

Inhibition of Secretory Phospholipase A₂-Induced Cytokine Production in Human Lung Macrophages by Budesonide

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Key Words

Budesonide • Macrophages • Cytokines • Secretory phospholipases A₂

Abstract

Background: Secretory phospholipases A₂ (sPLA₂) are an emerging class of mediators of inflammation. These enzymes are released in vivo in patients with systemic inflammatory diseases and allergic disorders. sPLA₂s may activate inflammatory cells by both enzymatic and nonenzymatic mechanisms. The aim of this study was to evaluate the effect of the inhaled glucocorticoid budesonide on sPLA₂-induced activation of primary human macrophages. **Methods:** Macrophages isolated from human lung tissue were preincubated (3–18 h) with budesonide (1–1,000 nM) before stimulation with 2 distinct sPLA₂s (group IA and group X). At the end of incubation the release of TNF- α , IL-6 and IL-8 was assessed by ELISA. Specific mRNA for these products was determined by quantitative RT-PCR. Activation of mitogen-activated kinases ERK 1/2 and p38 was assessed by Western blot. **Results:** Budesonide inhibited the release of TNF- α , IL-6 and IL-8 from sPLA₂-stimulated macrophages in a concentration-

dependent manner. The inhibitory effect of budesonide was due to a reduction of gene expression and was complete after 18 h of preincubation. Budesonide had no effect on sPLA₂-induced arachidonic acid mobilization and exocytosis, assessed as β -glucuronidase release. Suppression of cytokine/chemokine production by budesonide was associated with inhibition of sPLA₂-induced ERK 1/2 and p38 activation. **Conclusions:** Budesonide inhibits the production of proinflammatory cytokines/chemokines from human lung macrophages activated by sPLA₂. Budesonide represents the first example of a drug able to block the nonenzymatic effects of sPLA₂ on human inflammatory cells and, therefore, may provide a useful therapeutic options for diseases associated with enhanced release of sPLA₂s in vivo.

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Introduction

Phospholipases A₂ (PLA₂) are a family of enzymes responsible for mobilization of arachidonic acid (AA) from membrane phospholipids. This family includes both cy-

tosolic (cPLA₂) and secretory (sPLA₂) forms of phospholipases [1]. Secretory PLA₂s are enzymes released in the extracellular environment during inflammatory reactions [2]. At least 10 forms of sPLA₂s have been identified in mammalian cells and classified in different groups and subgroups [3]. In this text, PLA₂ groups are denoted with the letter 'G' followed by a Roman numeral indicating the group number and a capital letter indicating the subgroup (e.g. GIB = group IB PLA₂).

Increased levels of sPLA₂ are detected in plasma or biological fluids of patients with inflammatory and autoimmune diseases (e.g. acute pancreatitis, septic shock, rheumatoid arthritis, Crohn's disease and ulcerative colitis) [4]. sPLA₂ activity is detectable in the bronchoalveolar lavage fluid of healthy individuals [5], both smokers and nonsmokers [6]. This activity is significantly increased in the airways of patients with various inflammatory diseases of the lung (pneumonia, septic shock, trauma and adult respiratory distress syndrome) [7–9]. Moreover, sPLA₂ activity increases 3- to 5-fold upon specific antigen challenge in the upper and lower respiratory tract in patients with allergic rhinitis and bronchial asthma, respectively [10–12]. While certain sPLA₂s (GIB, GIIA, GIID, GV and GX) are overexpressed in biopsies from patients with lung inflammation [13] or rhinitis [14], the isoforms of sPLA₂ released in the airways of patients with inflammatory lung diseases have not been fully characterized.

sPLA₂s exert multiple proinflammatory effects related to their enzymatic activity as well as to their capacity to stimulate inflammatory cells by nonenzymatic mechanisms [15]. As enzymes, sPLA₂s hydrolyze membrane phospholipids and produce free AA and lysophospholipids, which are substrates for the biosynthesis of proinflammatory lipid mediators (prostaglandins, leukotrienes, lipoxins and platelet-activating factor) [1]. However, sPLA₂s induce other biological effects, such as activation of exocytosis, cytokine/chemokine production, expression of surface molecules and nitric oxide generation, by nonenzymatic mechanisms involving the interaction with binding molecules (peptidoglycans) and specific or promiscuous receptors expressed on inflammatory and immune cells [16–20].

Lung macrophages are a major cellular target of sPLA₂s released in human airways. Concentrations of GIA, GIB, GIIA and GX sPLA₂s comparable to those found in the bronchoalveolar fluid in vivo induce the release of preformed mediators (β-glucuronidase) and the production of various proinflammatory cytokines and chemokines [20–22]. Macrophages are the predominant

immunocompetent and inflammatory cells in the human lung, where they play an important role in the pathogenesis of chronic lung diseases [23]. The ability of sPLA₂s released in the airways of patients with inflammatory lung diseases to activate several functions of macrophages supports the role of these molecules in lung inflammation and remodeling.

Inhaled glucocorticoids are widely used in the treatment of inflammatory lung diseases such as asthma [24] and chronic obstructive pulmonary disease [25]. The therapeutic effects of these drugs are primarily attributed to their ability to reduce airway inflammation by inhibiting the production of proinflammatory cytokines and chemokines and by preventing recruitment and activation of inflammatory cells [26]. Glucocorticoids have been shown to modulate eicosanoid production by regulating the activity of cPLA₂s, which are the main enzymes responsible for AA generation in inflammatory cells [27]. While cPLA₂s appear to be a main target of the anti-inflammatory effect of glucocorticoids, little is known about the effect of this class of drug on the secretory isoforms of PLA₂. There is evidence that glucocorticoids may indirectly influence the proinflammatory activity of sPLA₂s by reducing the inducible expression of certain sPLA₂ isoforms [28]. For example, dexamethasone inhibits the de novo expression of GIIA in alveolar macrophages stimulated by formylated peptides [29]. However, it is not known whether glucocorticoids may directly influence the proinflammatory activities of sPLA₂s exerted by either enzymatic or nonenzymatic mechanisms. To address this issue we examined the effects of the inhaled glucocorticoid budesonide on the activation of primary human lung macrophages induced by 2 distinct sPLA₂s.

Materials and Methods

Reagents

The following were purchased: group IA sPLA₂ (svGIA; from *Naja mossambica mossambica* venom), fatty acid-free human serum albumin, BSA, piperazine-*N,N'*-bis-2-ethanesulphonic acid (PIPES), L-glutamine, antibiotic-antimycotic solution (10,000 UI/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B), dimethyl sulfoxide (DMSO), actinomycin D, cycloheximide (CHX), Percoll®, Tryton X-100 and phenolphthalein glucuronide (Sigma); RPMI-1640, fetal calf serum (FCS) and guanidine hydrochloride (MP Biomedicals Europe); arachidonic acid (Biomol); [3,6,8,9,11,12,14,15-³H] arachidonic acid ([³H]AA; 100 Ci/mmol; Du Pont NEN Products); rabbit antibodies (Abs) anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-phospho-p38 (Thr180/Tyr182), and the compound PD98059 (Cell Signaling); the compound SB203580 (Calbiochem); rabbit Abs anti-ERK2 (C-14) and anti-p38 (C-20), Horseradish peroxidase-conjugated donkey anti-rab-

bit Ab (Santa Cruz Biotechnology). Human recombinant group X sPLA₂ (hGX) was a generous gift from Michael H. Gelb (Departments of Chemistry and Biochemistry, University of Washington, Seattle, Wash., USA). Budesonide was kindly donated by Astra-Zeneca (Italy).

Isolation and Purification of Human Lung Macrophages

Macrophages were obtained from the lung parenchyma of patients undergoing thoracic surgery for lung carcinoma, as previously reported [20]. The study protocol was approved from the ethics committee of the University of Naples Federico II and informed consent was obtained from patients undergoing thoracic surgery. Briefly, macroscopically normal tissue was minced finely with scissors, washed extensively with PIPES buffer [20] over Nytex cloth (120- μ m pore size; Sefar Italia) and the dispersed cells recovered. The macrophage suspension was enriched (75–85%) by flotation over Percoll[®] density gradients. The cells were suspended (10⁶ cells/ml) in RPMI-1640 containing 5% FCS, 2 mM L-glutamine, and 1% antibiotic-antimycotic solution and incubated in 6- to 24-well plates (Falcon; Becton Dickinson) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 12 h, more than 98% of adherent cells were macrophages, as assessed by α -naphthylacetate esterase staining [30]. In the experiments with freshly isolated macrophages, the enriched macrophage suspension was layered onto additional Percoll[®] gradients to reach a purity ranging between 95 and 99%, as assessed by CD14/CD71/HLA-DR triple staining by flow cytometry [31].

Cell Incubations

Adherent macrophages were incubated (37°C, 3–24 h) in RPMI-1640 containing DMSO diluent or various concentrations (1–1,000 nM) of budesonide in the presence or the absence of 5% FCS. At the end of the incubations, washed cells were incubated (37°C, 2–6 h) in RPMI-1640 alone or with svGIA (30 nM) or hGX (50 nM) sPLA₂s. Commercial preparations of sPLA₂ were repurified by size exclusion chromatography [18] before use. sPLA₂ preparations were discarded if LPS contamination was detected (*Limulus amebocyte* test; MP Biomedical). At the end of the experiment, the supernatant was removed, centrifuged twice (1,000 g, 4°C, 5 min) and stored up to 72 h at –80°C for subsequent determination of β -glucuronidase, TNF- α , IL-6 and IL-8/CXCL8 release. The cells remaining in the plates were lysed with 0.1% Triton X-100 for the determination of total cellular content of proteins and β -glucuronidase.

ELISA for TNF- α , IL-6 and IL-8

Cytokine/chemokine release into the supernatant of macrophages was measured in duplicate determinations using ELISA kits for TNF- α , IL-6 and IL-8/CXCL-8 (Euro Clone). The linearity range of the assay was 20–800 pg/ml (TNF- α), 6–200 pg/ml (IL-6) and 30–2,000 pg/ml (IL-8/CXCL8). The results were normalized for the total protein content in each well determined in the cell lysates (0.1% Triton X-100) by a Bradford-based assay (Bio-rad).

RT-PCR for TNF- α , IL-6 and IL-8

Adherent macrophages (4 $\times$ 10⁶/2 ml) were incubated (37°C, 3–18 h) in RPMI-1640 containing DMSO diluent or budesonide (1 μ M) in the presence of 5% FCS. Washed cells were incubated (37°C, 3 h) in RPMI-1640 alone or with svGIA (30 nM) or hGX

(50 nM) sPLA₂s. In selected experiments, actinomycin D (5 μ g/ml) was subsequently added to cells at baseline and for 2 and 4 h. In other experiments, HLM were preincubated (37°C, 2 h) with CHX (30 μ g/ml) before incubations with budesonide and sPLA₂s. Total cellular RNA was isolated from macrophages by SV total RNA isolation system (Promega), treated with RNase-free DNase I and suspended in DEPC water. RNA concentrations and quality were assessed by spectroscopy using a Biophotometer (Eppendorf). One microgram of total RNA were reversely transcribed by 25 mM MgCl₂, 50 μ M oligo(dT) and 200 U of Superscript III Reverse Transcriptase (Invitrogen).

For semi-quantitative PCR, equivalent templates of cDNAs were amplified as previously described using target-specific primers for TNF- α , IL-6 and IL-8/CXCL8 and β -actin [18, 22]. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and visualized under the image analysis system Chemidoc XRS (Biorad). Densitometry was performed by measuring the signal intensity of each sample with the Quantity One 4.6 software (Biorad). The percent of the initial signal was obtained by calculating the ratio versus untreated samples of the signals normalized for β -actin.

Real-time quantitative PCR was performed on the iCycler (Bio-Rad) using the SYBR[®] Green qPCR Kits (Invitrogen) and target-specific primers for TNF- α , IL-6 and GAPDH as previously reported [22]. Primers for IL-8/CXCL-8 suitable for real-time PCR were designed using the Beacon Designer 3.0 (IL-8 forward 5'-ACGGGAGTCTCGCTCTTTC-3' and reverse 5'-GCA-GGAGAATGGCGTGAAC-3'). The data were analyzed with iCycler iQ analysis software (Biorad), the mRNA signals in each sample were normalized to that of the GAPDH mRNA, and the changes in TNF- α , IL-6 and IL-8/CXCL8 mRNA were expressed as fold changes versus that in untreated cells.

β -Glucuronidase Release

β -Glucuronidase release in cell-free supernatants or in cell lysates was measured by a colorimetric assay [20]. Briefly, supernatants or cell lysates were incubated (37°C, 18 h) with 0.1 M acetate buffer (pH 4.5) containing phenolphthalein glucuronide. At the end of the incubation, glycine buffer was added to each tube and the mixture was subjected to optical density reading at 540 nm. β -Glucuronidase release was expressed as the percentage of the total cellular content. All experiments were carried out in triplicate determinations.

Arachidonic Acid Release

Arachidonic acid release was determined as previously described [22] in [³H]AA labeled macrophages. Briefly, to label the endogenous pools of AA at equilibrium [30], adherent macrophages (10⁶ cells/well) were incubated (24 h, 37°C) in RPMI-1640 supplemented with 5% FCS, 2 mM L-glutamine, 1% antibiotic-antimycotic solution, and [³H]AA (1 μ Ci/10⁶ cells) in the presence of DMSO diluent or various concentrations (1–1,000 nM) of budesonide. Washed cells were then incubated (37°C, 2 h) with svGIA (30 nM) and hGX (50 nM) sPLA₂s. Radioactive AA released from the cells was isolated by thin layer chromatography and quantitated by liquid scintillation counting (2800 TR Liquid Scintillation Analyzer; Canberra Packard) as previously described [22]. [³H]AA release was expressed as the percentage of the total cellular [³H]AA determined in cell lysates.

Western Blot Analysis

Freshly isolated macrophages were incubated (37°C, 18 h) in RPMI-1640 containing DMSO diluent or increasing concentrations (1–1,000 nM) of budesonide in the presence of 5% FCS. Washed cells (1.5×10^6 per sample) were then incubated (37°C, 5–120 min) in PCG buffer alone or with svGIA (30 nM) [21]. At the end of incubation the reactions were stopped by adding ice-cold PIPES buffer and the samples were microfuged. Cell pellets were lysed and protein content was assessed by BCA Protein Assay Kit (Novagen). The remaining protein extracts were diluted in 2× lithium dodecyl-sulfate sample buffer (LSB, Novex) containing 2.5% 2β-mercaptoethanol and boiled for 5 min. Equal protein extracts (30 µg per sample) were separated on 10% Bis-Tris Gels (NuPAGE®, Novex), transferred to a nitrocellulose membrane (Schleicher & Schuell) and immunoblotted with anti-phospho-ERK1/2 or anti-phospho-p38 Abs as previously reported [21]. Membrane-bound Abs were visualized with the ECL Plus Western blotting detection system (GE Healthcare) under the Chemidoc XRS. Densitometric measurement of the signal intensity was performed with the Quantity One 4.6 software (Biorad) and kinase phosphorylation rate at the various time-points was obtained by calculating the ratio versus untreated samples. Normalization was accomplished by calculating the signal intensity of the unphosphorylated forms of the various kinases.

Statistical Analysis

The data are expressed as the mean $\pm$ SE of the indicated number of experiments. Statistical analysis was done by 1-way analysis of variance (ANOVA) followed by Dunnett's test by using Analyse-it 1.71 software (Analyse-it Ltd.). A p value of 0.05 or less was considered significant.

Results

Effect of Budesonide on sPLA₂-Induced Cytokine and Chemokine Production from Macrophages

Initial experiments were designed to examine the effect of budesonide on the release of 2 major proinflammatory cytokines (TNF-α and IL-6) and a chemokine (IL-8/CXCL8) produced by human macrophages stimulated with 2 structurally distinct sPLA₂s. We have previously shown that svGIA and hGX are the most potent sPLA₂ isoforms that activate cytokine and chemokine production in human macrophages [21, 22]. The cells were preincubated (18 h) with budesonide before stimulation (6 h) with equipotent concentrations of svGIA (30 nM) and hGX (50 nM). Figure 1 shows that budesonide inhibited sPLA₂-induced release of TNF-α, IL-6 and IL-8 in a concentration-dependent manner. The inhibitory effect of budesonide on TNF-α production was significant at a concentration of 10 nM. Production of all cytokines/chemokines induced by sPLA₂s was completely inhibited at 1,000 nM. The IC₅₀ values of budesonide (fig. 1) were comparable for all the 3 mediators analyzed and not

significantly different between the type of sPLA₂ used (svGIA or hGX). It should be noted that in 3 independent experiments, budesonide (1,000 nM) also significantly inhibited (up to 55%) the basal (spontaneous) release of TNF-α (untreated 2.28 ± 0.39 ng/mg of protein; budesonide 1.03 ± 0.17 ; $p < 0.05$) and IL-8 (untreated 20.77 ± 2.36 ; budesonide 11.85 ± 1.50 ; $p < 0.05$), but not of IL-6 (untreated 1.02 ± 0.36 ; budesonide 1.17 ± 0.27). The presence of 5% FCS during the 18 h of incubation with budesonide did not influence the inhibitory effect on either spontaneous or sPLA₂-induced cytokine/chemokine release (data not shown).

Activation of cytokine production in human macrophages by sPLA₂s requires the de novo gene expression [20–22]. Inhibition of gene expression is the primary mechanism by which glucocorticoids regulate proinflammatory mediator release [26, 32]. Thus, in the next group of experiments we examined the effect of budesonide on TNF-α, IL-6 and IL-8 mRNA expression by real-time PCR analysis of 3 experiments in which macrophages were stimulated with hGX. In these experiments we evaluated the time course of the effects of budesonide by examining cells preincubated with the drug for 3, 8 or 18 h before stimulation with the sPLA₂. Figure 2 shows that budesonide inhibited hGX-induced cytokine/chemokine mRNA expression in a time-dependent manner. In the case of TNF-α mRNA, the effect of budesonide was significant already after 3 h of preincubation ($53.3 \pm 7.8\%$ of the maximum response, $p < 0.05$) whereas it required 8 h of preincubation for IL-6 ($63.2 \pm 9.6\%$, $p < 0.05$) and IL-8 ($54.0 \pm 8.3\%$, $p < 0.05$). At the 18 h time-point budesonide elicited its maximum effect by reducing mRNA expression of TNF-α, IL-6 and IL-8 to near control values ($0.9 \pm 0.4\%$, $11.7 \pm 2.9\%$ and $7.5 \pm 1.8\%$ of the maximum response, respectively). These data indicate that budesonide inhibits sPLA₂-induced cytokine/chemokine production by reducing their specific gene expression. In agreement with the data on basal cytokine/chemokine secretion, the results obtained with cells not exposed to hGX (unstimulated) showed that budesonide also significantly reduced the basal mRNA expression of TNF-α (0.37 ± 0.03 fold changes, $p < 0.05$) and IL-8 (0.55 ± 0.05 , $p < 0.05$), but not IL-6 (0.95 ± 0.11).

Effect of Actinomycin D and CHX on the Inhibition of sPLA₂-Induced Cytokine/Chemokine mRNA Expression by Budesonide

It has been reported that glucocorticoids may regulate gene expression in inflammatory cells by reducing mRNA stability [33–35]. To determine whether inhibition of

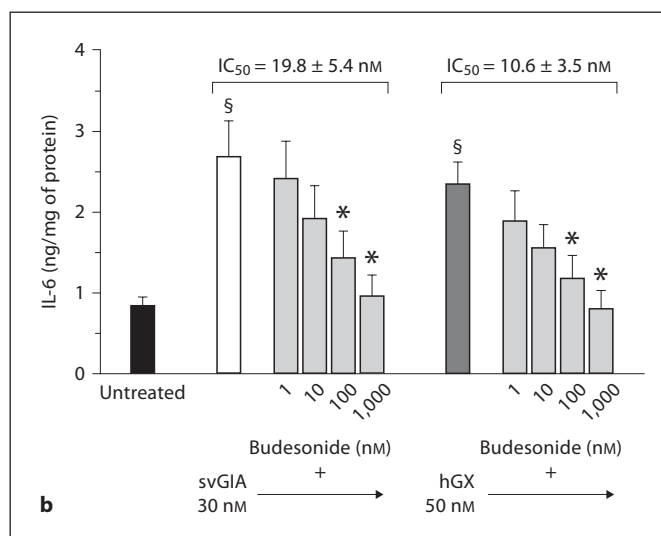
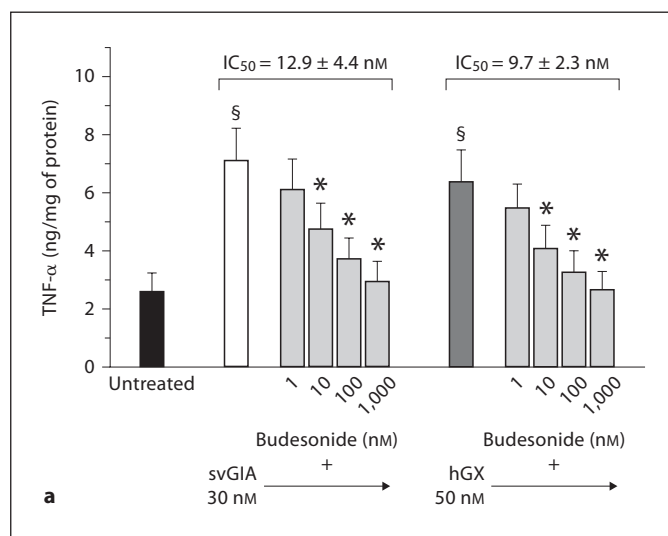
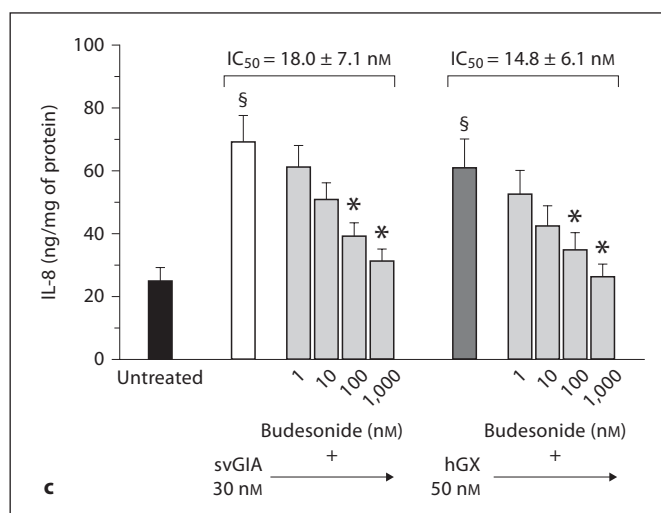


Fig. 1. Effect of increasing concentrations of budesonide on sPLA₂-induced release of cytokines and chemokines from human lung macrophages. The cells were incubated (37°C, 18 h) with increasing concentrations (1–1,000 nM) of budesonide or vehicle alone. At the end of the incubations the medium was removed and washed cells were then incubated (37°C, 6 h) with svGIA (30 nM) or hGX (50 nM) sPLA₂s. At the end of the incubation, TNF-α (a), IL-6 (b) and IL-8/CXCL-8 (c) release was determined by ELISA. The values are expressed as nanograms of cytokine-chemokine/milligram of protein. The data are the means ± SE of 4 experiments. § p < 0.01 vs. untreated; * p < 0.05 vs. respective sPLA₂ alone.



sPLA₂-induced cytokine production by budesonide was due to such effect, macrophages were preincubated (18 h) with 1,000 nM of budesonide before stimulation with hGX. Cells were then treated with 5 µg/ml of actinomycin D for 0, 2 or 4 h. At the end of the treatment, expression of mRNA for TNF-α was evaluated by RT-PCR. As shown in figure 3, the amount of TNF-α mRNA induced by hGX decreased to 41.5 ± 7.7% of the initial signal after treatment with actinomycin D for 4 h. Budesonide did not significantly alter the rate of degradation of TNF-α mRNA (31.7 ± 7.4% of the initial signal, p = 0.14). These results suggest that budesonide inhibits sPLA₂-induced TNF-α expression without influencing the stability of its mRNA.

Glucocorticoids can inhibit cytokine gene expression by inducing inhibitory proteins [35]. To examine this possibility, macrophages were preincubated (37°C, 2 h) with CHX (30 µg/ml) before incubation (37°C, 18 h) with budesonide (1,000 nM) and subsequent stimulation (37°C, 6 h) with hGX. Incubation longer than 2 h with CHX was unsuitable because of cytotoxicity. At the end of treatment, expression of mRNA for TNF-α, IL-8 and IL-6 was evaluated by RT-PCR. The results of 2 independent experiments indicated that CHX does not influence the inhibitory effect of budesonide on hGX-induced mRNA expression for TNF-α, IL-8 and IL-6 (data not shown), thereby excluding the possibility that budesonide inhibits cytokine/chemokine release primarily by inducing an inhibitory protein.

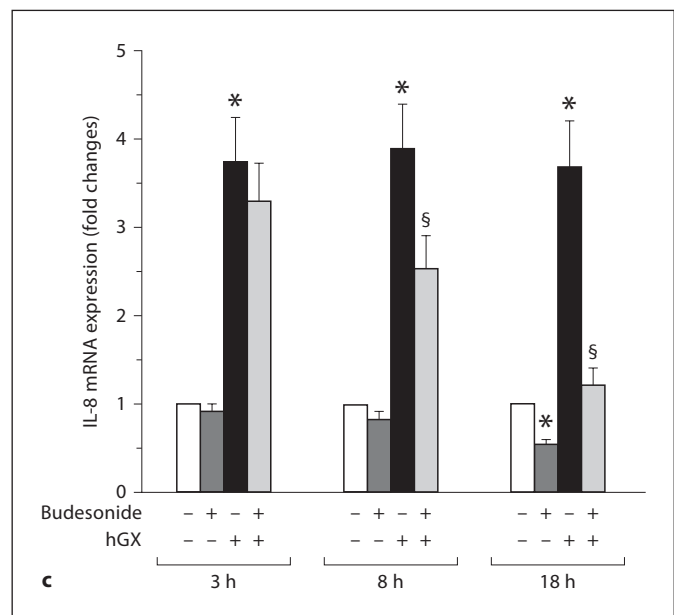
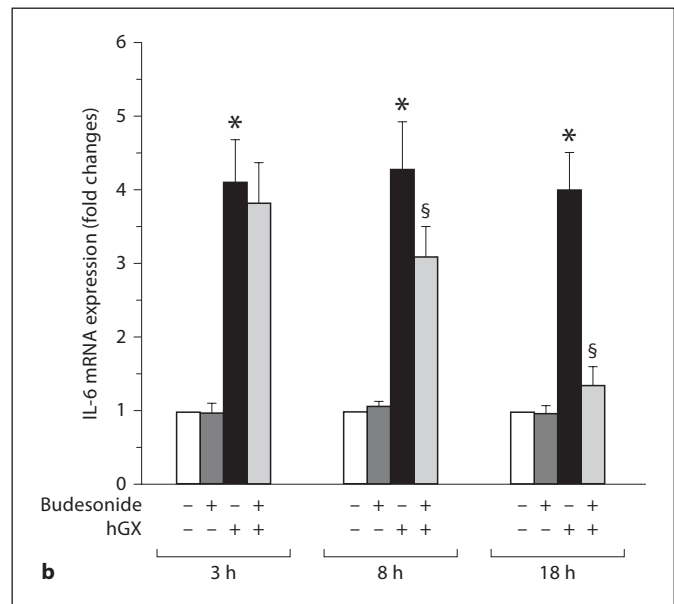
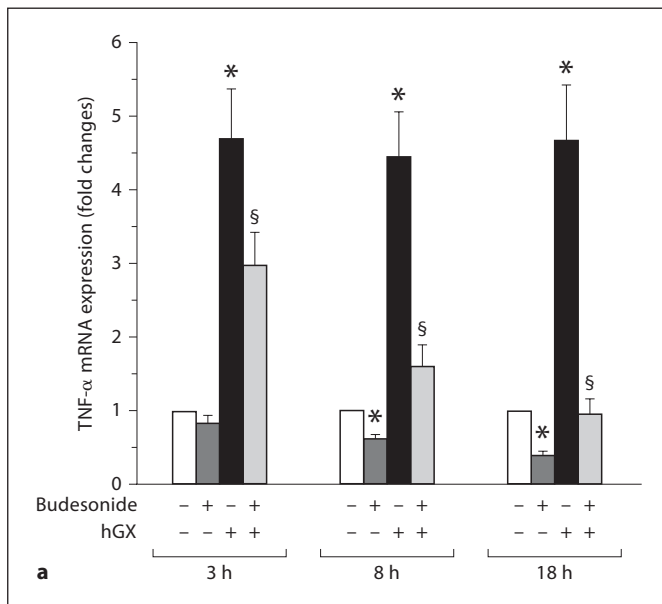


Fig. 2. Kinetics of the effects of budesonide on sPLA₂-induced mRNA expression of cytokines and chemokines in human lung macrophages. The cells were incubated (37°C) for different time periods (3, 8 or 18 h) with budesonide (1 μ M) or vehicle alone and, after washing, were stimulated (37°C, 3 h) with hGX (50 nM). At the end of the incubation total RNA was extracted and mRNA levels for TNF- α (a), IL-6 (b) and IL-8 (c) were quantitated by real-time PCR. Expression of TNF- α , IL-6, and IL-8 (normalized for GAPDH) was expressed as fold changes vs. untreated cells. Data are the means $\pm$ SE of 3 experiments. * $p < 0.05$ vs. untreated; § $p < 0.05$ vs. hGX alone.

Effect of Budesonide on the Release of Arachidonic Acid and β -Glucuronidase

Glucocorticoids may modulate cellular responses other than cytokine/chemokine production [27, 36]. These include the release of preformed mediators (e.g. lysosomal enzymes and granule proteins) and lipid mediator production. We evaluated the effect of budesonide on sPLA₂-induced release of AA, the precursor of eicosanoids, and of β -glucuronidase, a lysosomal enzyme used as a marker of macrophage exocytosis. As previously shown [20,

22] both svGIA and hGX induced AA mobilization and β -glucuronidase release from macrophages (table 1). Budesonide, at concentrations up to 1,000 nM, did not significantly modify the release of AA induced by svGIA and hGX from [³H]AA-labeled macrophages. Similarly, budesonide had no effect on the release of β -glucuronidase induced by both sPLA₂s. These results indicate that sPLA₂-induced production of lipid mediators and exocytosis in human macrophages were not sensitive to the inhibitory effect of glucocorticoids.

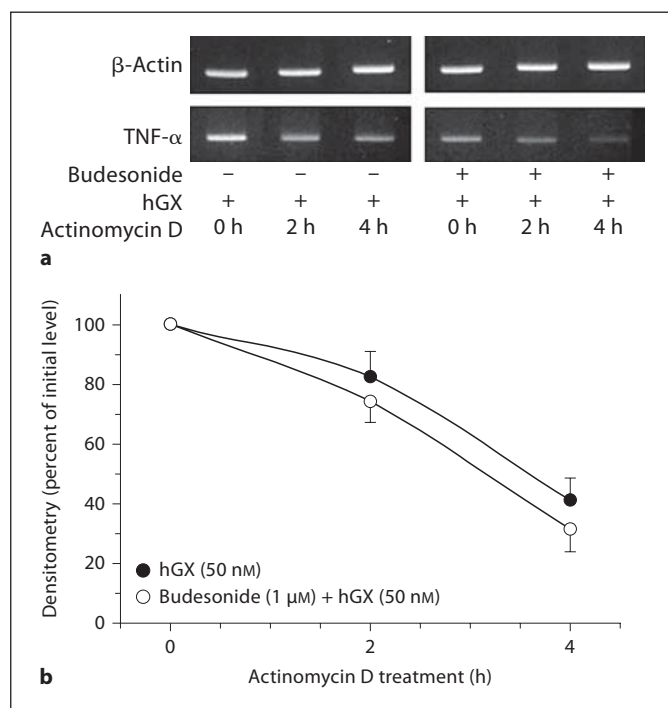


Fig. 3. Effect of budesonide on TNF- α mRNA stability in human lung macrophages. **a** β -actin and TNF- α specific RT-PCR amplification products from a representative experiment in which macrophages were incubated (37°C, 18 h) with budesonide (1 μ M) or vehicle alone. Washed cells were then stimulated (37°C, 3 h) with svGIA (30 nM) sPLA₂. Actinomycin D (5 μ g/ml) was subsequently added to the cells at baseline (0 h) and for 2 and 4 h. Adequate normalization of RNA for each sample was confirmed by the equality of RT-PCR amplification products for β -actin gene expression. **b** Densitometric analysis of the kinetics of degradation of TNF- α mRNA was performed by measuring the signal intensity at the various time-points and the percent of the initial signal was obtained by calculating the ratio vs. respective baseline samples of the signals normalized for β -actin. The data shown in **b** are the means $\pm$ SE of the 3 experiments.

Effect of Budesonide on sPLA₂-Induced Activation of ERK1/2 and p38 in Macrophages

The data reported in the previous sections suggested that budesonide inhibited steps crucial for sPLA₂-induced cytokine/chemokine production, but that are not involved in the process of exocytosis. Mitogen-activated protein kinases (MAPKs) ERK1/2 and p38 are potential candidates given their important role in cytokine/chemokine production in human macrophages [37]. Previous studies have shown that stimulation of human lung macrophages with both hGX and svGIA induces phosphorylation of both ERK1/2 and p38 MAPKs [19, 21, 22]. To understand whether these 2 MAPKs are involved in

cytokine/chemokine production induced by sPLA₂ we examined the effects of specific inhibitors of ERK1/2 (PD98059) and p38 (SB203580) on hGX-induced release of TNF- α , IL-6 and IL-8. Figure 4 shows that optimal concentrations of PD98059 and SB203580 significantly inhibit the release of all 3 cytokines/chemokines induced by hGX. Preincubation with a combination of both inhibitors completely suppressed TNF- α , IL-6 and IL-8 release, indicating that activation of MAPKs is required for sPLA₂-induced cytokine/chemokine production. We then tested the ability of budesonide to inhibit MAPK activation in sPLA₂-stimulated macrophages. Because of the limited availability of hGX protein, these experiments were performed with svGIA. Previous data have shown that activation of both ERK1/2 and p38 induced by svGIA is comparable to that induced by hGX [21, 22]. In these experiments, the cells were preincubated (18 h) with increasing concentrations (1–1,000 nM) of budesonide before stimulation with sPLA₂ for 60 min. At the end of the incubation phosphorylation of ERK1/2 and p38 MAPKs was assessed by Western blot. Figure 5a shows a typical result representative of 3 experiments in which budesonide concentration-dependently inhibited the phosphorylation of ERK1/2 (first gel) and p38 (third gel) in macrophages stimulated with svGIA. Densitometric analysis of the 3 experiments (fig. 5b) indicated that budesonide significantly inhibited ERK1/2 and p38 phosphorylation at a concentration of 10 nM with a maximum effect at 1,000 nM (inhibition of ERK1/2 = $89.3 \pm 5.7\%$, inhibition of p38 = $84 \pm 4.2\%$). In another group of experiments we examined the effect of optimal concentrations of budesonide on the kinetics of phosphorylation of ERK1/2 and p38 in sPLA₂-stimulated HLM. Figure 6a shows that svGIA induced a time-dependent phosphorylation of both ERK1/2 (first row, left gel) and p38 (third row, left gel) with maximum activation of the 2 kinases 60 min after stimulation. Budesonide inhibited phosphorylation of both ERK1/2 (first row, right gel) and p38 (third row, right gel) in macrophages stimulated with sPLA₂. Figure 5b reports the densitometric analysis of 3 experiments demonstrating that activation of MAPKs is already evident after 5 min of stimulation and peaks at 60 min. Budesonide significantly inhibited activation of ERK1/2 at 5, 15 and 60 min and that of p38 at 15 and 60 min. Together, these data indicate that budesonide prevents activation of 2 MAPKs that are essential for the production of cytokines and chemokines in sPLA₂-stimulated macrophages.

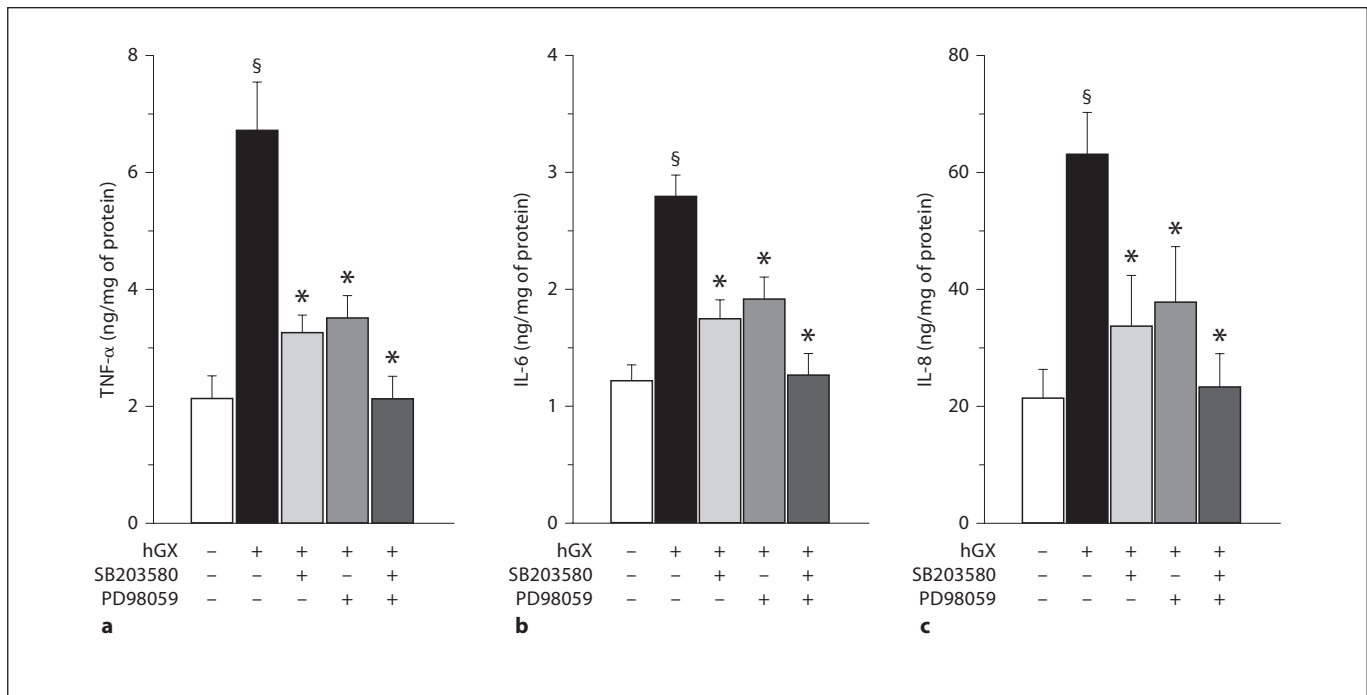


Fig. 4. Effect of ERK1/2 and p38 inhibitors on sPLA₂-induced TNF-α and IL-8 release. The cells were preincubated (37°C, 1 h) with SB203580 (30 μM), PD98059 (50 μM) or both (30 μM SB203580 + 50 μM PD98059) and then stimulated (37°C, 6 h) with hGX (50 nM). At the end of the incubation, TNF-α (**a**), IL-6 (**b**) and IL-8/CXCL-8 (**c**) release was determined by ELISA. The values are expressed as nanograms of cytokine-chemokine/milligram of protein. The data are the means ± SE of 3 experiments. § p < 0.01 vs. untreated; * p < 0.05 vs. hGX alone.

Table 1. Effect of budesonide on sPLA₂-induced arachidonic acid (AA) and β-glucuronidase release from macrophages

First incubation	Second incubation	AA release ^a (% of the total cellular [³ H]AA)	β-Glucuronidase release ^b (% of the total cellular content)
None	None	3.12 ± 0.38	3.73 ± 0.45
None	svGIA (30 nM)	5.83 ± 0.70*	7.35 ± 0.85*
Budesonide, 10 nM	svGIA (30 nM)	5.74 ± 0.77*	7.62 ± 0.77*
Budesonide, 100 nM	svGIA (30 nM)	5.65 ± 0.69*	7.47 ± 0.80*
Budesonide 1,000 nM	svGIA (30 nM)	5.91 ± 0.57*	7.56 ± 0.91*
None	hGX (50 nM)	6.16 ± 0.69*	8.10 ± 0.98*
Budesonide, 10 nM	hGX (50 nM)	5.98 ± 0.75*	7.83 ± 0.82*
Budesonide, 100 nM	hGX (50 nM)	6.07 ± 0.72*	8.02 ± 0.87*
Budesonide, 1,000 nM	hGX (50 nM)	6.04 ± 0.62*	7.91 ± 0.79*

^a The cells were prelabeled (24 h, 37°C) with [³H]AA and subsequently incubated (first incubation 37°C, 18 h) with the indicated concentrations of budesonide. At the end of the first incubation, the cells were washed and stimulated (second incubation: 37°C, 2 h) with the sPLA₂s. The release of [³H]AA was determined as described under 'Materials and Methods' and expressed as the percentage of the total [³H]AA incorporated into the cells. The data are expressed as the means ± SE of 3 experiments.

^b The cells were incubated (first incubation 37°C, 18 h) with the indicated concentrations of budesonide. At the end of the first incubation, the cells were washed and stimulated (second incubation 37°C, 2 h) with the sPLA₂s. The release of β-glucuronidase was determined as described under 'Materials and Methods' and expressed as the percentage of the total cellular content of β-glucuronidase. The data are expressed as the means ± SE of 3 experiments.

* p < 0.05 vs. controls (none/none).

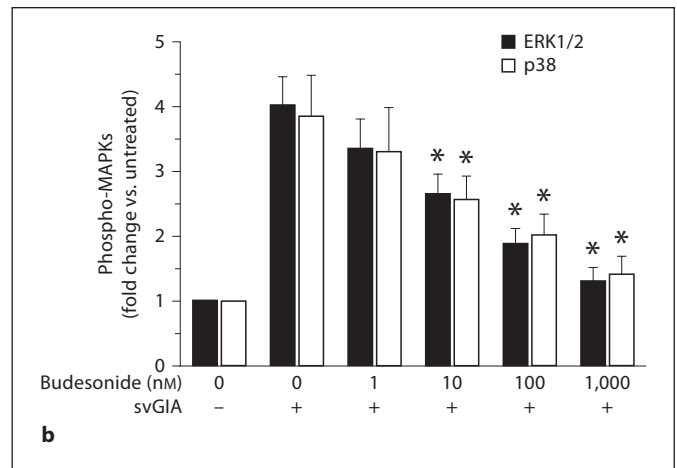
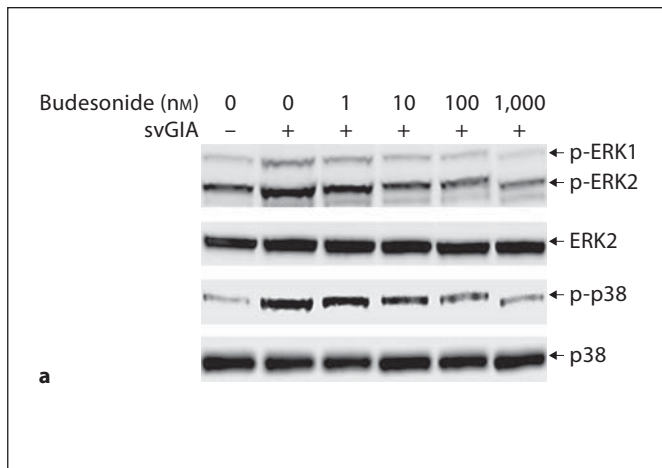
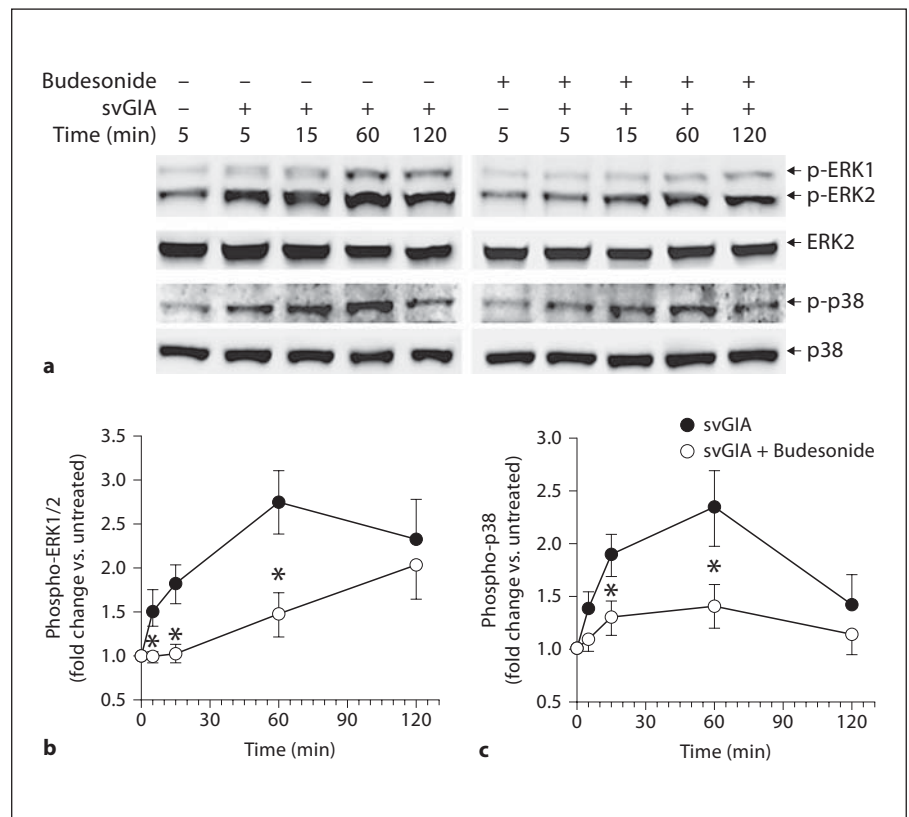


Fig. 5. Effect of increasing concentrations of budesonide on sPLA₂-induced phosphorylation of ERK1/2 and p38 in human lung macrophages. **a** The cells were incubated (37°C, 18 h) with increasing concentrations (1–1,000 nM) of budesonide or vehicle alone. Washed cells (1.5×10^6 per sample) were then stimulated with svGIA (30 nM) for 60 min. Cellular protein extracts (30 µg per sample) were subjected to Western blot analysis with phospho-ERK1/2 (first gel) Ab and anti phospho-p38 (third gel) Ab, as described under 'Materials and Methods'. Stripped membranes

were reblotted with anti-ERK2 (second gel), and anti-p38 (fourth gel) to confirm equal protein content of each sample. The Western blots shown are representative of 3 separate experiments. **b** Densitometric analysis of ERK1/2 and p38 phosphorylation was performed by measuring the signal intensity of each sample and by calculating the ratio vs. untreated cells. Adequate normalization was accomplished by measuring the signal intensity of unphosphorylated forms of ERK2 and p38. The data shown in B are the means $\pm$ SE of 3 experiments. * $p < 0.05$ vs. svGIA alone.

Fig. 6. Effect of budesonide on phosphorylation kinetics of ERK1/2 and p38 induced by sPLA₂ in human lung macrophages. **a** The cells were incubated (37°C, 18 h) with budesonide (1 µM) or vehicle alone. Washed cells (1.5×10^6 per sample) were then stimulated with svGIA (30 nM) for the times indicated. Cellular protein extracts (30 µg per sample) were subjected to Western blot analysis with phospho-ERK1/2 (first row) Ab, and anti-phospho-p38 (third row) Ab as described under 'Materials and Methods'. Stripped membranes were reblotted with anti-ERK2 (second row), and anti-p38 (fourth row) to confirm equal protein content of each sample. The Western blots shown are representative of 4 separate experiments. **b, c** Densitometric analysis of the phosphorylation kinetics of ERK1/2 and p38 was performed by measuring the signal intensity at the various time-points and by calculating the ratio vs. untreated samples. Adequate normalization was accomplished by measuring the signal intensity of unphosphorylated forms of p38 and ERK2. The data shown are the means $\pm$ SE of 4 experiments. * $p < 0.05$ vs. svGIA.



Discussion

Budesonide is an inhaled glucocorticoid widely used for the treatment of asthma [38]. This glucocorticoid exerts anti-inflammatory effects primarily by inhibiting the expression of several proinflammatory genes encoding for cytokines, chemokines, and adhesion molecules [33, 34, 39]. In this study we demonstrate that pharmacological concentrations of budesonide inhibit the production of TNF- α , IL-6 and IL-8 by blocking gene expression in human lung macrophages stimulated by sPLA₂s.

The role of sPLA₂s in tissue inflammation and remodeling is increasingly being appreciated [40]. These molecules are involved in the pathogenesis of inflammatory and allergic diseases by exerting pleiotropic effects related to their enzymatic activity and to their capacity to activate specific (M-type and N-type) or promiscuous receptors (mannose receptor, DEC 205) [15]. One of the major effects induced by several isoforms of sPLA₂ is the production of cytokines and chemokines from inflammatory cells [19]. These effects are also induced by sPLA₂s lacking the enzymatic activity and are mediated by interaction with membrane receptors [16, 18, 22, 41]. Most of the effects of sPLA₂s on human macrophages are mediated by the activation of the M-type receptor [22]. Binding of sPLA₂s to this receptor induces the activation of intracellular signaling pathways including ERK1/2, p38, the phosphatidylinositol 3-kinase/Akt system and NF- κ B which, in turn, lead to cytokine/chemokine gene expression [21]. The wide proinflammatory potential of sPLA₂s makes these molecules an attractive pharmacological target for anti-inflammatory drugs. However, the effects of glucocorticoids on sPLA₂-induced proinflammatory activities have not so far been examined.

Our data demonstrate that budesonide effectively inhibits the production of 3 major cytokines and chemokines induced by sPLA₂s in human macrophages. The inhibitory effect of budesonide on sPLA₂-induced production of cytokines and chemokines requires hours to become evident and is related to the inhibition of mRNA expression. Although in other cell systems glucocorticoids have been shown to inhibit production of proinflammatory molecules by accelerating their mRNA degradation [33–35] or by inducing the synthesis of inhibitory proteins [35], data obtained with actinomycin D and CHX indicate that the reduction of mRNA for cytokines/chemokines in sPLA₂-stimulated macrophages is related to genomic effects rather than to decreased mRNA stability or induction of an inhibitory protein. Inhibition by budesonide follows different kinetics, with TNF- α being reduced ear-

lier than IL-6 and IL-8. This observation indicates that glucocorticoids differentially modulate cytokine/chemokine expression by interfering with distinct regulatory pathways. This concept is supported also by our observation that budesonide inhibits the basal mRNA expression and protein release of TNF- α and IL-8, but not of IL-6. Previous studies have shown that dexamethasone inhibits TNF- α expression in human monocytic THP-1 cells by suppressing transactivation mediated by NF- κ B and c-Jun/ATF-2 [42] whereas inhibition of IL-6 expression in HeLa cells appears to be mediated primarily by transrepression of NF- κ B and AP-1 and by interaction with a negative glucocorticoid responsive element [43]. Another possibility to explain the different kinetics of inhibition is that TNF- α may be, at least in part, involved in IL-6 and IL-8 production in sPLA₂-stimulated macrophages. It is known that TNF- α induces IL-6 and IL-8 production in human inflammatory cells [44]. Furthermore, we demonstrated that the production of TNF- α in sPLA₂-stimulated macrophages precedes that of IL-6 [22] and IL-8 (unpubl. observation). Thus, early inhibition of TNF- α release by budesonide might be needed for subsequent reduction of IL-6 and IL-8 expression. It should also be mentioned that various signal transduction intermediates (e.g. phosphatidylinositol 3-kinase, Akt, Btk) differentially contribute to the production of TNF- α , IL-8 and IL-6 in human macrophages [21, 45]. Therefore, the possibility exists that budesonide preferentially interferes with earlier pathways selectively involved in the production of TNF- α .

Glucocorticoids reduce the biosynthesis of eicosanoids in several cells and tissues. This effect is primarily due to the inhibition of the cPLA₂s [27]. While the contribution of sPLA₂s to eicosanoid generation in inflammatory cells is still unclear [1], our data demonstrate that budesonide does not influence the mobilization of AA induced by exogenous sPLA₂s. This means that budesonide has no effect on the enzymatic activity of sPLA₂s and, therefore, it is not expected to reduce eicosanoid production dependent on sPLA₂ activity.

Budesonide does not alter the release of β -glucuronidase induced by sPLA₂s at concentrations at which the production of cytokines is completely suppressed. The release of lysosomal enzymes, like cytokine and chemokine production, is a nonenzymatic effect of sPLA₂s mediated by the activation of the M-type receptor [18, 20, 46]. Therefore, the observation that the release of β -glucuronidase is not inhibited by budesonide suggests that the inhibition of cytokine/chemokine production is not due to down-regulation or desensitization of the M-type receptor. Indeed, the divergent effect of budesonide on

sPLA₂s-induced responses (inhibition of cytokine/chemokine production without changes in β -glucuronidase release) point to a selective interference of this glucocorticoid with the intracellular signaling involved in cytokine/chemokine production. Phosphorylation of ERK1/2 and p38 is induced by stimulation of human macrophages by sPLA₂s [21, 22]. Activation of these MAPKs is crucial for cytokine and chemokine generation [21, 22] (fig. 4), but it is not involved in β -glucuronidase release (unpubl. observation). Our data demonstrate that budesonide reduces ERK1/2 and p38 phosphorylation in macrophages activated by sPLA₂s, and therefore blocks a crucial intracellular pathway leading to cytokine/chemokine gene expression. Although it is difficult to establish unambiguously a cause-effect relationship between inhibition of MAPKs and suppression of cytokine/chemokine generation by budesonide, the fact that budesonide inhibits MAPK activation and cytokine/chemokine production with similar concentration response curves (fig. 1, 5) supports this hypothesis. In this context, our results are in agreement with previous data obtained with mast cells [47] and fibroblasts [48] showing that anti-inflammatory activity of glucocorticoid is due, at least in part, to the inhibition of MAPK-regulated gene expression. The proposed mechanism by which glucocorticoids inhibit MAPK activation would be an increased expression and/or a decreased degradation of the MAPK phosphatase-1, an enzyme involved in dephosphorylation and inactivation of MAPKs [49]. While our

data with CHX do not support a role for an increased expression of MAPK phosphatase-1, further studies will examine whether a decreased degradation of this protein is involved in the inhibitory effect of budesonide on sPLA₂s-induced cytokine/chemokine production.

Together, the results of our study indicate that budesonide is a potent inhibitor of major proinflammatory activities of sPLA₂s. This is the first evidence that a glucocorticoid suppresses the nonenzymatic effects of sPLA₂s without influencing their enzymatic activity. It should be noted that specific inhibitors of sPLA₂ enzymatic activity have been used in clinical trials in patients with bronchial asthma and rheumatoid arthritis [50, 51]. These trials have been discontinued because of the lack of efficacy of these drugs, which suggests that blocking only the enzymatic activity of sPLA₂s is not sufficient to reduce their proinflammatory potential. Glucocorticoids such as budesonide, by inhibiting the receptor-mediated effects of sPLA₂s, may therefore represent a better therapeutic option to reduce inflammation in those diseases associated with enhanced production of sPLA₂s.

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