

Allergen-induced proteolytic cleavage of annexin-1 and activation of cytosolic phospholipase A₂ in the lungs of a mouse model of asthma

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To identify proteins that might play an important role in allergen-induced asthma, we analyzed lung extracts prepared from allergen (ovalbumin)-challenged animals in a mouse model of this condition. The combination of two-dimensional gel electrophoresis and mass spectrometry revealed that annexin-1, a 37 kDa anti-inflammatory protein that inhibits the activity of cytosolic phospholipase A₂ (cPLA₂), was down-regulated by allergen challenge in the lungs of ovalbumin-sensitized mice. Immunoblot analysis showed that this effect of ovalbumin challenge was attributable to proteolytic cleavage of annexin-1. The ovalbumin-induced degradation of annexin-1 was blocked by pretreatment of mice with the antioxidant *N*-acetylcysteine (NAC) or with sodium selenite, both of which have previously been shown to exert anti-inflammatory effects in this asthma model. Ovalbumin challenge also both increased the expression of cPLA₂ in lung tissue and reduced the extent of the interaction between cPLA₂ and annexin-1, and these effects were inhibited by NAC or selenite. Moreover, the concentrations of cysteinyl leukotrienes in bronchoalveolar lavage fluid and of leukotriene B₄ in lung tissue were increased by ovalbumin challenge in a NAC- or selenite-sensitive manner. Together, these results suggest that allergen-induced oxidative stress results in proteolysis of annexin-1 and consequent up-regulation of cPLA₂ activity and leukotriene production in this mouse model of asthma, and that the anti-inflammatory effects of selenite may provide a basis for the development of new antiasthmatic drugs.

Keywords: Annexin-1 / Asthma / Cytosolic phospholipase A₂ / Matrix-assisted laser desorption/ionization-time of flight / Two-dimensional gel electrophoresis

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1 Introduction

Asthma is a chronic respiratory disease characterized by degranulation of mast cells and infiltration of eosinophils and other inflammatory cells in the lungs [1]. The infiltrated eosinophils release cytotoxic products, including reactive oxygen species (ROS), at the sites of inflammation, result-

ing in injury to airway tissue [2]. The oxidative stress and redox imbalance induced by allergens in the lungs trigger the activation of redox-sensitive transcription factors, such as nuclear factor- κ B (NF- κ B) and activator protein-1 [3, 4]. Recently, we and others have shown that selenite or the small-molecule inhibitors MOL 294 or PNRI-299 protect mice from allergen-induced asthma by blocking the action of these redox-sensitive transcription factors [5–7].

Annexin-1 (lipocortin-1, calpactin-II) is a 37 kDa glucocorticoid-inducible protein [8, 9] that inhibits the activity of cytosolic phospholipase A₂ (cPLA₂). Given that cPLA₂ plays a key role in the release of arachidonic acid for the production of lipid inflammatory mediators such as prostaglandins and leukotrienes, the inhibition of this enzyme by annexin-1 is thought to contribute to its anti-inflammatory activity [10]. We previously showed that an

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Abbreviations: BAL, bronchoalveolar lavage; cPLA₂, cytosolic phospholipase A₂; cysLTs, cysteinyl leukotrienes; LTB₄, leukotriene B₄; NAC, *N*-acetylcysteine; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species

increase in glutathione peroxidase activity in selenite-treated mice was associated with inhibition both of allergen-induced activation of NF- κ B as well as of the consequent inflammatory gene expression and eosinophil recruitment to the airways [5]. We have now applied a proteomics approach to identify asthma-related proteins in the lung tissue of allergen (ovalbumin)-challenged mice. The abundance of annexin-1 was found to be reduced in the allergen-challenged animals and this effect was prevented by pretreatment of the mice with selenite or *N*-acetylcysteine (NAC), suggesting that the down-regulation of annexin-1 was dependent on oxidative stress. In addition, allergen challenge increased both the amount of cPLA₂ (total cPLA₂ and the active form not bound to annexin-1) as well as the production of cysteinyl leukotrienes (cysLTs) and leukotriene B₄ (LTB₄). These results thus suggest that the increased generation of leukotrienes induced by allergen challenge in this mouse asthma model is attributable to down-regulation of annexin-1 and consequent activation of cPLA₂.

2 Materials and methods

2.1 Allergen sensitization and challenge of mice

The sensitization and challenge of mice was achieved with a modified version of a method described previously [11]. In brief, C57BL/6 mice (6 weeks of age) were injected intraperitoneally with 200 μ g of ovalbumin (Sigma, St. Louis, MO, USA) and 1 mg of Imject Alum (Pierce Chemical, Rockford, IL, USA). A second intraperitoneal injection of 20 μ g of ovalbumin adsorbed to adjuvant was administered 10 d after the first injection. After an additional 10 d, the mice were exposed to an aerosol of 1% ovalbumin in water for 30 min at 1 d intervals for 3 d. Two days after the final exposure to the aerosolized ovalbumin, the animals were challenged with a nebula of 10% ovalbumin in water. Nebulization was performed in a plastic chamber connected to an ultrasonic nebulizer (Omron, Vernon Hills, IL, USA) that allows entry of an ovalbumin aerosol. NAC (150 mg *per* kilogram of body mass) or sodium selenite (2.5 mg/kg) was injected into the peritoneum of mice 1 and 24 h before ovalbumin challenge.

2.2 Collection of bronchoalveolar fluid

Bronchoalveolar lavage (BAL) fluid was collected and the lungs were removed from mice 48 h after ovalbumin challenge. BAL fluid and sections of lung tissue were stained with Diff-Quick (Merck, Whitehouse Station, NJ, USA) and hematoxylin-eosin (Sigma), respectively, for examination of eosinophil recruitment and histology as described previously [12].

2.3 2-DE

For analysis by 2-DE, lung tissue was homogenized in a solution containing 8 M urea, 4% CHAPS detergent, 13 mM and 2% Pharmalyte (pH 3–10; Amersham Biosciences, Uppsala, Sweden). The homogenate was maintained on ice for 20 min before centrifugation at 20 000 $\times g$ for 30 min at 4°C. The resulting supernatant was stored at –70°C until analysis. Tissue extract (50 μ g of protein) was mixed with 450 μ L of rehydration solution (8 M urea, 2% CHAPS, 0.5% Pharmalyte (pH 3–10), 0.002% bromophenol blue) and then applied to a 24 cm precast IPG DryStrip (linear pH gradient of 3 to 10; Amersham Biosciences) that had been rehydrated for 6 h in a ceramic strip holder. IEF was performed at 20°C at a maximal current setting of 50 μ A *per* strip with an Ettan IPGphor IEF system (Amersham Biosciences); the conditions were 30 V for 6 h, 500 V for 1 h, 1000 V for 1 h, and 75 000 Vh. The strips were then equilibrated first with a solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, and 1% DTT and then with the same solution lacking the DTT, after which they were transferred to 12.5% polyacrylamide gels (thickness, 1.0 mm) and sealed in place with 0.5% agarose. SDS-PAGE was performed at 16°C and at a constant power first of 2.5 W *per* gel for 30 min and then of 17 W *per* gel with an Ettan Dalt six electrophoresis system (Amersham Biosciences). The gels were then stained with silver nitrate as recommended by the manufacturer (Amersham Biosciences). The pattern of the spots was analyzed with 2D Elite software (Amersham Biosciences).

2.4 MALDI-TOF MS

Protein spots were excised from the silver-stained 2-D gels and incubated first for 10 min with a solution containing 15 mM K₃Fe(CN)₆ and 50 mM Na₂S₂O₃ to remove silver and then for 20 min with 50 mM NH₄HCO₃. The pieces of gel were dehydrated with ACN, dried by vacuum centrifugation, and incubated for 16 h at 37°C with modified porcine trypsin (10 μ g/mL) (sequencing grade; Promega, Madison, WI, USA) in 100 μ L of 50 mM NH₄HCO₃. Peptides were recovered from the incubation mixture by extraction three times with 40 μ L of 5% TFA in 50% ACN. The pooled extract was dried by vacuum centrifugation, resuspended in 10 μ L of 0.5% TFA, desalted with a ZipTip C₁₈ column (Millipore, Bedford, MA, USA), and crystallized in a matrix of CHCA. The peptides were then fractionated with a Voyager DE-STR mass spectrometer (Framingham, MA, USA). All MALDI-TOF spectra were externally and internally calibrated with two standard peptide mixtures. Databases for protein identification were searched at <http://prospector.ucsf.edu>.

2.5 Immunoprecipitation and immunoblot analysis

Lungs were washed with PBS and homogenized with a pestle in a solution containing 10 mM HEPES-NaOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, aprotinin (2 µg/mL), leupeptin (2 µg/mL), and 0.3% NP-40. The homogenate was incubated for 30 min on ice and then centrifuged at $13\,000 \times g$ for 5 min at 4°C. Portions (10 µg of protein) of the resulting supernatant were then fractionated by SDS-PAGE on a 10% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies (Abs) to annexin-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse mAb to cPLA₂ (Santa Cruz Biotechnology), or mouse mAbs to β-actin (Sigma). Immune complexes were detected with appropriate secondary Abs and enhanced chemiluminescence reagents (Pierce). For immunoprecipitation, lung extracts prepared as described above were incubated with Abs to cPLA₂ and then with protein A-agarose (Amersham Biosciences); the resulting immunoprecipitates were then subjected to immunoblot analysis with Abs to annexin-1.

2.6 RNA isolation and RT-PCR

Total RNA was extracted from the lung tissues according to the manufacturer's instructions using TRIzol Reagent (Invitrogen, Leek, The Netherlands). One microgram of the extracted RNA was denatured for 5 min at 65°C and reverse transcribed by incubating it for 1 h at 42°C in reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM dNTP (Takara, Shiga, Japan), 1 U/µL ribonuclease inhibitor (Takara), and 2.5 mM random hexamer. For the PCR, an equivalent of 1 µg mRNA was used as template in the mixture of AccuPower PCR PreMix from Bioneer (Taejon, Korea) and a 30-cycles PCR reaction was performed in a MiniCycler (MJ Research, Waltham, MA, USA) using the following primers designed for detection of annexin-1; forward, 5'-GCGGCGATCCATGGTCAATGGTATCAGAATTCCTC-3'; and reverse, 5'-GCGGCGTCGACTAGTTTCCTCCACAAAGAGCCAC-3'. As control, the following primers were designed for glyceraldehyde-3-phosphate dehydrogenase; forward, 5'-AGGTCTGGAGTCAACGGATTT-3'; and reverse, 5'-AGGTGGAGGAGTGGGTGTCG-3'.

2.7 Assay for leukotrienes

BAL fluid and lung tissue were assayed for cysLTs (LTC₄, LTD₄, LTE₄) and LTB₄, respectively, with the Bio-trak enzyme immunoassay system (Amersham Biosciences). In brief, samples were incubated with rat

antisera to leukotrienes for 2 h and then with a leukotriene-peroxidase conjugate for 3 h at room temperature in a 96-well plate that had been coated with goat Abs to rat immunoglobulin G. After washing each well, the amount of bound peroxidase-labeled leukotriene was determined by the addition of tetramethylbenzidine and hydrogen peroxide, termination of the reaction, and measurement of absorbance at 450 nm with a microplate reader (Bio-Rad).

3 Results and discussion

3.1 Inhibition of eosinophil recruitment in ovalbumin-challenged mice by NAC

We first examined the effect of pretreatment with NAC (150 mg/kg), a glutathione precursor and ROS scavenger with antioxidant activity, on the allergen-induced recruitment of eosinophils to the airways of ovalbumin-sensitized mice. Examination of BAL fluid collected from mice 48 h after ovalbumin challenge revealed that the allergen induced a marked increase in the number of eosinophils, compared with that apparent for control animals, and that this effect was substantially inhibited by pretreatment with NAC (Fig. 1).

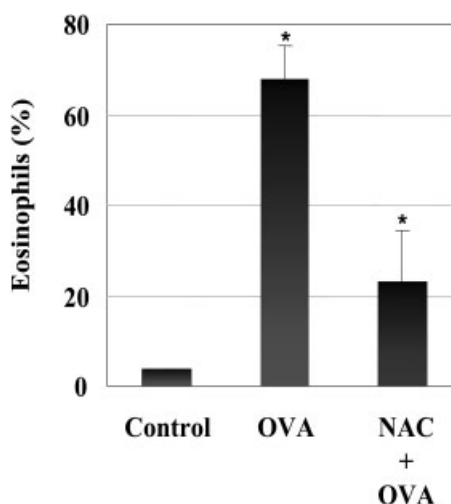


Figure 1. Eosinophil recruitment to the airways of ovalbumin-challenged mice and its inhibition by NAC. Ovalbumin-sensitized mice were treated without or with NAC (150 mg/kg) before challenge with allergen. BAL fluid was collected 48 h after challenge and was stained with Diff-Quick for microscopic detection of eosinophils. The number of eosinophils in BAL fluid of mice was determined as a percentage of total cells. Data are means $\pm$ SD of values obtained from four animals *per* group. * $p < 0.001$ versus OVA (Student's *t*-test).

3.2 Identification of proteins whose expression is affected by ovalbumin challenge

Lung tissue homogenates prepared from control mice and from ovalbumin-challenged mice that had been pretreated or not with NAC were fractionated by 2-DE and stained with silver nitrate. The abundance of three proteins (spots 1, 2, and 4) was increased and that of two proteins (spots 3 and 5) was decreased in ovalbumin-challenged mice compared with controls (Fig. 2). The effects of ovalbumin challenge on each of these five proteins were prevented by pretreatment of the mice with NAC. These five proteins were identified by MALDI-TOF mass spectrometric analysis (Table 1). One of the five proteins (spot 4) was identified as vascular cell adhesion molecule-1, the expression of which we had previously shown to be similarly modified by ovalbumin and NAC in this animal model [5]. In this study, we chose spot 5, identified as annexin-1, a Ca²⁺- and phospholipid-binding protein that modulates anti-inflammatory responses, for further analysis. The amount of annexin-1 has been shown to be reduced both in BAL fluid of individuals with lung diseases [13], and in irradiated rat lung [14].

3.3 Proteolytic cleavage of annexin-1 in ovalbumin-challenged mice

We next examined the effects of ovalbumin challenge and NAC pretreatment on the abundance of annexin-1 in lung tissue by immunoblot analysis. Ovalbumin challenge resulted in an ~50% decrease in the amount of annexin-1 compared with that in control animals and this effect of allergen was blocked by pretreatment of mice with NAC (Fig. 3A). The decrease in abundance of annexin-1 induced by ovalbumin challenge was also prevented by pretreatment of animals with sodium selenite (2.5 mg/kg). We previously showed that selenite inhibits allergen-induced inflammatory signaling, possibly by increasing the activity of glutathione peroxidase and by mediating direct oxidation of thiol groups of NF- κ B [5]. RT-PCR revealed that the effect of ovalbumin challenge on the amount of annexin-1 in lung tissue was not mediated at the level of annexin-1 mRNA (Fig. 3B). Our immunoblot analysis showed that a protein (~33 kDa) that was smaller than full-length annexin-1 (37 kDa) and reactive with the Abs to annexin-1 was present in the lung tissue of ovalbumin-challenged mice but not in that of control animals, and that the challenge-induced production of this protein was inhibited by pretreatment of mice with NAC or selenite (Fig. 3A). A cleaved form (~33 to 34 kDa) of annexin-1 has previously been detected in the lung tissue of indi-

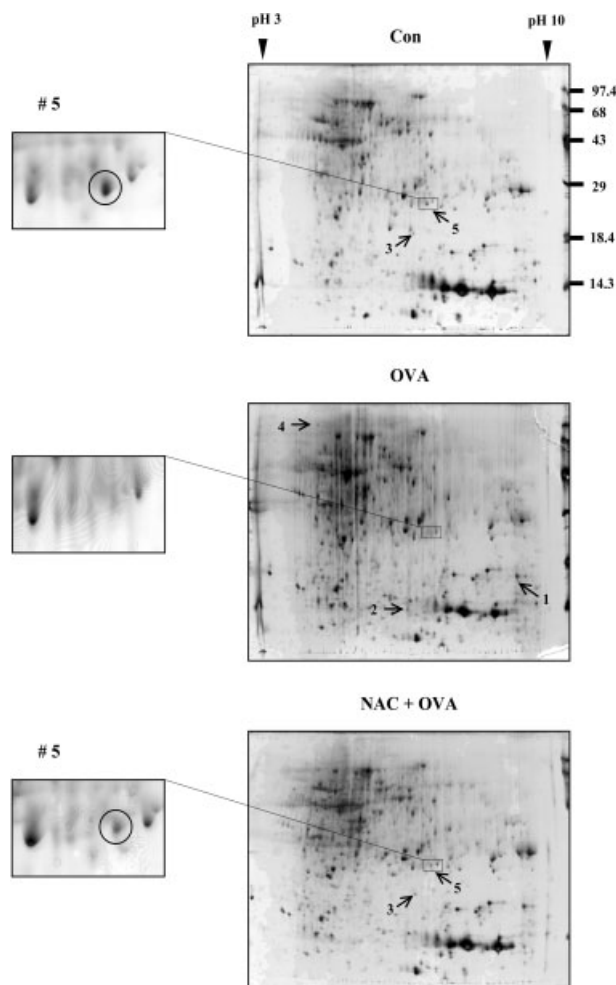


Figure 2. 2-DE of lung tissue from ovalbumin-challenged mice. Lung extracts were prepared 48 h after challenge and subjected to 2-DE ($n = 3$). The gels were stained with silver nitrate. The intensity of spots designated 1, 2, and 4 was increased and that of spots 3 and 5 was reduced by ovalbumin challenge, and these effects of allergen were inhibited by pretreatment of mice with NAC. The regions of the gels containing spot 5 (circled) are shown enlarged on the left.

viduals with inflammatory lung disease [13, 15]. Our results thus suggest that the down-regulation of annexin-1 expression in the lungs induced by ovalbumin challenge in this mouse model of asthma is mediated by proteolysis. The cleavage of annexin-1 in its NH₂-terminal region yields a truncated protein that exhibits an intracellular distribution and catalytic properties distinct from those of the full-length protein [16, 17]. The existence of a specific enzyme (lipocortinase) that cleaves annexin-1 has been proposed [18] and the responsible enzyme was suggested to be a serine protease [15].

Table 1. Designation of proteins identified following 2-DE IEF/SDS-PAGE of OVA-challenged mouse lung

Spot no.	Identified protein	Accession no.	Size (kDa)	pI	Coverage score	Change pattern	
						+ OVA	+ NAC
1	Protein C6orf62 homolog	13277384	27.1	9.0	740	Appeared > 300%	Disappeared 47%
2	ADP-ribosylation factor-like protein 2	Q9DOJ4	20.9	5.7	150	Increased ^{a)} 78%	Decreased > 300%
3	Heme oxygenase 1	6754212	32.9	6.1	58.2	Decreased	Increased ^{b)} 86%
4	Vascular cell adhesion molecule-1 (VCAM 1)	459893	81.3	5.2	90.3	Appeared	Decreased ^{c)}
5	Annexin-1	113945	38.7	7.0	398	Disappeared	Appeared

Spot numbers refer to the designation of peptide spots on the 2-DE gel shown in Fig. 2. Proteins were identified by MS and were searched on the Swiss-Prot and NCBI nr database. Student's *t*-test showed significant differences

a) $p < 0.05$ versus control group

b), c) $p < 0.05$ versus OVA-treated group

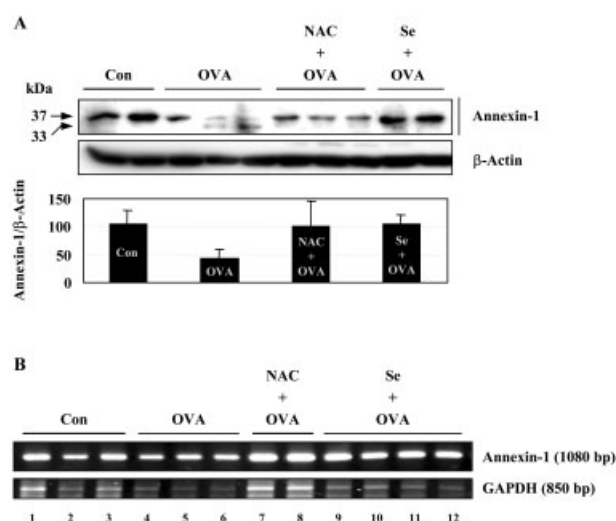


Figure 3. Degradation of annexin-1 induced by ovalbumin challenge. Ovalbumin-sensitized mice were treated with NAC (150 mg/kg), or sodium selenite (2.5 mg/kg) before challenge with allergen, as indicated. (A) Lung tissue extracts were prepared 48 h after allergen challenge and subjected to immunoblot analysis with Abs to annexin-1 or to β -actin (internal control). Data for two or three animals of each group are shown in the upper panel; the ratio of annexin-1 to β -actin was also determined by densitometry and the values for each group are shown as means $\pm$ SD from three independent experiments in the lower panel. (B) Transcriptional level of annexin-1 and glyceraldehyde-3-phosphate dehydrogenase (internal control) were examined using the RNAs extracted from the ovalbumin-sensitized mice. RT-PCR was carried out as described in section 2.6.

3.4 Increased expression and activation of cPLA₂ in ovalbumin-challenged mice

Given that the anti-inflammatory activity of annexin-1 is attributable mostly to its ability to inhibit cPLA₂ [19], we next examined the effect of allergen challenge on the expression of cPLA₂ in the lungs of ovalbumin-sensitized mice. Because cPLA₂ exists at a low level in cells [20], we could not detect the protein on 2-D gels by silver-staining. However immunoblot analysis revealed that the abundance of cPLA₂, which is expressed in various forms with molecular sizes of 85 to 105 kDa, was increased by ovalbumin challenge and that this effect was inhibited by pretreatment of mice with NAC or selenite (Fig. 4A). Although the mechanism by which annexin-1 inhibits cPLA₂ activity is not fully understood, two models have been proposed. According to the substrate depletion model, annexin-1 binds in a Ca^{2+} -dependent manner to phospholipid substrates of cPLA₂ and thereby renders them inaccessible to cPLA₂ [19]. According to the specific interaction model, annexin-1 inhibits cPLA₂ by protein-protein interaction [21]. We detected a specific interaction between annexin-1 and cPLA₂ in lung tissue by immunoprecipitation and immunoblot analysis (Fig. 4B). Furthermore, the amount of annexin-1 that coprecipitated with cPLA₂ was markedly reduced by allergen challenge in ovalbumin-sensitized mice, and this effect was inhibited by pretreatment of mice with selenite (Fig. 4B) or with NAC (data not shown). The expression of cPLA₂ was recently shown to be increased in mice that lack annexin-1 [22]. It is thus possible that the up-regulation of cPLA₂

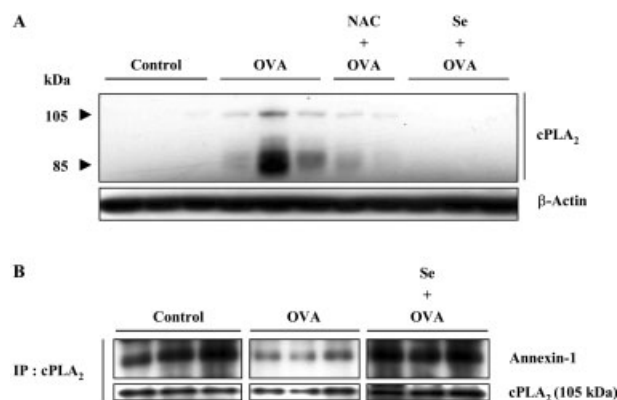


Figure 4. Expression of cPLA₂ and its interaction with annexin-1 in the lungs of ovalbumin-challenged mice. (A) Lung tissue extracts were prepared 48 h after allergen challenge and were subjected to immunoblot analysis with Abs to cPLA₂ or to β-Actin. Each lane corresponds to tissue extract from an individual mouse. (B) Lung tissue extracts prepared from mice treated as in (A) were subjected to immunoprecipitation (IP) with Abs to cPLA₂, and the resulting precipitates were subjected to immunoblot analysis with Abs to annexin-1 or to cPLA₂.

expression in the lungs of ovalbumin-sensitized mice induced by allergen challenge is due to relief of annexin-1 mediated inhibition of cPLA₂ gene transcription.

3.5 Increased cPLA₂-mediated production of leukotrienes in ovalbumin-challenged mice

To examine further the role of cPLA₂ in ovalbumin-induced inflammation, we determined the concentrations of cysLTs in BAL fluid and of LTB₄ in lung tissue. These leukotrienes are derived from arachidonic acid released from phospholipids by the catalytic action of cPLA₂ and exert a broad range of biological effects, including the promotion of chemotaxis and smooth muscle contraction and the activation of inflammatory cells [23]. Several recent studies have also implicated ROS in cPLA₂ mediated inflammatory signaling [24–26]. Ovalbumin challenge induced marked increases in the concentrations both of cysLTs in BAL fluid (Fig. 5A) and of LTB₄ in lung tissue (Fig. 5B) of sensitized mice. Furthermore, these effects of allergen challenge were prevented by pretreatment of animals with NAC or selenite. These results thus suggest that ROS produced by ovalbumin challenge play an important role in the cPLA₂-mediated production of leukotrienes in this mouse model of asthma. We also examined the effects of NAC and selenite on allergic airway inflammation by histological analysis of lung sections stained with hematoxylin-eosin. Ovalbumin challenge induced marked infiltration of the airways by eosinophils and other inflammatory cells in sensitized mice. Pretreat-

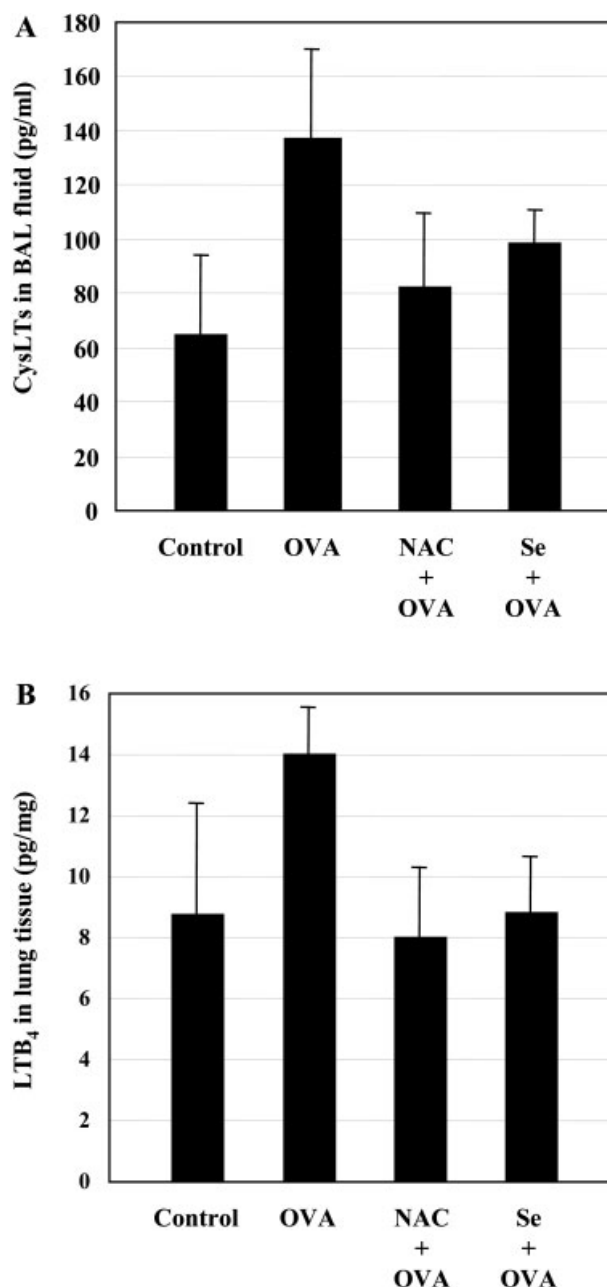


Figure 5. Leukotriene production in ovalbumin-challenged mice. The concentrations of cysLTs (A) and LTB₄ (B) were determined in BAL fluid and lung tissue, respectively, harvested from the animals 48 h after allergen challenge. Data are expressed as picograms per milliliter of BAL fluid or as picograms per milligram of wet lung tissue and are means ± SD of values from three (A) or four (B) animals per group.

ment of mice with NAC or selenite greatly reduced this allergen-induced influx of inflammatory cells as we reported previously [5].

4 Concluding remarks

Together, our present data suggest that allergen-induced oxidative stress results in degradation of the anti-inflammatory protein annexin-1 and a consequent increase in the expression and activity of cPLA₂ and leukotriene production. Selenite was effective in blocking the effects of allergen on annexin-1 proteolysis, cPLA₂ expression and activation, leukotriene production, and airway infiltration by inflammatory cells, possibly providing a basis for the development of new drugs to treat asthma.

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