

Inhibition of Poly(ADP-Ribose) Polymerase Prevents Allergen-Induced Asthma-Like Reaction in Sensitized Guinea Pigs

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Received June 10, 2004; accepted July 9, 2004

ABSTRACT

Poly(ADP-ribose) polymerase (PARP) plays an important role in tissue injury in conditions associated with oxidative stress and inflammation. Because asthma is a chronic inflammatory disorder of the airways, we designed the present experimental study to evaluate the effects of PARP inhibition on allergen-induced asthma-like reaction in ovalbumin-sensitized guinea pigs. Cough and dyspnea in response to ovalbumin aerosol were absent in naive guinea pigs, whereas they became severe in the sensitized animals. In the latter ones, ovalbumin aerosol also induced a rapid increase in PARP activity, bronchiolar constriction, pulmonary air space inflation, mast cell degranulation, poly(ADP-ribose) and nitrotyrosine immunostaining, my-

eloperoxidase activity, and malondialdehyde in lung tissue, as well as a rise in the amounts of nitrites and tumor necrosis factor- α in bronchoalveolar lavage fluid. Pretreatment with the PARP inhibitors 3-aminobenzamide (10 mg/kg b.wt.) or 5-aminoisoquinolinone (0.5 mg/kg b.wt.) given i.p. 3 h before ovalbumin challenge significantly reduced the severity of cough and the occurrence of dyspnea and delayed the onset of respiratory abnormalities. Both PARP inhibitors were also able to prevent the above morphological and biochemical changes of lung tissue or bronchoalveolar lavage fluid induced by ovalbumin challenge. Conversely, *p*-aminobenzoic acid, the inactive analog of 3-aminobenzamide, had no effects.

Poly(ADP-ribose) polymerase (PARP; E.C.2.4.2.30) identifies a family of ubiquitous nuclear enzymes involved in many physiological and pathophysiological events, whose best studied member is PARP-1 (molecular mass 113 kDa) (Shieh et al., 1998; Smith et al., 1998; Amè et al., 1999; Johansson, 1999; Kickhoefer et al., 1999; Sallmann et al., 2000). PARP-1, using NAD⁺ as a substrate, attaches polymers of ADP-ribose to acceptor proteins including histones, transcription factors, and PARP-1 itself. Despite its abundant presence in the chromatin fractions, PARP-1 is normally inactive. Only when DNA damage occurs, under any circumstance, this enzyme is rapidly activated (De Murcia and Shall, 2000; Burkle, 2001a) because it requires nicked DNA to exert the catalytic activity (Benjamin and Gill, 1980). The physiological roles of PARP-1, which are strictly related to this enzymatic characteristic, include DNA repair and genotoxic stress resistance, signal-

ing of DNA damage, regulation of genomic stability in cells under genotoxic stress, transcriptional regulation, stimulation of nuclear proteosomal function, aging, and longevity (Burkle, 2001b). However, PARP-1 activation is a double-edged sword because it burns out massive amounts of NAD⁺ and ATP, thereby causing rapid depletion of metabolic substrates and energy failure of the cells (Amè et al., 1999; De Murcia and Shall, 2000; Burkle, 2001b). In fact, several studies have offered evidence for an involvement of PARP-1 in the pathophysiology of diabetes mellitus, ischemia reperfusion-induced damage in the brain, heart, kidney, and bowel, septic and hemorrhagic shock, acute and chronic inflammatory disorders, and induction of cell apoptosis (Szabò and Dawson, 1998). Regarding the role of PARP-1 in inflammation, it has been recently pointed out that PARP-1 may cooperate with nitric-oxide synthase in causing the inflammatory tissue damage (Szabò and Dawson, 1998). In fact, massive amounts of nitric oxide (NO), produced by inducible nitric-oxide synthase under proinflammatory conditions by various cells (such as macrophages, epithelial cells, etc.), can react with superoxide to yield highly toxic peroxynitrite (Szabò, 2003) that, in turn, induces DNA strand break and rapid PARP-1

This study was supported by a grant from the Italian Ministry of Education, University and Research (MIUR, PRIN 2002 to H.S. and D.B.) and by funds from the Universities of Verona and Florence.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.072546.

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; NO, nitric oxide; PBS, phosphate-buffered saline; ANOVA, analysis of variance; TNF, tumor necrosis factor.

activation, with consequent energy crisis and cell death (Szabò and Dawson, 1998; Szabò, 2003).

On these grounds, an increasing body of literature indicates the therapeutic potential of PARP inhibitors as anti-inflammatory agents, as observed, for instance, in the inflammatory response to ischemia/reperfusion-induced injury that triggers the production of cytokines and free radicals (Thiemermann et al., 1997; Zingarelli et al., 1997; Szabò and Dawson, 1998; Cuzzocrea et al., 2002; Virag and Szabò, 2002). One of the most widely used PARP inhibitors, 3-aminobenzamide, reduced the infarct size of heart or skeletal muscle after ischemia/reperfusion insult (Thiemermann et al., 1997; Zingarelli et al., 1997). A novel water-soluble PARP-1 inhibitor, 5-aminoisoquinolinone, reduced the degree of lung injury and attenuated the expression of P-selectin and ICAM-1 as well as the recruitment of neutrophils into the injured lung (Cuzzocrea et al., 2002). This is in keeping with the recent report on the critical role played by activation of PARP-1 in lipopolysaccharide-induced acute lung inflammation (Albertini et al., 2000).

The increased expression of adhesion molecules, such as P-selectin, VCAM-1, and ICAM-1, and the enhanced recruitment of leukocytes are some of the pathophysiological hallmarks of asthma, a chronic inflammatory disorder of the airways whose prevalence is increasing everywhere, especially among children. Asthma is also characterized by bronchial hyperresponsiveness, airway constriction associated with bronchial edema, mucosal inflammation, and increased production of mucus, all events leading to airflow limitation and mild to severe respiratory symptoms, including dyspnea and cough.

The aim of the present study was to provide insight into the possible role of PARP-1 activation in the pathophysiology of asthma by investigating the effects of substances able to inhibit PARP-1 in an experimental model of asthma-like reaction induced by airborne allergen in sensitized guinea pigs. Our findings show that systemic administration of PARP-1 inhibitors such as 3-aminobenzamide and 5-aminoisoquinolinone to the sensitized animals prevents efficiently allergen-elicited respiratory abnormalities as well as histological and biochemical changes of the lungs. Therefore, pharmacological inactivation of PARP-1 might be a therapeutic strategy to limit lung injury in allergic airway chronic inflammation.

Materials and Methods

Animals. Male Hartley albino guinea pigs were used. They were purchased from a commercial dealer (Rodentia, Bergamo, Italy) and quarantined for 7 days at 22–24°C with a 12-h light/dark cycle before use. Standard laboratory chow (Rodentia), fresh vegetables, and water were available ad libitum. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and in agreement with the Good Laboratory Practice. It was approved by the animal care committee of the University of Florence (Florence, Italy). At the end of the treatment, the animals weighed 350 to 400 g.

Animal Sensitization and Treatments. The guinea pigs were divided into six experimental groups, as detailed below.

Group 1 ($n = 8$) guinea pigs were injected with phosphate-buffered saline (PBS; 5 ml/kg i.p. plus 5 ml/kg s.c.) 21 days before entering the further experiments. They are referred to as naive animals and were the negative controls.

The remaining guinea pigs were sensitized with 100 mg/kg i.p. plus 100 mg/kg s.c. ovalbumin, suspended in PBS (20 mg/ml). After 21 days, they were divided into five further groups and treated as indicated below.

Group 2 ($n = 8$) animals had no further treatment. These animals are referred to as sensitized not challenged and were used as controls for the following experimental groups in the biochemical and morphological studies on lung tissue samples.

Group 3 ($n = 8$) animals received PBS (1 ml), the drug vehicle, given by subcutaneous injection.

Group 4 ($n = 8$) animals received the PARP inhibitor 3-aminobenzamide (10 mg/kg; Sigma-Aldrich, St. Louis, MO) in 1 ml of PBS s.c. This dose was chosen because it has been previously found to exert a clear-cut PARP inhibition (Thiemermann et al., 1997).

Group 5 ($n = 8$) animals received the PARP inhibitor 5-aminoisoquinolinone [1 mg/kg; 5-aminoisoquinolin-1(2H)-one; Alexis Corporation, Läufelfingen, Switzerland] in 1 ml of PBS s.c. This dose was also chosen as being able to effectively inhibit PARP (Cuzzocrea et al., 2002).

Group 6 ($n = 8$) received the inactive compound *p*-aminobenzoic acid (3 mg/kg; Sigma-Aldrich) in 1 ml of PBS s.c. This is chemically similar to 3-aminobenzamide but lacks PARP inhibitory activity.

Three hours after injections, the animals of all groups, except group 2, underwent challenge with the inhaled antigen, as described below.

Challenge with Inhaled Ovalbumin and Evaluation of Respiratory Activity. The guinea pigs were individually placed in an airtight transparent whole-body chamber, as previously described (Bani et al., 1997). The changes in inner pressure in the respiratory chamber induced by breathing were monitored with a high-sensitivity pressure transducer (Battaglia-Rangoni, Bologna, Italy) connected to a PC2400A channel polygraph (Battaglia-Rangoni). Upon breath stabilization, usually occurring within 30 to 60 s, guinea pigs were challenged with an aerosol of ovalbumin (Fluka, Buchs, Switzerland), 5 mg/ml in saline, for 10 s. The naive guinea pigs (group 1) were included in the antigen challenge to reveal possible breath alterations due to nonspecific stimulation of the airways by the aerosol droplets. The respiratory activity of the animals subjected to the different treatments was registered for 10 min after the onset of aerosol administration and classified according to the criteria reported previously (Bani et al., 1997; Xiang et al., 1998; Liu et al., 2001). Namely, cough was detected as a transient change in the pressure (a rapid inspiration followed by a rapid expiration), whereas dyspnea was detected as a series of irregular breaths of abnormal frequency and amplitude or as repeated gasping. During the experiments, the guinea pigs were visually monitored by two trained observers, who were blinded to group assignment of the animals. In this way, any motion- and sneezing-related changes in the inner pressure of the body chamber could also be disregarded. The following parameters were evaluated (Bani et al., 1997; Xiang et al., 1998; Liu et al., 2001): latency time (seconds) for the first cough stroke or dyspnea episode, cough severity, the product of cough frequency (cough strokes per minute) and mean cough amplitude (excess pressure over the normal breath, in mm Hg), and overall duration of dyspnea (seconds).

At the end of the experiments, the animals of all groups were killed by decapitation, and, upon thoracotomy, the gross appearance of the lungs was examined. In five animals from each group, lung tissue samples from the middle and the lower lobes were taken for biochemical and morphological analyses. Before opening the thorax, three animals from each group were subjected to bronchoalveolar lavage. Briefly, the trachea was cannulated, and bronchoalveolar lavage was performed by the intratracheal installation of 3 ml of PBS, pH 7.4, into the exposed lungs, maintained within the thoracic cavity. Bronchi were washed three times before final collection of bronchoalveolar lavage fluid, which was then centrifuged at 1100g for 30 min. The cell-free supernatant was collected, and its volume measured and frozen at -70°C until needed.

Assay of PARP Activity. This was measured using methods described by Berton et al. (1991). Tissues from control (groups 1 and 2) and sensitized, ovalbumin-challenged guinea pigs (group 3) were homogenized in 50 mM Tris HCl, pH 8, 4°C, containing 0.1% NP-40, 200 mM KCl, 2 mM MgCl₂, 50 μM ZnCl₂, 2 mM dithiothreitol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μl/ml leupeptin, and antipain). Samples were then centrifuged, and 10 μl of each supernatant was incubated for 5 min at 25°C with 2 μl of [³H]NAD⁺ (specific activity 25 Ci/nmol) in 50 mM Tris HCl, pH 8, containing 20 mM MgCl₂, 1 mM dithiothreitol, and 20 μM NAD⁺, in the absence or presence of activated calf thymus DNA, in a final volume of 100 μl. The reaction was stopped by the addition of 5% trichloroacetic acid. Samples were filtered, and radioactivity in the acid-insoluble fraction was counted by a Beckman LS1801 liquid scintillation spectrometer. PARP activity estimated without activated DNA in the mixture was referred to as endogenous activity. Activity estimated in the presence of activated DNA in the assay mixture was referred to as total activity of PARP. Ratio between endogenous and total activities was considered as the measure of PARP activity in the tissues.

Histological and Morphometric Analyses. Small tissue samples, two from each animal, were fixed by immersion in Mota fluid (50% ethanol, 50% H₂O, 0.5% acetic acid, and 1% basic lead acetate), dehydrated in graded ethanol and embedded in paraffin. This fixative medium allows a rapid infiltration of the tissue and provides artifact-free tissue morphology and optimal mast cell preservation. Sections (5 μm thick) were cut and stained with hematoxylin and eosin for conventional histology and morphometry of lung alveoli and small-sized bronchi or with Astra blue (Fluka) to reveal mast cell granules.

A first series of determinations was carried out on hematoxylin and eosin-stained sections to evaluate the surface area of alveolar aerial spaces. In each guinea pig, determinations were performed on tissue sections cut from the two different lung samples, examined with a ×10 objective. Four randomly chosen microscopic fields per animal (two fields per section) were analyzed. At the chosen magnification, each field corresponds to a tissue area of 570,224 μm² that includes an average of 300 alveolar profiles. The same tissue sections were used to evaluate the surface area of bronchial lumina, selected by: histological appearance of small-sized, muscular bronchi; and transverse or slightly oblique cross-section. In each guinea pig, measurements were carried out on four to six randomly chosen bronchi from the tissue sections cut from the two different lung samples, examined with a ×20 objective. For both alveolar and bronchial luminal areas, the microscopic fields to be analyzed were registered by a video camera (S 75D1; World Precision Instruments, Inc., Sarasota, FL) applied to a Reichert-Jung Microstar IV light microscope (Cambridge Instruments, Buffalo, NY) and interfaced with a personal computer through a Matrox Marvel G400-TV digitizing card (Matrox Graphics, Dorval, Canada). On the digitized images, surface area measurements were carried out using the Scion Image Beta 4.0.2 image analysis program (Scion Corporation, Frederick, MD) upon appropriate thresholding to include only blank, tissue-free aerial spaces. The mean values (±S.E.M.) 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 the content of secretory granules. In each guinea pig, determinations were performed on tissue sections cut from the two different lung samples, according to the method described previously for similar purposes (Bani et al., 1997). Mast cells were viewed by the same image analysis device described above, using a ×100 oil immersion objective. In particular, the digitizing card allows the measurement of the light transmitted across the microscopic slide within a range of 256 gray levels (0, black; 255, white) and the reproduction of a digitized image based on the measured values. Determinations of optical density were carried out on selected mast cell profiles using the Scion Image Beta 4.0.2 image

analysis program. In each animal, 30 different mast cells, 15 from each lung sample, were analyzed, and the mean optical density (±S.E.M.) was then calculated for the entire experimental group.

Immunohistochemistry for Poly(ADP-Ribose) and Nitrotyrosine. This was carried out on histological sections, 5 μm thick, of Mota-fixed, paraffin-embedded lung tissue fragments. Sections were treated with 0.3% (v/v) H₂O₂ 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 goat polyclonal anti-poly(ADP-ribose) antibodies (1:500 in PBS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Immune reaction was revealed by indirect immunoperoxidase method (Vectastain Elite kit; Vector Laboratories, Burlingame, CA), using 3,3'-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used.

Peroxyntirite, the harmful coupling product of nitric oxide and superoxide anion, is a major cause for oxidative tissue damage and DNA strand break during inflammation (Zingarelli et al., 1996). Nitrotyrosine, a marker of peroxyntirite reaction with tissue substrates, was detected in lung tissue by rabbit polyclonal anti-nitrotyrosine antibodies (1:1000 in PBS; Upstate Biotechnology, Buckingham, UK) using the same protocol described above.

Evaluation of Myeloperoxidase Activity. This was used as a marker for leukocyte accumulation in tissues, according to Bradley et al. (1982). Briefly, 100 mg of lung tissue were homogenized in 1.5 ml of 50 mM potassium phosphate buffer, pH 6. One milliliter of the homogenate was centrifuged at 10,000g for 10 min and the pellet suspended in 1 ml of potassium phosphate buffer (50 mM), pH 6, containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) to negate the pseudoperoxidase activity of hemoglobin and to solubilize membrane-bound myeloperoxidase. The suspensions were freeze-thawed, sonicated, and centrifuged at 12,000g for 10 min. Myeloperoxidase activity was determined by mixing 0.1 ml of the supernatant with 2.9 ml of potassium phosphate buffer (50 mM), pH 6, containing 0.19 mg/ml *o*-dianisidine chloride and 0.0005% H₂O₂ as a substrate for myeloperoxidase. Oxidized *o*-dianisidine forms a stable chromophore absorbing at a 460-nm wavelength. The absorbance was determined spectrophotometrically over 2 min. The values of tissue myeloperoxidase activity were obtained by comparison with standard concentrations of *o*-dianisidine in the presence of excess H₂O₂. One unit of myeloperoxidase activity is defined as that required to degrade 1 μmol of H₂O₂/min at 25°C. Protein concentration was determined with the Bradford method. The results are expressed as milliunits per milligram of protein.

Determination of Malondialdehyde. Malondialdehyde, an end-product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals, is considered a reliable marker of inflammatory tissue damage and was determined by measurement of the chromogen obtained from the reaction of malondialdehyde with 2-thiobarbituric acid, according to Aruoma et al. (1989). About 100 mg of lung tissue were homogenized with 1 ml of 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4. Then, 0.5 ml of 2-thiobarbituric acid (1% w/v) in 0.05 mol/l NaOH and 0.5 ml of HCl (25% w/v in water) were added to 0.5 ml of sample. The mixture was boiled for 10 min and cooled. The chromogen was extracted in 3 ml of 1-butanol, and the organic phase separated by centrifugation at 2000g for 10 min. The absorbance of the organic phase was read spectrophotometrically at a 532-nm wavelength. Protein concentration was determined with the Bradford method. The values are expressed as nanomoles of thiobarbituric acid-reactive substances (malondialdehyde equivalents) per milligram of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

Nitrite Determination in Bronchoalveolar Lavage Fluid. The intrinsic production of NO was determined by the measurement of nitrites, the end-product of NO metabolism, in the bronchoalveolar lavage fluid. Briefly, nitrates were reduced to nitrites by incubation of the samples with nitrate reductase (270 mU/ml; Sigma-Aldrich) and NADPH (150 mM) at room temperature for 1 h. The amount of

nitrite was determined spectrophotometrically by the Griess reaction adapted for a 96-well plate reader (Failli et al., 2002). Briefly, 100 μ l of sample were allowed to react with 100 μ l of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]ethylenediamine in 5% phosphoric acid) for 30 min in the dark. The optical density at a 546-nm wavelength was measured with a Bio-Rad 550 microplate reader. Nitrite concentrations in the supernatants were calculated by comparison with standard concentrations of NaNO₂ dissolved in PBS. The reported values are expressed as nanomoles per milliliter of bronchoalveolar lavage fluid.

Determination of TNF- α in Bronchoalveolar Lavage Fluid.

Production of TNF- α was measured using a commercial enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI), following the protocol provided by the manufacturer. Results are expressed as nanograms per milliliter of bronchoalveolar lavage fluid.

Statistical Analysis. Unless otherwise stated, the reported data are expressed as mean $\pm$ S.E.M. of the individual values of the animals in each group. Statistical comparison of differences between the different groups was carried out using one-way ANOVA test followed by Student-Newman-Keuls multiple comparison test. $p \leq 0.05$ was considered significant. Calculations were done using a GraphPad Prism 2.0 statistical program (GraphPad Software Inc., San Diego, CA).

Results

Respiratory Activity. The values of the respiratory parameters assayed are reported in Fig. 1, A to C. There were no substantial abnormalities in the nonsensitized guinea pigs after inhalation of ovalbumin aerosol (group 1), apart from sporadic cough strokes appearing about 2 min after the onset of the aerosol. Challenge of vehicle-treated sensitized guinea pigs with the ovalbumin aerosol (group 3) resulted in striking abnormalities of the respiratory pattern, consisting of a significant reduction of the latency time for cough and/or dyspnea and a significant increase in the severity of cough and in the occurrence and duration of dyspnea. Conversely, pretreatment with the PARP inhibitors 3-aminobenzamide and 5-aminoisoquinolinone of sensitized, ovalbumin-challenged guinea pigs (groups 4 and 5) resulted in a marked reduction of the respiratory abnormalities compared with the vehicle-treated sensitized animals (group 3). In particular, cough latency time was significantly increased, and cough severity was significantly reduced. Clear-cut signs of dyspnea were not detected in the breath recordings in any of the animals of these groups. Administration of *p*-aminobenzoic acid in the place of 3-aminobenzamide to sensitized, ovalbumin-challenged guinea pigs (group 6) resulted in clear-cut respiratory abnormalities, similar to those of the vehicle-treated animals (group 3).

PARP Activity. In the lung tissues of naive guinea pigs (group 1, 0.2 ± 0.2), PARP activity was estimated to be almost the same as that measured in sensitized, nonchallenged animals (group 2, 0.26 ± 0.3). Ovalbumin challenge of the sensitized guinea pigs (group 3, 0.7 ± 0.8) induced a 3.5-fold increase in PARP activity over naive animals.

Lung Morphology and Morphometry. Macroscopic examination of the lungs showed prominent changes in the sensitized, ovalbumin-challenged guinea pigs (group 3) compared with the nonsensitized guinea pigs (group 1) or the sensitized animals not subjected to ovalbumin aerosol (group 2). These changes mainly consisted of marked swelling of the pulmonary lobes due to accumulation of air. Sectioning of trachea or of main bronchi did not cause lung deflation, thus indicating that peripheral airway obstruction had occurred.

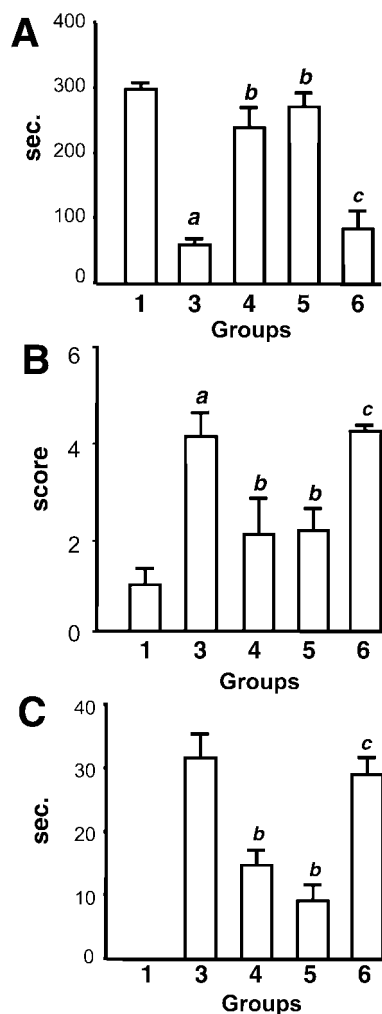


Fig. 1. Occurrence of respiratory abnormalities in guinea pigs of the different experimental groups. A, latency for the onset of cough; B, severity of cough; C, overall duration of dyspnea. Compared with the sensitized, ovalbumin-challenged animals (group 3), pretreatment with 3-aminobenzamide (group 4) or 5-aminoisoquinolinone (group 5) causes a clear-cut, significant improvement of the respiratory parameters assayed. Significance of differences between groups (one-way ANOVA): a, $p > 0.01$ versus sensitized, nonchallenged controls (group 1); b, $p < 0.01$ versus ovalbumin-challenged animals (group 3); and c, $p < 0.01$ versus 3-aminobenzamide-pretreated animals (group 4).

Lung inflation could not be observed in the sensitized, ovalbumin-challenged guinea pigs pretreated with either 3-aminobenzamide or 5-aminoisoquinolinone (groups 4 and 5), whereas it could in most of the animals given *p*-aminobenzoic acid in place of 3-aminobenzamide (group 6).

Light microscopy of lung tissue (Fig. 2) showed that the intrapulmonary bronchi and respiratory air spaces of control guinea pigs (groups 1 and 2) had a normal appearance. In particular, intrapulmonary bronchi showed open lumens with bronchial mucosa forming short folds, and most respiratory air spaces were small sized (Fig. 2, A and B). Conversely, lung tissue from the sensitized, ovalbumin-challenged guinea pigs (group 3) mostly showed a reduction of the lumen of intrapulmonary bronchi, with long mucosal folds expanding into the lumen. Moreover, in large areas of the lung parenchyma, the respiratory air spaces were markedly dilated (Fig. 2C). In the sensitized, ovalbumin-challenged guinea pigs pretreated with 3-aminobenzamide (group 4) or

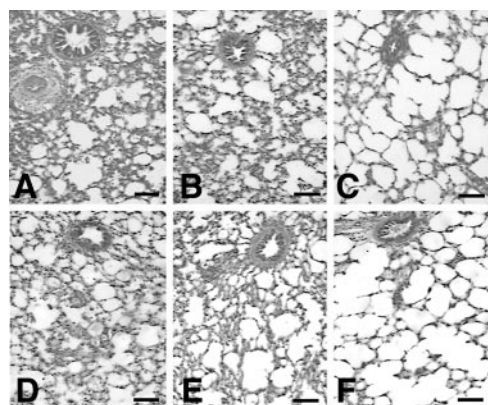


Fig. 2. Lung tissue from guinea pigs of the different experimental groups. A, nonsensitized, ovalbumin-challenged controls (group 1); B, sensitized, nonchallenged controls (group 2); sensitized, ovalbumin-challenged, pretreated with: C, PBS (group 3); D, 3-aminobenzamide (group 4); E, 5-aminoisoquinolinone (group 5); and F, *p*-aminobenzoic acid (group 6). Note the peculiar features of alveolar aerial spaces and small-sized bronchi in groups 3 and 6 (right panels), consistent with alveolar air inflation and bronchoconstriction. Hematoxylin and eosin. Bar = 100 μ m.

5-aminoisoquinolinone (group 5), the histological lung abnormalities were minimal or even absent. In fact, the intrapulmonary bronchi usually showed no appreciable signs of constriction, and most respiratory air spaces were not dilated (Fig. 2, D and E). Only in small areas of the lung parenchyma were attenuated signs of bronchoconstriction and dilation of respiratory air spaces observed. In the sensitized, ovalbumin-challenged guinea pigs treated with *p*-aminobenzoic acid in place of 3-aminobenzamide (group 6), the histological features of the lung tissue were substantially similar to those of the sensitized, ovalbumin-challenged animals of group 3 (Fig. 2F).

The visual observations were objectified by morphometric analysis, whose results are given in Fig. 3. As compared with the control guinea pigs (groups 1 and 2), the sensitized, ovalbumin-challenged guinea pigs (group 3) showed a significant increase in the mean surface area of alveolar aerial spaces (Fig. 3A) and a significant decrease in the mean surface area of bronchial lumens (Fig. 3B). In the sensitized, ovalbumin-challenged guinea pigs treated with 3-aminobenzamide (group 4) or 5-aminoisoquinolinone (group 5), but not with *p*-aminobenzoic acid (group 6), these parameters nearly returned to the control values, 5-aminoisoquinolinone being slightly more potent than 3-aminobenzamide.

Lung Mast Cell Densitometry. This assay (Fig. 3C) revealed a marked, significant decrease in optical density, indicating a decrease in the overall amount of Astra blue-stained secretion granules, in mast cells from the sensitized, ovalbumin-challenged guinea pigs (group 3) compared with those from the control animals (groups 1 and 2). In the mast cells of the sensitized, ovalbumin-challenged guinea pigs pretreated with 3-aminobenzamide (group 4), and even more in those treated with 5-aminoisoquinolinone (group 5), optical density was significantly higher than in the animals of group 3, thus attaining values similar to those in the control animals (groups 1 and 2). Conversely, in the sensitized, ovalbumin-challenged guinea pigs pretreated with *p*-aminobenzoic acid (group 6), the optical density was fairly decreased.

Immunohistochemical Localization of Poly(ADP-Ribose) and Nitrotyrosine. Detection of nuclear poly-ADP ribosylated DNA sites, a marker for PARP activity, in lung

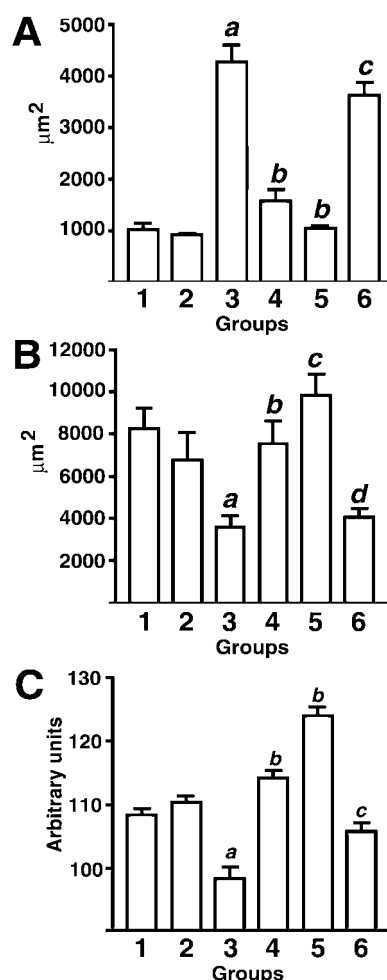
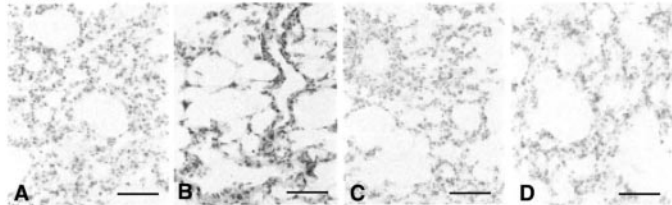


Fig. 3. A, surface area of alveolar aerial spaces in the lungs of guinea pigs from the different experimental groups. Significance of differences between groups (one-way ANOVA): a, $p > 0.001$ versus both controls (groups 1 and 2); b, $p < 0.001$ versus sensitized, ovalbumin-challenged animals (group 3); and c, $p < 0.001$ versus 3-aminobenzamide-pretreated animals (group 4). B, surface area of small-sized bronchial lumens in the lungs of guinea pigs from the different experimental groups. Significance of differences between groups (one-way ANOVA): a, $p > 0.05$ versus both controls (groups 1 and 2); b, $p < 0.05$; c, $p < 0.01$ versus sensitized, ovalbumin-challenged animals (group 3); and d, $p < 0.001$ versus 3-aminobenzamide-pretreated animals (group 4). C, optical density of Astra blue-stained lung mast cells in guinea pigs from the different experimental groups. Significance of differences between groups (one-way ANOVA): a, $p < 0.001$ versus both controls (groups 1 and 2); b, $p < 0.001$ versus ovalbumin-challenged animals (group 3); and c, $p < 0.01$ versus 3-aminobenzamide-pretreated animals (group 4).

tissue cells (Fig. 4A) showed no apparent staining in the nonsensitized guinea pigs (group 1), whereas a few, scattered positive cells could be observed in the sensitized, nonchallenged animals (group 2). Immunostained cells increased markedly in the sensitized, ovalbumin-challenged guinea pigs (group 3). They were mostly located in the airway epithelium and the inflammatory infiltrate within the stromal septa. In the sensitized, ovalbumin-challenged guinea pigs pretreated with 3-aminobenzamide (group 4) or 5-aminoisoquinolinone (group 5), immunostained cells were not detected. Similar findings were observed in the lung tissue sections immunostained to reveal nitrotyrosine (Fig. 4B), a marker of peroxynitrite generation and free-radical tissue injury. No immunostaining was detected in both the control guinea pigs (groups 1 and 2), whereas a clear-cut immuno-

PAR



NT

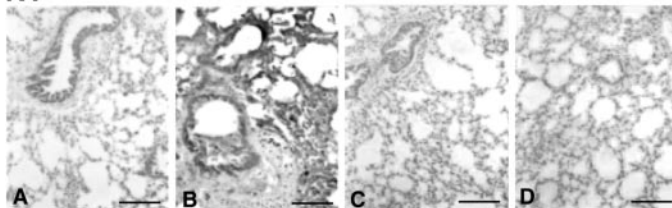


Fig. 4. Immunocytochemical detection of poly-ADP ribosylated DNA (PAR) and nitrotyrosine (NT) in lung tissue from guinea pigs of the different experimental groups. A, nonsensitized, ovalbumin-challenged controls (group 1); B, sensitized, ovalbumin-challenged, pretreated with: PBS (group 3); C, 3-aminobenzamide (group 4); and D, 5-aminoisoquinolinone (group 5). Poly-ADP ribosylated DNA and nitrotyrosine immunoreactivities are intense in the sensitized, ovalbumin-challenged guinea pigs pretreated with the vehicle (group 3), especially in the bronchiolar and alveolar epithelium. Conversely, immunostaining was nearly absent in the sensitized, ovalbumin-challenged animals pretreated with 3-aminobenzamide or 5-aminoisoquinolinone (groups 4 and 5). Counterstaining with hematoxylin. Bar = 100 μ m.

reactivity, distributed evenly in the tissue and especially in the airway epithelium, could be detected in the sensitized, ovalbumin-challenged guinea pigs challenged with ovalbumin (group 3). In the sensitized, ovalbumin-challenged guinea pigs treated with 3-aminobenzamide (group 4) or 5-aminoisoquinolinone (group 5), nitrotyrosine immunostaining was not observed, whereas it was in the guinea pigs given *p*-aminobenzoic acid (group 6).

Myeloperoxidase Activity and Malondialdehyde Production. Both myeloperoxidase activity (Fig. 5A), a marker of leukocyte infiltration into inflamed tissues, and malondialdehyde (Fig. 5B), a marker of free radical-mediated inflammatory damage, underwent a marked, significant increase in the lung tissue from sensitized, ovalbumin-challenged guinea pigs (group 3) as compared with the control animals (groups 1 and 2). In the sensitized, ovalbumin-challenged guinea pigs treated with 3-aminobenzamide and 5-aminoisoquinolinone (groups 4 and 5), myeloperoxidase activity and malondialdehyde were markedly and significantly decreased compared with the animals of group 3, thus attaining values similar to those in the control animals (groups 1 and 2). Conversely, in the guinea pigs given *p*-aminobenzoic acid (group 6), myeloperoxidase activity and malondialdehyde remained high.

Assays on Bronchoalveolar Lavage Fluid. Both nitrites (Fig. 6A), the end-products of NO, and TNF- α (Fig. 6B), an inflammatory cytokine, were increased in the sensitized, ovalbumin-challenged guinea pigs (group 3) as compared with the controls (groups 1 and 2). In the sensitized, ovalbumin-challenged guinea pigs treated with 3-aminobenzamide and 5-aminoisoquinolinone (groups 4 and 5), but not in those given *p*-aminobenzoic acid (group 6), the values of nitrites, as well as those of TNF- α , were significantly lower than in the animals of group 3.

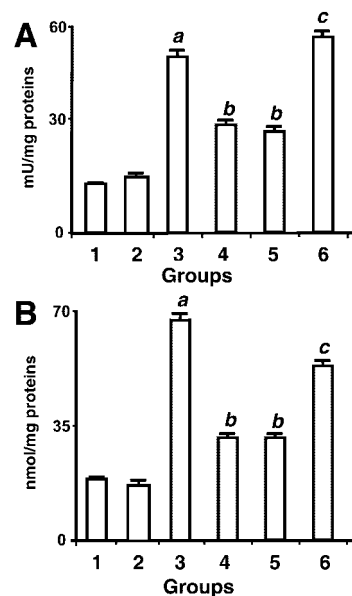


Fig. 5. Myeloperoxidase activity (A) and malondialdehyde production (B) in lung tissue of guinea pigs from the different experimental groups. Significance of differences between groups (one-way ANOVA): a, $p > 0.001$ versus both controls (groups 1 and 2); b, $p < 0.001$ versus sensitized, ovalbumin-challenged animals (group 3); and c, $p < 0.001$ versus 3-aminobenzamide-pretreated animals (group 4).

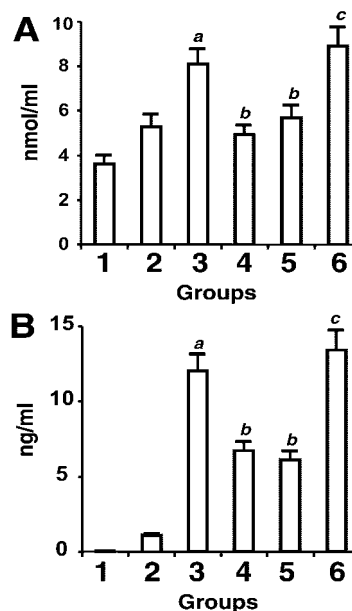


Fig. 6. Nitrite amounts (A) and TNF- α release (B) in bronchoalveolar lavage fluid of guinea pigs from the different experimental groups. Significance of differences between groups (one-way ANOVA): a, $p > 0.01$ versus both controls (groups 1 and 2); b, $p < 0.01$ versus sensitized, ovalbumin-challenged animals (group 3); and c, $p < 0.001$ versus 3-aminobenzamide-pretreated animals (group 4).

Discussion

Asthma is the most common airway inflammatory disease characterized by recurrent exacerbations and atopy as the main predisposing factor (Kitch et al., 2000; Holgate, 2002; Holt and Sly, 2002). In asthmatic patients, chronic airway inflammation is associated with airway hyperresponsiveness, bronchoconstriction, bronchial mucus plug formation, swelling of aerial air spaces, and airway remodeling, all

events concurring to produce airflow limitation and respiratory symptoms (Djukanovic, 2000). Inflammation, remodeling, and altered neural control of the airways are deemed responsible for both recurrent exacerbations of asthma and permanent airflow obstruction (King, 1999; Holt and Sly, 2002; Maddox and Schwartz, 2002). Histopathologically, airway inflammation in asthma is characterized by increased number of activated eosinophils, mast cells, macrophages, and T-lymphocytes in the airway mucosa and lumen. Many different cell types and inflammatory mediators are involved, giving rise to an interlocked cascade of events that results in the characteristic inflammatory and tissue remodeling processes of asthma (Lemanske, 2000; Renauld, 2001; Maddox and Schwartz, 2002).

At present, no definitive cure for asthma has yet been found, although its symptoms can be controlled in most patients by several therapeutic strategies, basically relying on two milestones, glucocorticoids and β_2 -agonists. Inhaled glucocorticoids are the most effective treatment since they are able, in the long term, to markedly reduce the frequency and severity of exacerbations (Lemanske, 2000; von Mutius, 2000). On the other hand, inhaled β_2 -agonists, due to their rapid action, are the drugs of choice to counteract acute asthmatic symptoms (von Mutius, 2000). Alternative treatments, like those based on theophyllines, chromones, immunosuppressors, and the recently developed lipoxygenase inhibitors have not been able to replace the standard therapy of asthma (von Mutius, 2000).

The results presented in this study, using a guinea pig model of allergen-induced asthma-like reaction, offer in vivo evidence for the critical role played by PARP at the very early phase of the response to allergen inhalation by the sensitized animals. In fact, ovalbumin challenge of sensitized guinea pigs caused a marked activation of PARP, as shown by PARP activity assay and immunocytochemistry for poly-ADP ribosylated DNA sites in the lung tissue. Accordingly, the current study also shows that a systemic pretreatment of ovalbumin-sensitized guinea pigs with the PARP inhibitors 3-aminobenzamide and 5-aminoisoquinolinone, at doses and exposure times found previously to afford a complete inhibition of PARP (Thiemermann et al., 1997; Cuzzocrea et al., 2002), results in a clear-cut, statistically significant reduction of respiratory abnormalities induced by the inhaled allergen. In particular, the latency for the onset of cough and/or dyspnea is strongly increased, whereas cough severity and dyspnea are markedly blunted by 5-aminoisoquinolinone (1 mg/kg b.wt.) and, with a slightly lower efficiency, by 3-aminobenzamide (10 mg/kg b.wt.). The beneficial effects of the assayed PARP inhibitors on the respiratory abnormalities of asthma-like reaction can be explained by the marked reduction of the signs of lung inflammation, bronchoconstriction, and air entrapment, as shown by both histopathological and biochemical analyses. As compared with the sensitized, ovalbumin-challenged guinea pigs pretreated with vehicle alone, the lungs from the animals pretreated with either 3-aminobenzamide or 5-aminoisoquinolinone show a significantly lower degree of bronchial constriction, alveolar inflation, and mast cell degranulation, as well as a significant reduction of myeloperoxidase, a marker of leukocyte infiltration, and malondialdehyde and nitrotyrosine, both markers of oxidative inflammatory tissue injury. Moreover, a drop of NO (as nitrite amount) and TNF- α , both inflammation-related molecules,

was also observed in the bronchoalveolar lavage from the animals treated with the PARP inhibitors. The anti-inflammatory effects of 3-aminobenzamide and 5-aminoisoquinolinone appear to rely on their specific PARP-inhibitory properties, as can be argued by the almost complete absence of immunostaining for poly-ADP ribosylated DNA sites in the lung tissue from guinea pigs pretreated with the two inhibitors. Further clues in favor of a primary role of PARP inhibition in the anti-inflammatory mechanism of the assayed inhibitors also come from the findings that: 5-aminoisoquinolinone, a more potent and specific PARP inhibitor than 3-aminobenzamide (Cuzzocrea et al., 2002), also has the most prominent beneficial effects on asthma-like response to ovalbumin; and *p*-aminobenzoic acid, which is similar to 3-aminobenzamide except for ability to inhibit PARP, has no protective effects. The present findings fit well with a recent report by Smulson's group (Boulares et al., 2003) describing the protection from ovalbumin-induced inflammatory lung response in PARP-1^{-/-} knockout mice because both studies strongly suggest that PARP-1 plays a pivotal role in the pathophysiology of asthma-like reaction. However, the possibility of the involvement of other PARP isoforms (Amè et al., 1999; Johansson, 1999; Kickhoefer et al., 1999) should not be ruled out.

The possible causal relationships between PARP inhibition and protection by asthma-like reaction remain to be elucidated. Nonetheless, if it is assumed that, in the very first steps of allergen-induced inflammatory response, PARP may be activated by DNA damage induced by the local generation of harmful reactive oxygen species, it is reasonable to hypothesize that PARP inhibition could reduce the metabolic and oxidative lung cell injury occurring upon massive NAD⁺ and ATP depletion due to activation of PARP. On the other hand, we deem unlikely that the beneficial effects of PARP inhibitors on asthma-like reaction could involve a direct inhibition of allergen-induced activation of lung mast cells, which are known to initiate inflammatory allergic reaction (Bradding, 2003).

Over the last decade, many studies have highlighted the role of PARP activation in the pathophysiology of a wide range of diseases. Furthermore, numerous experiments have proven that PARP inhibition, by both pharmacological tools or by selective disruption of PARP gene, may represent an effective approach to reduce inflammatory tissue injury in animal models of ischemia and reperfusion (Thiemermann et al., 1997; Moroni et al. 2001; Mota-Filipe et al., 2002; for review, see Virag and Szabò, 2002) and of chemically or immunologically induced lung inflammation (Cuzzocrea et al., 2002; Boulares et al., 2003; Bradding, 2003). Of note, the allergen-induced asthma-like reaction in sensitized guinea pigs used in the present study is deemed a reliable model to study the effects of antiasthmatic drugs due to its similarity to human asthmatic disease (Wanner et al., 1990; Pretolani and Vargaftig, 1993). Hence, the present findings suggest that use of PARP inhibitors may be a promising approach to alleviate asthma attack and may provide background for future clinical trials to evaluate the possible antiasthmatic potential of PARP inhibitors, conceivably in synergism with the current therapeutic protocols based on glucocorticoids and β_2 -agonists.

Acknowledgments

We thank T. Sugimura, S. Shall, and G. Piacentini for helpful discussion in the course of this study and critical reading of the manuscript.

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