Significance of differences (one-way ANOVA): $***P < 0.001$ *versus* OV; $+++$ $P < 0.001$ *versus* CP.

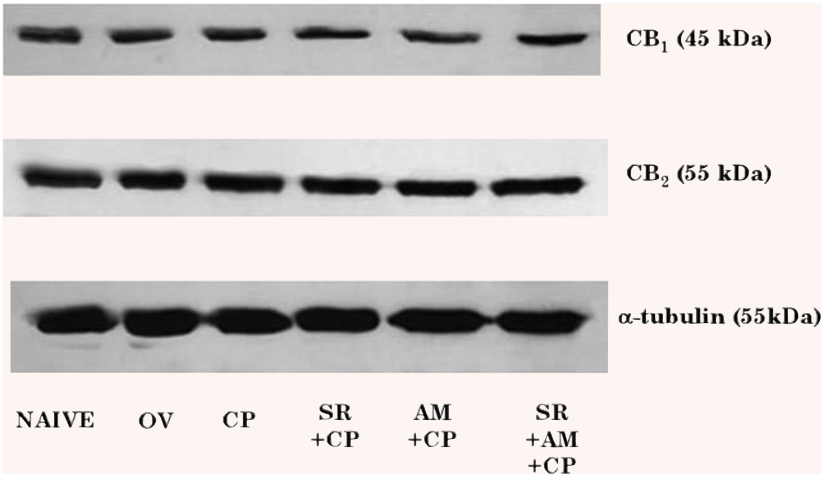

Fig. 10 Western blot analysis of CB$_1$ and CB$_2$ cannabinoid receptors in lung tissue of the guinea pigs from the different experimental groups. The treatment with the CB$_1$/CB$_2$ receptor agonist CP (group 3), alone or in combination with the CB$_1$ receptor antagonist SR (SR+CP, group 4), the CB$_2$ receptor antagonist AM (AM+CP, group 5) or both (SR+AM+CP, group 6) did not cause any modification in the receptor protein expression when compared to the naive (group 1) or the untreated, OV-challenged animals (group 2).

with CP nor pre-treatment with the noted antagonists resulted in any modification in the receptor protein expression (Fig. 10).

Discussion

This study provides evidence that the cannabinoid receptor agonist CP55,940 (CP) is able to counteract the allergen-induced functional, biochemical and histopathological lung changes in a guinea pig model of allergic asthma-like reaction to OV aerosol. Systemic pre-treatment of OV-sensitized guinea pigs with CP 3 hrs before OV challenge caused a significant reduction of the occurrence of respiratory abnormalities (cough and dyspnea), bronchial lumen restriction, alveolar hyperinflation, leukocyte and eosinophilic infiltration, mast cell activation and free radical-induced DNA injury compared with the vehicle-treated OV-challenged controls. Moreover, a drop of the inflammation-related molecules TNFα and PGD$_2$ was observed in BAL fluid from the animals treated with CP. A schematic diagram summarizing the data and highlighting the hypotheses on how CP may work in the current asthmatic model is given in Fig. 11. Briefly, we suggest that CP may promote bronchodilation by stimulation of CB$_1$ receptors on bronchial smooth muscle cells and nerve endings and, in

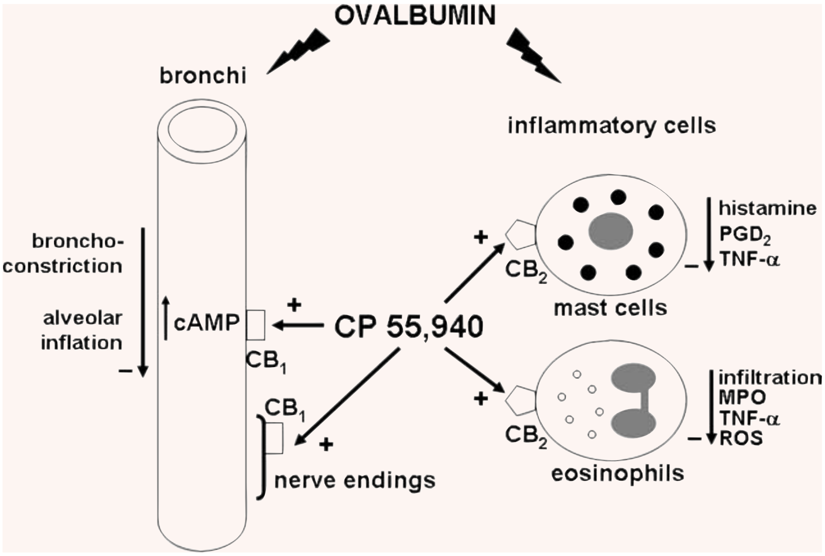

Fig. 11 Schematic diagram summarizing the effects of the CB$_1$/CB$_2$ receptor agonist CP55,940 in the current model of allergic asthma-like reaction. CB$_1$, CB$_2$, cannabinoid receptors 1 and 2; MPO, myeloperoxidase; ROS, reactive oxygen species.

the meantime, exert inhibitory effects on lung tissue mast cells and eosinophils recruited by allergen challenge by stimulation of CB$_2$ receptors.

The actions of cannabis on airway functions have been among the effects first explored for potential therapeutic use [24]. Smoked marijuana and ingested Δ^9-tetrahydrocannabinol (THC) were found to decrease airway resistance and to increase airway conductance both in healthy and asthmatic subjects [3]. However, even though these findings may suggest that THC could be therapeutically used as a bronchodilator in asthma, it should be underlined that smoke-borne delivery of cannabinoids to asthmatic patients is contraindicated because of the noxious gaseous and particulate substances in smoke, whose long-term effects include chronic airway irritation and tumourigenesis [24]. Moreover, oral THC is not suitable for therapeutic use in asthma due to its adverse toxic effects on the central nervous system and positive cardiac chronotropism, opposed to its modest bronchodilator properties [4, 25].

The mechanisms of cannabinoid-induced bronchodilation have been recently studied but not yet completely understood [15]. In the present *in vivo* study, we have shown that the cannabinoid agonist CP has marked anti-inflammatory effects. Our study also confirms that both CB$_1$ and CB$_2$ receptors are involved in lung response to CP, as indicated by the increase of the lung tissue levels of cAMP, the second messenger of CB receptor activation, upon CP administration to the guinea pigs. Western blot analysis demonstrated that CP causes no modification of the receptor protein expression.

Mast cells are known as key players during the early phase of allergy, since allergen-induced cross-linking of their high affinity IgE receptors culminates in massive degranulation and release of pro-inflammatory mediators, such as histamine, TNFα and PGD$_2$, with potent and rapid effects on blood vessels and bronchial smooth muscle cells. This explains the majority of symptoms experienced by asthmatics during the early phase [26]. Our findings indicate that the observed decrease of mast cell granule release afforded by CP in OV-challenged animals was CB$_1$/CB$_2$ mediated. Moreover, both CB1 and CB2 antagonists were able to blunt the inhibitory effect of CP on the release of TNFα and PGD2 in BAL fluid, demonstrating that both receptors are involved. In this context, the localization of CB1 receptors on nerve endings in close proximity to airway smooth muscle cells [15] could explain not only bronchodilation but also the anti-inflammatory effects of CP. In fact, mast cells have been found in close contact with nerve fibres [27], suggesting a neural control on mast cell function [28–30]. The influence between nerves and mast cells is reciprocal: neurons change their firing rate upon mast cell degranulation [29], and mast cells may activate upon neural stimulation [30]. A feedback has been also suggested for mast cells and non-myelinated sensory C-fibres [31], the major vagal afferents to the airways [32], which have been involved in lung vascular and bronchial changes caused by allergy [33] and by inhalation of chemical irritants and air pollutants [34]. Our data suggest that CP may directly reduce the activation of mast cells *via* their CB$_2$ receptors, as we previously demonstrated *in vitro* [6], and also indirectly *via* the CB$_1$ receptors located on nerve endings distributing to the bronchial smooth muscle coat [15].

The present study also shows that CB$_2$ receptors are likely involved in the decrease in myeloperoxidase (MPO) activity and eMBP-positive eosinophils induced by CP. This indicates that CP exerts its anti-inflammatory activity mainly through CB$_2$ receptors, in keeping with our previous results [6, 35]. As activated leukocytes are the main source for oxidizing free radicals, it appears that inhibition by CP of CB$_2$-dependent leukocyte recruitment into the allergen-challenged lung tissue is directly related to the reduction of oxidative DNA damage, as indicated by the reduced levels of 8OHdG. Multiple mechanisms have been demonstrated for cannabinoids, including receptor-dependent and -independent effects [10, 36]. Among the latter ones, the indirect, antioxidant effects of cannabinoids may turn out very useful in asthma, considering that increased levels of oxygen-derived free radicals and high-energy oxidants produced by inflammatory and epithelial cells in the airways are deemed important pathogenic mediators of asthma [37, 38].

In conclusion, this study provides background to the concept that novel non-psychoactive cannabinoid analogues with bronchodilator and anti-inflammatory activity could find a therapeutical use as adjuncts in the treatment of allergic asthma, especially as preventative agents which could blunt the severity of asthmatic attacks and in patients who are at risk for adverse side effects by the major anti-asthmatic drugs. In particular, cannabinoid derivatives selectively targeting CB_2 receptors appear as the most promising drugs, as they should be free of central side effects. However, based on the putative role of CB_1 receptors shown by the current study, CB_1 agonists could also be worthy of attention. Of note, vapourizing devices are now commercially available which could be used for safe cannabinoid delivery [39]. Such tools could meet the requirement for high lung cannabinoid uptake, while avoiding the respiratory disadvantage of smoking and reducing the risk for adverse cardiovascular and neural side effects.

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