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Epigallocatechin-3-Gallate Reduces Allergen-Induced Asthma-Like Reaction in Sensitized Guinea Pigs

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ABSTRACT

In this study, we have evaluated the effects of the polyphenol epigallocatechin-3-gallate (EGCG), an antioxidant molecule that also enhances constitutive nitric-oxide synthase (NOS) activity, on antigen-induced asthma-like reaction in sensitized guinea pigs. For comparison, we used epicatechin, which shares antioxidant but not NOS-modulating properties with EGCG. Ovalbumin-sensitized guinea pigs placed in a respiratory chamber were challenged with ovalbumin. EGCG (25 mg/kg b.wt.) or epicatechin (25 mg/kg b.wt.) was given i.p. 20 min before ovalbumin challenge. We analyzed latency time for the onset of respiratory abnormalities, cough severity, duration of dyspnea, lung tissue histopathology, mast cell activation (by granule release), leukocyte/eosinophilic infiltration (by major basic protein and myeloperoxidase), oxygen free radical-mediated injury (by nitrotyrosine and 8-hydroxy-2-deoxyguanosine and superoxide dismutase), NOS activity, and bronchial inflammatory response [by tumor necrosis factor-α in bronchoalveolar lavage (BAL)]. In the sensitized animals, severe respiratory abnormalities appeared soon after the antigen challenge, accompanied by bronchoconstriction, alveolar inflation, and a marked increase in the assayed parameters of inflammatory cell recruitment, free radical lung injury, and release of proinflammatory molecules in BAL fluid. This was associated with marked depression of constitutive NOS activity. Pretreatment with EGCG, but not epicatechin, significantly reduced all the above parameters and sustained endothelial-type NOS activity. These findings provide evidence that EGCG, probably by modulating NOS activity, can counteract allergic asthma-like reaction in sensitized guinea pigs and suggest its possible future use for the treatment of asthma.

Asthma is a common inflammatory airway disease whose prevalence is ever-increasing, especially among children (Kitch et al., 2000), and whose major pathophysiological hallmarks are mast cell activation, increased endothelial expression of adhesion molecules, and enhanced leukocyte recruitment (Maddox and Schwartz, 2002). The therapy mainly relies on β_2-agonists for acute episodes and glucocorticoids for long-term treatment (Von Mutius, 2000). Understanding the factors regulating transition between acute and chronic asthma and airway remodeling, or perpetuation of any of these states, may help identification of new targets for asthma therapy.

In many cell types, nitric oxide (NO) is generated by three NO synthase (NOS) isoenzymes. The neuronal-type (nNOS) and the endothelial-type (eNOS) isoforms are Ca^{2+}/calmodulin-dependent and expressed constitutively. The inducible NOS (iNOS) is Ca^{2+}/calmodulin-independent and is expressed mainly on inflammation (Moncada et al., 1991). Induction of iNOS transcription is mediated by early events, including the activation of nuclear factor κB by lipopolysaccharide, interleukin 1β, and tumor necrosis factor (TNF)-α, as well as of signal transducer and activator of transcription 1 by interferon-γ. Constitutive NOS generate low NO amounts (nanomolar) involved in the regulation of physiological events (Moncada et al., 1991). Conversely, iNOS expression yields high NO levels (micromolar) involved in beneficial functions (host defense) but also deleterious side effects (inflammatory tissue damage) (Moncada et al., 1991). According to the recent literature, n/eNOS-derived NO is crucial to prevent nuclear factor κB activation and iNOS expression; in fact, in early inflammation, a sharp decline in n/eNOS activity and a decrease in endogenous NO often occur. Thus, counteracting the early decrease in physiological NO trig-

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; TNF, tumor necrosis factor; EGCG, epigallocatechin-3-gallate; PBS, phosphate-buffered saline; BAL, bronchoalveolar lavage; eMBP, major basic protein; 8OHdG, 8-hydroxy-2-deoxyguanosine; SOD, superoxide dismutase; NBT, nitro blue tetrazolium; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance.

gered by inflammation could be a potential anti-inflammation strategy (Colasanti and Suzuki, 2000; Mariotto et al., 2004).

Many laboratory and clinical studies show that NO plays a role in asthma (Coleman, 2002). However, the pathophysiological connection between NO and asthma remains uncertain, as does the question of whether NO is primarily beneficial or harmful. Exhaled NO is increased in asthma (Kharitonov et al., 1994) thus being proposed as a marker to monitor underlying inflammation (Horvath et al., 1998). Exogenous NO has bronchodilator and bronchoprotective properties, and endogenous NO release by n/eNOS blunts bronchoconstrictor stimuli. Moreover, NO inhibits mast cell-dependent inflammation mediated by histamine, prostaglandin D_2, leukotriene C_4, and TNF-α. In acute asthma, low NO amounts, such as those released by n/eNOS, are sufficient to exert protection (Kharitonov and Barnes, 2000), probably by scavenging reactive oxygen species (Dweik et al., 2001). Conversely, high NO levels, reflecting iNOS up-regulation by proinflammatory cytokines, can produce deleterious effects, including increased vascular permeability, airway epithelium damage, and leukocyte infiltration (Gaston et al., 1994). Nonetheless, evidence that inhibition of iNOS could be therapeutic in asthma is limited (Mulrennan and Redington, 2004). The conflicting reports on the role played by NO in asthma could be explained by a defect in putative cross-talk between n/eNOS activity and iNOS expression.

In this study, we used an animal model of asthma-like reaction, i.e., ovalbumin challenge to ovalbumin-sensitized guinea pigs, to provide insight into the possible role of n/eNOS-derived NO in the pathophysiology of early asthma and to test the possible therapeutic effect of epigallocatechin-3-gallate (EGCG), a polyphenol that enhances n/eNOS activity.

Materials and Methods

Animals

Male adult albino guinea pigs were used. They were purchased from a commercial dealer (Harlan, Milan, Italy) and quarantined for 7 days at 22–24°C on a 12-h light/12-h dark cycle before use. Standard laboratory chow, fresh vegetables, and water were available ad libitum. The experimental protocol was basically the same used previously for similar purposes (Bani et al., 1997; Suzuki et al., 2004; Masini et al., 2005). 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 to 400 g.

Treatments

Group 1. Ten 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 (Fluka, Buchs, Switzerland) suspended in phosphate-buffered saline (PBS) (5 mg/ml). They are referred to as unsensitized controls.

Other guinea pigs were sensitized with ovalbumin (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 aerosol of ovalbumin (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 airway hyper-responsiveness to the inhaled antigen are referred to as sensitized animals. With the chosen protocol, only two of 42 animals (4.8% of total) failed to develop sensitization. After 4 to 8 days, the sensitized animals were randomly divided in four further groups, 10 animals each, and treated as indicated below.

Group 2. No further treatment: these animals are referred to as sensitized not challenged and were used as sensitized controls for the following experimental groups in the biochemical and morphological studies on lung tissue samples.

Group 3. Treatment with an s.c. injection of 1 ml of PBS: 30 min later, the animals underwent challenge with ovalbumin aerosol, as described below.

Group 4. Treatment with an s.c. injection of EGCG (25 mg/kg b.wt.; Sigma, Milan, Italy) dissolved in 1 ml of PBS: 30 min later, the animals underwent challenge with ovalbumin aerosol, as described below. These dose and exposure times were chosen according to our recent work in experimental rat colitis model (Mazzon et al., 2005).

Group 5. Treatment with an s.c. injection of epicatechin (25 mg/kg b.wt.; Sigma) dissolved in 1 ml of PBS: 30 min later, the guinea pigs underwent challenge with ovalbumin aerosol, as described below.

Evaluation of Respiratory Activity

The guinea pigs of all the groups, except group 2, were placed one by one in a whole body respiratory chamber, as described previously (Bani et al., 1997; Suzuki et al., 2004; Masini et al., 2005). The changes in inner pressure in the respiratory chamber induced by breathing were monitored with a high sensitivity pressure transducer (pressure and linearity ranges from −10 to +10 mm Hg; Battaglia-Rangoni, Comerio, Italy) connected with a multichannel polygraph (Battaglia-Rangoni). On stabilization of the breath pattern (usually occurring within 30–60 s), the guinea pigs were challenged with an aerosol of ovalbumin (5 mg/ml in water) for 10 s. With this device, very small aerosol particles can be obtained that can easily reach the lower respiratory airways, as assessed in previous tests carried out with aerosolization of trypan blue dye dissolved in water. The nonsensitized guinea pigs of group 1 were included in the aerosolic challenge to reveal possible alterations of the breath pattern caused by unspecific stimulation of the airways by the aerosol droplets. The changes in the respiratory activity of the animals subjected to the different treatments were recorded for 10 min after the aerosol administrations. Evaluation of the following parameters was achieved: latency time (s) for the appearance of respiratory abnormalities, assessed as the time between the onset of aerosolization and the first cough stroke, a cough stroke being assumed as a respiratory movement whose amplitude exceeded at least 10 that of normal breath preceding the cough stroke; and cough severity score, assessed as the product of cough frequency and mean cough amplitude, assuming as cough frequency the number of cough strokes per minute and as cough amplitude the excess pressure (mm Hg) over the normal breath preceding the cough stroke. In addition, the occurrence of dyspnea, recognized in breath recordings as a series of irregular breaths of abnormally elevated or reduced amplitude compared with the basal breath, was reported. Movements of 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.

Once extracted from the respiratory chamber, six guinea pigs per group were housed for a further 10 min and even-

tually killed by lethal i.p. injections of sodium thiopental (Abbott, Latina, Italy). Six sensitized guinea pigs of group 2, which were excluded from aerosol administration, underwent the same fate. At death, bronchoalveolar lavage (BAL) was carried out by insertion of a cannula into the trachea and instillation of 3 ml of PBS, pH 7.4. Bronchi were washed three times before collection of bronchoalveolar fluid, which was then centrifuged at 1100*g* for 30 min. The cell-free supernatant was collected, and its volume was measured and frozen at −70°C until needed. The remaining four guinea pigs from each group were removed from the chamber 5 min after the onset of ovalbumin aerosol; they were used to evaluate early NOS activity.

In all the animals, the thorax was then opened, allowing for the gross appearance of lungs to be examined. Tissue specimens from the middle and the lower lobes of the right lung were excised and processed for further analyses, as described in the following.

Histologic and Morphometric Analyses

Tissue samples, two from each animal, were fixed by immersion in Mota fluid, dehydrated in graded ethanol, and embedded in paraffin. This fixative solution allows a rapid infiltration of the tissue, with only minimal artifactual shrinking, thus providing a tissue morphology that is representative of the lung features in vivo. Sections (5 μm thick) were cut and stained with H&E 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 H&E-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 μm^2 that includes an average of 300 alveolar profiles. The same tissue sections were used to evaluate the surface area of bronchial lumina, selected by 1) histological appearance of small-sized, muscular bronchi, and 2) 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 lumenal areas, the microscopic fields to be analyzed were registered by a closed-circuit television camera (WPI, 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, QC, Canada). On the digitized images, surface area measurements were carried out using the Scion Image Beta 4.0.2 image analysis program (Scion Corp., Frederick, MD) on appropriate thresholding to include only blank, tissue-free aerial spaces. The mean values (±S.E.M.) of alveolar and bronchial lumenal 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, as described previously (Bani et al., 1997; Suzuki et al., 2004; Masini et al., 2005). The mast cells were viewed by the same image analysis device described above, using a ×100 oil immersion objective. In particular, the digitizing card allows for the light transmitted across the microscopic slide to be determined within a range of 256 gray levels, which are comprised between 0 (black level) and 255 (white level). The card also allows for a digitized image of mast cells to be reproduced based on the values estimated. Measurements 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 value (±S.E.M.) was then calculated for the entire experimental group.

Immunohistochemistry for Eosinophilic Major Basic Protein 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) 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 mouse monoclonal anti-major basic protein (eMBP) (clone BMK13; Chemicon, Temecula, CA), with a working dilution of 1:50 in PBS. Immune reaction was revealed by indirect immunoperoxidase method (Vectastain Elite kit; Vector, Burlingame, CA) 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 microscopic fields at a 200× final magnification (test area, 72,346 μm^2). Values obtained from two different observers were averaged.

Peroxynitrite, the harmful coupling product of NO and superoxide anion, is a major cause for oxidative tissue damage and DNA strand break during inflammation (Suzuki et al., 2004). Nitrotyrosine, a marker of peroxynitrite reaction with tissue substrates, was detected in lung tissue after microwave antigen retrieval in citrate buffer, pH 6, by rabbit polyclonal antinitrotyrosine antibodies (Upstate Biotechnology, Buckingham, UK) with a working dilution of 1:120 in PBS, applying the same detection procedure described above. The optical density of nitrotyrosine immunostaining in bronchial mucosa was assayed by the same densitometric method described above. In each animal, eight different bronchi, four from each lung sample, were analyzed, and the mean optical density value (±S.E.M.) was then calculated for the entire experimental group.

Evaluation of Myeloperoxidase Activity

Myeloperoxidase activity can be used as a marker for leukocyte accumulation in tissues. It was evaluated according to Bradley et al. (1982). Briefly, lung tissue samples were frozen in isopentane/liquid nitrogen and stored at −80°C until needed. Fragments weighing approximately 100 mg were thawed at room temperature and homogenized in 1.5 ml of 50 mM potassium phosphate buffer, pH 6. One milliliter of the homogenate was centrifuged at 10,000*g* for 10 min, and the pellet was suspended in 1 ml of potassium phosphate buffer (50 mM), pH 6, containing 0.5% hexadecyltrimethylammonium bromide (Sigma) to negate the pseudoperoxidase activ-

ity of hemoglobin and to solubilize membrane-bound myeloperoxidase. The suspensions were treated with three cycles of freezing/thawing, sonicated on ice for 10 s, and centrifuged at 12,000*g* for 10 min. Myeloperoxidase activity was determined in the supernatants: 0.1 ml of the supernatant was mixed with 2.9 ml of potassium phosphate buffer (50 mM), pH 6, containing 0.19 mg/ml *o*-dianisidine chloride and 0.0005% H_2O_2 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 H_2O_2. One unit of myeloperoxidase activity is defined as that required to degrade 1 μmol/min of H_2O_2 at 25°C. Protein concentration was determined with the Bradford method. The results are expressed as milliunit per milligram protein.

Determination of 8-Hydroxy-2′-Deoxyguanosine

Frozen tissue samples were thawed at room temperature, and cell DNA isolation was performed according to Lodovici et al. (2000) with minor modifications. The samples were homogenized in 1 ml of 10 mM PBS, pH 7.4, sonicated on ice for 1 min, added with 1 ml of 10 mM Tris-HCl buffer, pH 8, containing 10 mM EDTA, 10 mM NaCl, and 0.5% SDS, and incubated for 1 h at 37°C with 20 μg/ml RNase (Sigma). Then, the samples were incubated overnight at 37°C under oxygen-free conditions by insufflating argon in the presence of 100 μg/ml proteinase K (Sigma). After incubation, the mixture was extracted with chloroform/isoamyl alcohol (10:2 v/v). DNA was precipitated from the aqueous phase with 0.2 volume of 10 M ammonium acetate, solubilized in 200 μl of 20 mM acetate buffer, pH 5.3, and denatured at 90°C for 3 min. The extract was then supplemented with 10 IU of P1 nuclease in 10 μl and incubated for 1 h at 37°C with 5 IU of alkaline phosphatase in 0.4 M phosphate buffer, pH 8.8. All the procedures were performed in the dark under argon. The mixture was filtered by an Amicon Micropure-EZ filter (Amicon, MA), and 50 μl of each sample was used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) determination using a Bioxytech EIA kit (Oxis, Portland, OR), following the instructions provided by the manufacturer. The values are expressed as nanograms of 8-OHdG per milligram of protein.

Measurement of Superoxide Dismutase Activity

Consumption of endogenous antioxidant enzymes, like superoxide dismutase (SOD), is a typical effect of oxidative stress (Bowler and Crapo, 2002). Thus, measurement of SOD activity is a reliable marker of free radical-mediated lung tissue injury (Nishida et al., 2002). Frozen tissue samples were homogenized with 10 mM PBS, pH 7.4, sonicated on ice for 1 min, and centrifuged at 100*g* for 10 min. Supernatants were used for measurement of manganese-SOD (MnSOD) activity, as described previously (Nishida et al., 2002). This assay is based on SOD-induced inhibition of the conversion of nitro blue tetrazolium (NBT) into a blue tetrazolium salt mediated by superoxide radicals generated by xanthine oxidase. The reaction was performed in sodium carbonate buffer, 50 mM, pH 10.1, containing 0.1 mM EDTA, 25 μM NBT (Sigma), 0.1 mM xanthine, and 2 nM xanthine oxidase (Boehringer Ingelheim, Milan, Italy). The rate of reduction of NBT was monitored with a PerkinElmer (Boston, MA) spectrophotometer set at 560 nm. The amount required to inhibit the rate of reduction of NBT by 50% was defined as 1 unit of SOD activity. Specific MnSOD activity was calculated by inhibiting total SOD activity and preincubating the sample for 30 min with 2 mM NaCN. Values are expressed as milliunit per milligram protein.

Assay of NOS Activity

Because NOS activity may change soon after an inflammatory stimulus is applied, this was evaluated on guinea pigs withdrawn from the respiratory chamber 5 min (each group $n = 4$) or 30 min (each group $n = 6$) after the onset of ovalbumin challenge. Lung tissue specimens from groups 1, 3, 4, and 5 were frozen in isopentane/liquid nitrogen and stored at −80°C. Enzymatic activity of nNOS, iNOS, and eNOS was evaluated by analyzing the transformation of L-[2,3,4,5-^{3}H]-arginine into L-[2,3,4,5-^{3}H]-citrulline according to the modification of the method described by Bredt and Snyder (1990). Tissue samples were homogenized with an UltraTurrax device in lysis buffer containing 50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml antipain, and 1 mM phenylmethylsulphonyl fluoride. The homogenates were centrifuged (25,000*g* for 30 min at 4°C) to separate the cytosol containing nNOS and iNOS from the particulate sediment containing eNOS. The sediment samples, after twice washing with lysis buffer and solubilization with 20 mM Chaps, were centrifuged (25,000*g*, 30 min at 4°C), and the supernatant obtained was designated as particulate fractions. These fractions were further purified by affinity chromatography on 2′,5′-ADP-agarose and were eluted with 10 mM NADPH. An aliquot of the eluate was added to a reaction mixture of a final volume of 100 μl containing 50 mM HEPES, pH 7.4, 20 nM L-[2,3,4,5-^{3}H]arginine, 1 μM arginine, 1 mM NADPH, 1 mM EDTA, 10 μM FAD, 0.1 mM (6*R*)-5,6,7,8-tetrahydro-1-biopterin, and 1 mM dithiothreitol, either in the presence of 1.2 mM $CaCl_2$ and 1 μg/ml calmodulin (for nNOS and eNOS assay) or in the presence of 1 mM EGTA (for iNOS assay). The reaction was stopped by adding 0.4 ml (1:1) of slurry of Dowex AG50WX-8, Na^+ form (Bio-Rad, Hercules, CA) in 50 mM HEPES, pH 5.5, and after 15 min of shaking, the radioactivity in the supernatant was measured. Enzyme activity was linear up to 15-min incubation. For appropriate comparison between groups, data have been expressed as percentage change of NOS activity in the naive controls unless otherwise stated.

TNF-α Determination in BAL Fluid

The pulmonary 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 nanogram per milliliter of BAL fluid.

Statistical Analysis

Statistical comparison of differences between the experimental groups was carried out using one-way analysis of variance (ANOVA) test, followed by the Student-Newman-Keuls multiple comparison test. A $p \leq 0.05$ was considered significant. Calculations were done using a GraphPad Prism 2.0 statistical program (GraphPad Software, San Diego, CA).

Results

ECGC Reduces Ovalbumin-Induced Respiratory Abnormalities. The values of the respiratory parameters assayed are reported in Fig. 1, A through C. There were no substantial abnormalities in the unsensitized control guinea pigs after inhalation of ovalbumin aerosol (group 1), apart from sporadic cough strokes arising approximately 2 min after the onset of the aerosol. Challenge of PBS-pretreated, sensitized guinea pigs with the ovalbumin aerosol (group 3) resulted in striking abnormalities of the respiratory pattern, consisting of a significant reduction of the cough latency time and a significant increase in the severity of cough and in the occurrence and duration of dyspnea episodes.

Conversely, a 30-min pretreatment with EGCG of sensitized guinea pigs before antigen inhalation (group 4) resulted in a marked, statistically significant reduction of the respiratory abnormalities compared with those in the sensitized animals of group 3. In particular, the latency for cough increased and the cough severity decreased. No clear-cut signs of dyspnea were detected in any of the animals of these groups. Pretreatment of the sensitized guinea pigs with epicatechin in place of EGCG (group 5) afforded no protection to the occurrence of respiratory abnormalities on antigen challenge.

EGCG Reduces Ovalbumin-Induced Lung Histopathological Changes. Compared with the unsensitized control guinea pigs (group 1) or the sensitized animals not subjected to ovalbumin aerosol (group 2), macroscopic examination of the lungs showed prominent changes in the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3). The pulmonary lobes were swollen because of air entrapment, and focal subpleural hemorrhage foci could be 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 hemorrhage were not found in the sensitized guinea pigs pretreated with EGCG (group 4), whereas they were in most of the animals given epicatechin in place of EGCG (group 6).

By light microscopy (Fig. 2), the lung parenchyma of control guinea pigs in groups 1 and 2 had a normal appearance: intrapulmonary bronchi showed open lumina, and respiratory air spaces were mostly small-sized (A and B). Conversely, the lungs from the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3) mostly showed a reduction of the lumen of intrapulmonary bronchi, with long mucosal folds expanding into the lumen and markedly dilated respiratory air spaces (Fig. 2C). In the sensitized guinea pigs pretreated with EGCG (group 4), 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. 2D). In the sensitized guinea pigs pretreated with epicatechin in place of EGCG (group 5), the histological features of the lung tissue were substantially similar to those of the PBS-pretreated, sensitized animals of group 3 (Fig. 2E).

The visual observations were objectified by morphometric analysis, whose results are given in Fig. 3. Compared with the control guinea pigs in groups 1 and 2, the PBS-pretreated, sensitized guinea pigs challenged with the antigen (group 3) showed a significant increase in the mean surface area of alveolar air spaces (Fig. 3A) and a significant decrease in the mean surface area of bronchial lumina (Fig. 3B). In the sensitized guinea pigs pretreated with EGCG (group 4), but not with epicatechin (group 5), these parameters nearly returned to the control values.

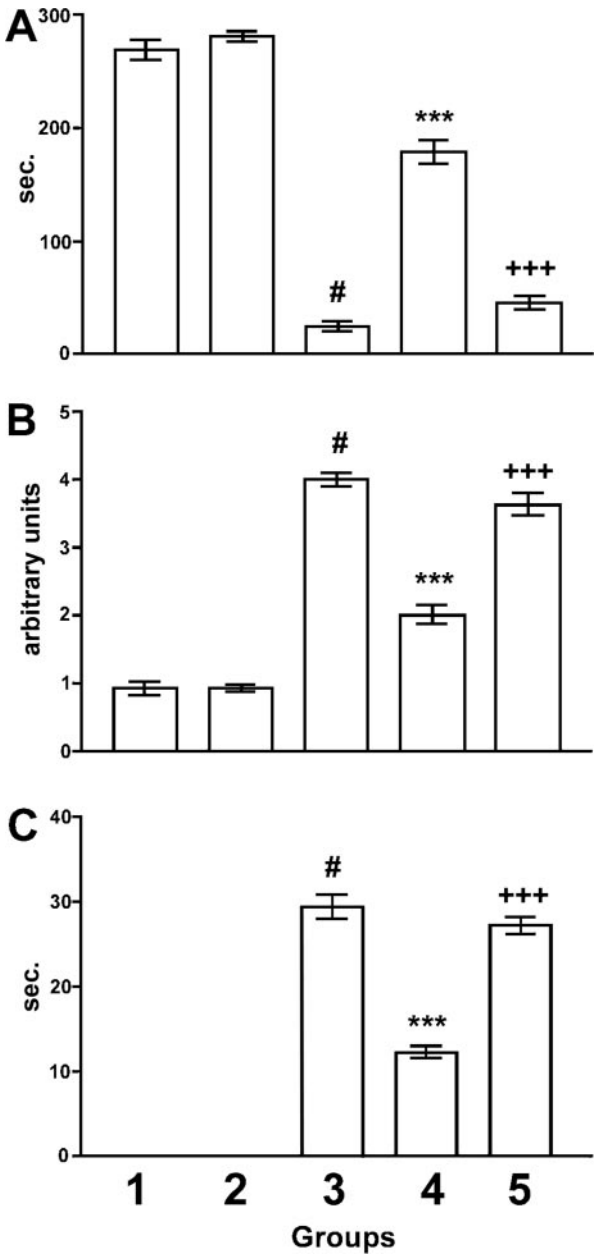

Fig. 1. Occurrence of respiratory abnormalities in guinea pigs of the different experimental groups. A, latency for the onset of respiratory abnormalities. B, severity of cough. C, duration of dyspnea. Compared with the sensitized, ovalbumin-challenged animals given PBS (group 3) pretreatment with EGCG causes a clear-cut, significant improvement of the respiratory parameters assayed. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.001$ versus controls (groups 1 and 2); ***, $p < 0.001$ versus PBS-pretreated (group 3); +++, $p < 0.001$ versus EGCG-pretreated animals (group 4).

EGCG Reduces Ovalbumin-Induced Mast Cell Granule Release. This assay (Fig. 3C) revealed a marked, significant decrease in optical density, indicating a decrease in secretion granule content, in mast cells from the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3) compared with those from the controls (groups 1 and 2). In the mast cells of the sensitized guinea pigs pre-

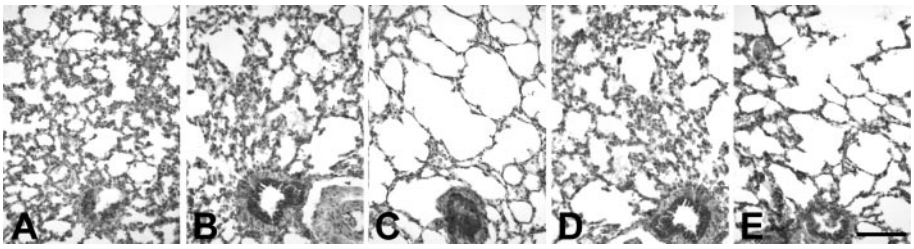

Fig. 2. Representative light micrographs of lung tissue from guinea pigs of the different experimental groups. A, unsensitized, ovalbumin-challenged controls (group 1). B, sensitized, not challenged controls (group 2). C, sensitized, ovalbumin-challenged, pretreated with PBS (group 3). D, sensitized, ovalbumin-challenged, pretreated with EGCG (group 4). E, sensitized, ovalbumin-challenged, pretreated with epicatechin (group 5). Note the peculiar features of alveolar aerial spaces and small-sized bronchi in groups 3 and 5, consistent with alveolar air inflation and bronchoconstriction. H&E; bar, 100 μm.

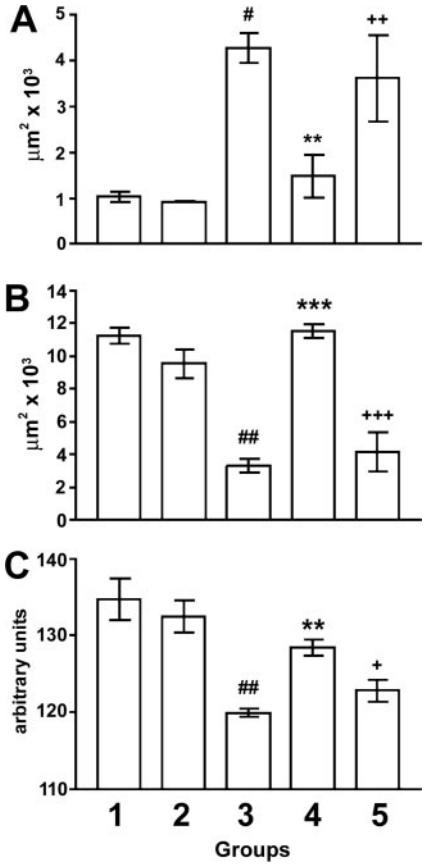

Fig. 3. Surface area of alveolar aerial spaces (A), of small-sized bronchial lumina (B), and optical density of Astra blue-stained lung mast cells (C) in the lungs of guinea pigs from the different experimental groups. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.01$, ##, $p < 0.001$ versus the controls (groups 1 and 2); **, $p < 0.01$, ***, $p < 0.001$ versus PBS-pretreated animals (group 3); +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$ versus EGCG-pretreated animals (group 4).

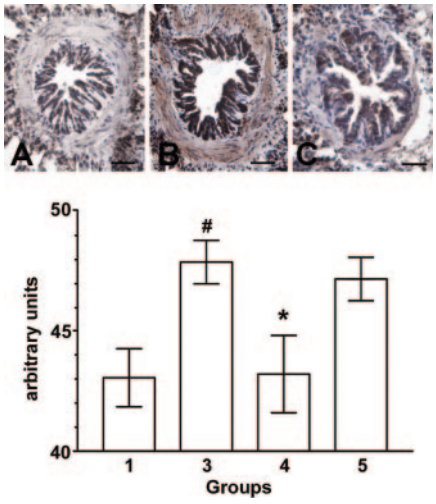

Fig. 4. Immunocytochemical detection of nitrotyrosine in lung tissue from guinea pigs of the different experimental groups. Top, representative light micrographs from (A) unsensitized, ovalbumin-challenged controls (group 1); (B) sensitized, ovalbumin-challenged, pretreated with PBS (group 3); and (C) sensitized, ovalbumin-challenged, pretreated with EGCG (group 4). Nitrotyrosine immunoreactivity is intense in the PBS-pretreated guinea pigs of group 3 but not in those pretreated with EGCG. Counterstaining with hematoxylin; bar, 100 μm. Bottom, densitometric evaluation of nitrotyrosine immunostaining. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.05$ versus controls (group 1); *, $p < 0.05$ versus PBS-pretreated animals (group 3).

treated with EGCG (group 4), optical density underwent a marked, significant increase compared with the animals of group 3, thus attaining values similar to those of the controls. On the other hand, in the guinea pigs given epicatechin (group 5), mast cell optical density was decreased.

EGCG Reduces Ovalbumin-Induced Free Radical Generation and Tissue Damage. Immunocytochemical detection of nitrotyrosine, the product of protein nitration by peroxynitrite, in the bronchial mucosa (Fig. 4) showed no staining in the control guinea pigs of groups 1 and 2. Immunostained cells increased markedly in the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3). They were mostly located in the airway epithelium and the inflammatory infiltrate within the tunica propria. In the sensitized guinea pigs pretreated with EGCG (group 4), nitrotyrosine immunostaining was very faint, at variance with the animals pretreated with epicatechin (group 5).

The assayed indicators of lung oxidative stress, e.g., the marker of free radical-induced DNA damage 8-OHdG (Fig. 5A), and the decrease in the activity of the antioxidant enzyme MnSOD (Fig. 5B) underwent clear-cut, significant changes in the lungs from PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3) compared with the controls (groups 1 and 2). In the guinea pigs pretreated with EGCG (group 4), 8-OHdG was markedly and significantly lower, whereas MnSOD activity was significantly higher than in the animals of group 3, thus attaining values similar to those of the controls. On the other hand, in the guinea pigs given epicatechin (group 5), 8-OHdG remained high, although at lower levels than in the sensitized animals of group 3, whereas MnSOD activity was markedly reduced.

EGCG Reduces Ovalbumin-Induced Lung Infiltration by Inflammatory Leukocytes. Myeloperoxidase ac-

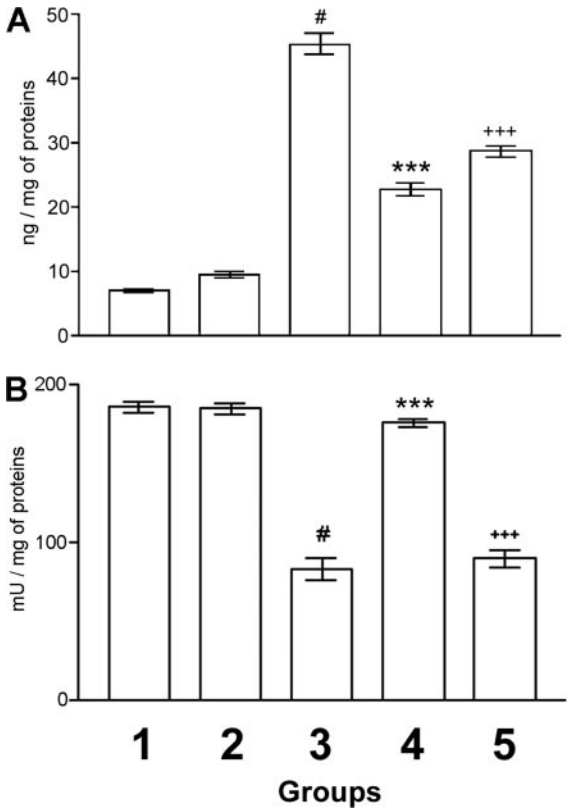

Fig. 5. 8-OHdG (A) and MnSOD activity (B) in lung tissue of guinea pigs from the different experimental groups. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.001$ versus controls (groups 1 and 2); ***, $p < 0.001$ versus sensitized, PBS-pretreated animals (group 3); +++, $p < 0.001$ versus EGCG-pretreated animals (group 4).

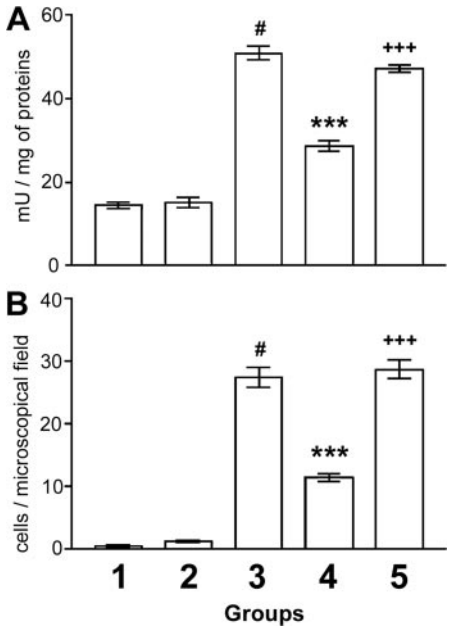

Fig. 6. Lung tissue myeloperoxidase (A) and number of eMBP-immunoreactive eosinophils (B) in the guinea pigs from the different experimental groups. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.001$ versus the controls (groups 1 and 2); ***, $p < 0.001$, versus PBS-pretreated animals (group 3); +++, $p < 0.001$ versus EGCG-pretreated animals (group 4).

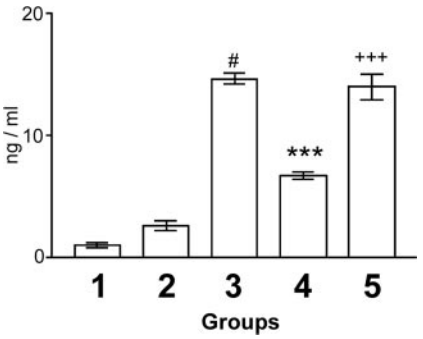

Fig. 7. TNF-α release in BAL fluid of guinea pigs from the different experimental groups. Significance of differences (one-way ANOVA; each group $n = 6$): #, $p < 0.001$ versus the controls (groups 1 and 2); ***, $p < 0.001$ versus PBS-pretreated animals (group 3); +++, $p < 0.001$ versus EGCG-pretreated animals (group 4).

tivity (Fig. 6A), a marker of leukocyte infiltration into inflamed tissues, as well as eMBP-positive eosinophils (Fig. 6B), underwent a marked, significant increase in the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3) compared with the controls (groups 1 and 2). In the sensitized guinea pigs pretreated with EGCG (group 4), but not in those given epicatechin (group 5), myeloperoxidase activity and the number of eMBP-positive eosinophils were markedly and significantly decreased compared with the animals of group 3, thus attaining values similar to those in the controls (groups 1 and 2).

EGCG Reduces Ovalbumin-Induced Release of TNF-α in BAL Fluid. The inflammatory cytokine TNF-α (Fig. 7) was increased in the PBS-pretreated, sensitized guinea pigs challenged with ovalbumin (group 3) compared with the controls (groups 1 and 2). In the sensitized guinea pigs pretreated with EGCG (group 4), but not in those given epicatechin (group 6), the values of TNF-α were significantly lower than in the animals of group 3.

EGCG Reverts Ovalbumin-Induced Inhibition of n/eNOS Activity. The lung tissues of unsensitized control guinea pigs (group 1) expressed Ca^{2+}-dependent NOS activity both in cytosolic and membrane-bound fractions (nNOS and eNOS, respectively), the latter being more prominent (Fig. 8A). There was no sign of Ca^{2+}-independent NOS activity, indicating the absence of iNOS expression. A 5-min challenge of PBS-pretreated, sensitized guinea pigs (group 3) with ovalbumin resulted in a dramatic decrease in both enzymatic activities, leading to nonmeasurable nNOS activity and to approximately 50% reduction of eNOS activity (Fig. 8A). At this time, no iNOS activity could be detected in PBS-pretreated, sensitized ovalbumin-challenged animals (group 3, data not shown). In the sensitized guinea pigs pretreated with EGCG (group 4), nNOS activity remained low, whereas eNOS activity was more than 20% higher than corresponding values in the unsensitized controls (group 1). Pretreatment of sensitized animals with epicatechin before ovalbumin challenge (group 5) caused a slight recovery of nNOS activity but completely failed to counteract the ovalbumin-induced decrease in eNOS activity. As a whole, EGCG pretreatment had little or no effect on nNOS activity in ovalbumin-challenged animals but strongly counteracted the ovalbumin-induced decrease of eNOS activity, thus maintaining more than 70% of the cumulative activity of constitutive NOS enzymes compared with the unsensitized, control animals (Fig. 8B).

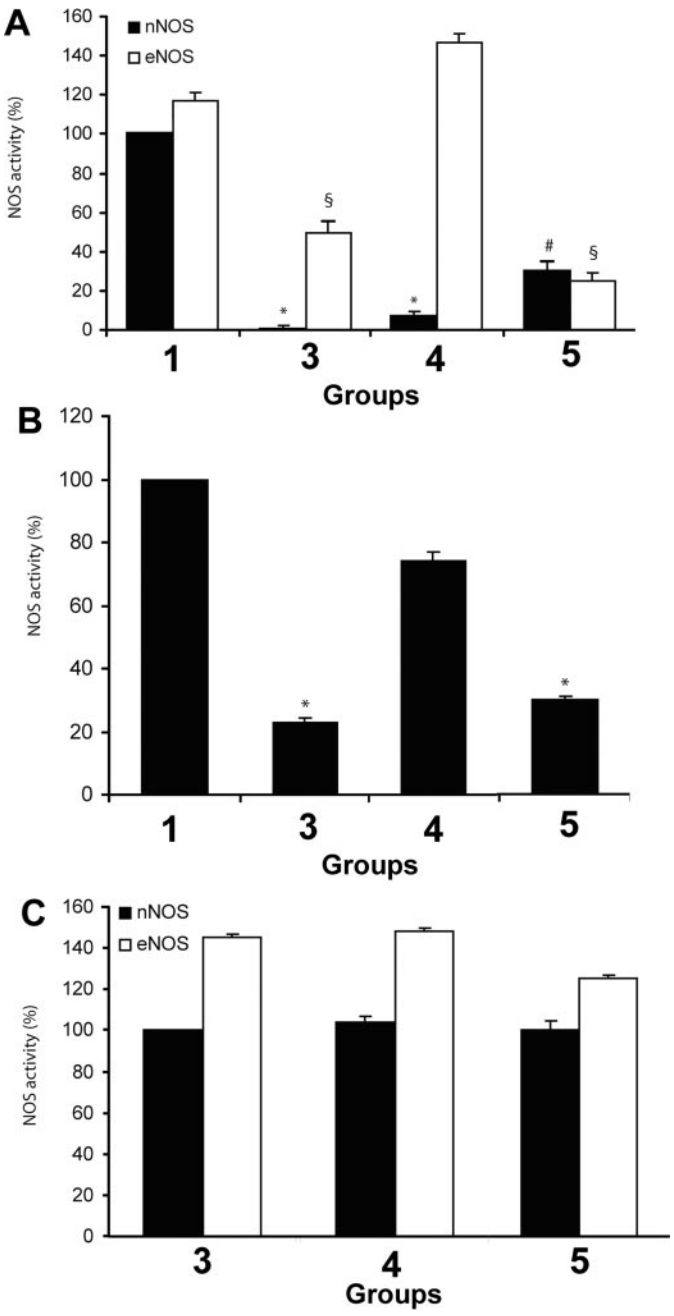

Fig. 8. NOS activity in the lung of ovalbumin-challenged guinea pigs. A, nNOS and eNOS activity in the lungs, measured 5 min after ovalbumin challenge, in the absence and presence of EGCG or epicatechin pretreatment. Data are presented as percentage of nNOS activity measured in the unsensitized control animals (group 1). Significance of differences (one-way ANOVA; each group $n = 4$): *, $p < 0.01$ and #, $p < 0.05$ versus nNOS in group 1 animals; §, $p < 0.05$ versus eNOS in group 1 animals. B, total constitutive NOS activity, calculated by the sum of nNOS and eNOS activities, measured 5 min after ovalbumin challenge. Data are presented as percentage of the value of unsensitized control animals (group 1). Significance of differences (one-way ANOVA; each group $n = 4$): *, $p < 0.005$ versus group 1 animals. C, nNOS and eNOS activity in the lung was measured 30 min after ovalbumin challenge in the absence and presence of EGCG or epicatechin pretreatment. Data are presented as percentage of nNOS activity measured in PBS-pretreated animals (group 3). Differences are not statistically significant (one-way ANOVA; each group $n = 6$).

Thirty minutes after ovalbumin challenge, the activity of both nNOS and eNOS in the lungs was similar in all the experimental groups (Fig. 8C), regardless of pretreatment with PBS (group 3), EGCG (group 4), or epicatechin (group 5). This indicates that the depressant effect of ovalbumin challenge on e/nNOS activity lasts less than 30 min. At this time point, iNOS activity was still undetectable (data not shown).

Discussion

This study provides evidence that EGCG, a main polyphenol present in the green tea leaves capable of enhancing n/eNOS activity, but not its analog epicatechin-lacking NOS-activating properties, is able to counteract the allergen-induced functional, biochemical, and histopathological lung changes in a guinea pig model of allergic asthma-like reaction, similar to that used previously to test potential antiasthmatic substances (Bani et al., 1997; Suzuki et al., 2004; Masini et al., 2005). Compared with the PBS-pretreated animals, systemic pretreatment of ovalbumin-sensitized guinea pigs with EGCG as early as 30 min before ovalbumin challenge caused a marked, significant reduction of the occurrence of respiratory abnormalities (cough and dyspnea), bronchial lumen reduction, alveolar hyperinflation, leukocyte and eosinophilic infiltration (eMBP-positive cells, myeloperoxidase), mast cell activation (granule release), free radical-induced tissue injury (nitrotyrosine, 8-OHdG), and consumption of endogenous antioxidant enzymes (MnSOD activity). Moreover, EGCG pretreatment resulted in almost complete prevention of the early decrease in lung eNOS activity occurring on ovalbumin challenge.

It has been reported that inflammatory cells produce large amounts of superoxide anion in asthma and that an inverse correlation exists between superoxide production from leukocytes and in 1 s, suggesting that airway obstruction in asthma is associated with increased production of superoxide anion by leukocytes (Jarjour and Calhoun, 1994). Indeed, scavenging of superoxide by SOD mimetics results in clear-cut protection against ovalbumin-induced acute bronchospasm, lung inflammation, and prostaglandin production in sensitized guinea pigs (Masini et al., 2005). In the present study, we have observed that lung leukocyte infiltration, evaluated as myeloperoxidase activity, and eosinophilic accumulation, evaluated as eMBP-positive cells, were significantly reduced in the guinea pigs treated with EGCG, thus confirming our previous results obtained with this substance in other models of inflammation (Townsend et al., 2004). As known, infiltration of leukocytes, especially eosinophils, is a prominent feature of asthmatic lungs, and inflammatory mediators released by these cells give a major contribution to the pathogenesis of allergic asthma (Kroegel et al., 1994). Therefore, the reduction of leukocyte infiltration may be one of the key mechanisms for the beneficial effect of EGCG. In this context, it is worth noting that NO has been shown to induce a dramatic reduction in the recruitment of inflammatory leukocytes by a down-regulation of endothelial cell adhesion molecules (Kubes et al., 1991; Sluiter et al., 1993; Laroux et al., 2000).

Acute airway response to allergens is known to depend on eosinophils and mast cells (Wardlaw et al., 1988). Besides leukocyte infiltration, pretreatment with EGCG also prevented mast cell activation, as judged by the marked reduction of granule discharge. In turn, mast cell mediators may cause bronchoconstriction and smooth muscle cell proliferation and may recruit other inflammatory cells, thereby

sparking a vicious cycle that may amplify the pathophysiological features of asthma. It is even possible that EGCG may slow down mast cell reactivity by inhibiting oxygen free radical-induced activation of high-affinity IgE receptors (Yoshimaru et al., 2002). Although evidence for mast cells as key players in acute allergic reactions is well documented, their role in the late-phase response is controversial. Studies in transgenic mast cell-deficient mice have shown that these cells may not be essential to the asthma-like reaction in mice but can represent an important local amplifier of antigen-dependent bronchoconstriction (Williams and Galli, 2000). In fact, even in mast cell-deficient mice, sensitization agents can produce strong nonspecific responses, most likely because of the use of artificial adjuvants in many sensitization protocols, which promote a strong antibody response and influence the features of this asthma-like model. In human asthmatic lungs, mast cell number is increased and bronchial hyper-responsiveness correlates with the number of these cells. Endobronchial biopsies show increased mast cell degranulation in asthmatic patients compared with nonasthmatic subjects (Beasley et al., 1989; Djukanovic et al., 1992; Pesci et al., 1993). Moreover, the levels of prostaglandin D_2, the major cyclo-oxygenase product generated by activated mast cells during allergic response (Bochenek et al., 2004), are elevated in urine of asthmatic patients after antigen exposure in both the early and late phases, suggesting an involvement of these cells in any phase of the asthmatic response (O'Sullivan, 1999).

A critical role played by NO in animal models of asthma has been reported in previous studies (Barnes, 1996; Coleman, 2002), which underlined the protective effect of low amounts of NO, as those released by constitutive NOS isoenzymes, at the early phase of inflammatory response, such as allergen-triggered mast cell degranulation. Consistently, inhibition of NOS enhanced allergen-induced histamine release in vivo (Masini et al., 1991). In line with this notion, the present findings show that ovalbumin challenge rapidly induces a decrease in both nNOS and eNOS activity in the lung tissue, up to 20% of the basal e/nNOS activity. In vitro studies with the human alveolar pulmonary cell line A549 showed that EGCG (50 μM), but not epicatechin (50 μM), time-dependently enhanced eNOS activity (A. Ciampa, unpublished data). This finding fits well with the current observation that administration of EGCG 30 min before ovalbumin aerosol to animals efficiently counteracts the early decrease of eNOS activity. Overall, in the ovalbumin-challenged animals receiving EGCG, the total constitutive NOS activity was approximately 70% of the values of the unsensitized controls, indicating that EGCG can afford an almost normal production of NO in the lung tissue despite ovalbumin challenge. The presence of nearly physiological level of NO in the lungs of the EGCG-treated animals has probably played a prominent role in the observed prevention of ovalbumin-induced mast cell activation.

The molecular mechanisms by which EGCG can sustain eNOS activity in the lungs of ovalbumin-challenged animals remain to be elucidated. Likely, the antioxidant properties of EGCG are only marginally involved because epicatechin, which is as potent as EGCG in antioxidant action, showed no effects on eNOS activity. Rather, it could be speculated that EGCG, by its multiple phenolic groups spontaneously oxidizing to diones, might donate to eNOS the electrons needed for catalysis of L-arginine to L-citrulline and NO, similarly to the endogenous eNOS cofactors NADH, FAD, and FMN (Griffith and Stuher, 1995). Anyhow, in keeping with the known action of eNOS-derived NO in regulating vascular function (Moncada et al., 1991), our findings suggest that eNOS rather than nNOS is critically involved in the local generation of NO required to afford protection of the lung from the noxious effects of inhaled allergens.

The effect of EGCG in counteracting the decrease in NOS activity on ovalbumin challenge fits well with its protective effect on very early asthma-like reactions such as bronchial lumen narrowing, alveolar inflation, increase in inflammatory cell recruitment, free radical lung injury, and release of proinflammatory molecules in BAL fluid, all of which can be elicited by mast cell-derived inflammatory mediators, including histamine, arachidonic acid metabolites, and cytokines. Although EGCG is a potent antioxidant, its effect on eNOS activity should not involve this property.

In summary, the present study shows that ovalbumin challenge induces a rapid decrease in lung n/eNOS activity and that the protective effect of EGCG pretreatment against ovalbumin-induced asthma-like reaction may be exerted through the ability of EGCG to sustain eNOS activity and to hold endogenous NO generation at physiological levels. This is in keeping with the previously reported findings that low amounts of NO are protective in inflammatory diseases, including asthma, because they seem to be required to counteract the activation of iNOS (Colasanti and Suzuki, 2000; Mariotto et al., 2004), which releases micromolar, harmful NO levels. In turn, excess NO can bind to nitrosylate and activate cyclo-oxygenase-2 to produce inflammatory prostaglandins (Kim et al., 2005), thereby sparking a vicious cycle leading to further iNOS induction and increasing generation of proinflammatory eicosanoids and cytokines. Taken together, the present findings strongly support EGCG as a valid therapeutic approach for the treatment of asthma, as well as of other inflammatory bronchopulmonary diseases involving an imbalance of NO/NOS biosynthetic pathway.

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