

Increased Levels of Hypoxia-sensitive Proteins in Allergic Airway Inflammation

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In this study we investigated the alterations in protein levels that are induced by allergic eosinophilic lung inflammation. Lung tissue eosinophilia and sequestration of inflammatory cells in airspaces were provoked by systemic sensitization with ovalbumin followed by repeated inhalation challenge with aerosolized ovalbumin. Proteome alterations in lung tissue and bronchoalveolar lavage fluid, respectively, were examined by two-dimensional gel electrophoresis followed by identification of proteins by mass spectrometry. Several proteins were markedly increased in inflamed tissue. In particular, several proteins that are known to be associated with hypoxia were elevated, for example, glycolytic enzymes, glucose-regulated protein 78 kD, prolyl-4-hydroxylase, peroxiredoxin 1, and arginase. Out of the identified proteins, Ym2 displayed the clearest increase, present at high levels in animals with lung eosinophilia, while being undetectable in control subjects. Furthermore, the levels of cathepsin S were markedly increased in inflamed tissue. Taken together, this study identifies a number of marker proteins associated with the pathogenesis of allergic lung inflammation and indicates a link between allergic airway inflammation and induction of hypoxia-related gene products.

Keywords: asthma; proteomics; hypoxia; allergy

Allergic asthma is a chronic inflammatory disease involving a multitude of cell types, for example, T cells, neutrophils, eosinophils, and mast cells. The typical clinical features of asthma include airway inflammation, bronchial hyperresponsiveness, and airway thickening caused by structural remodeling of the airway epithelium leading to obstruction of airflow (1). Although great efforts have been made to identify the pathogenesis of human allergic airway disease, the underlying mechanisms for the development of severe symptoms remain poorly defined. It is now established that hypersensitivity for environmental allergens is to a high degree hereditary determined (2), and several genes that may be associated with allergic asthma have been identified, for example, the genes encoding IgE, interleukin (IL)-4, IL-5, IL-13, CD14, tumor necrosis factor- α (2), and ADAM33 (3). The incidence and prevalence of asthma are subject to a dramatic increase in certain parts of the world. Thus, to cope with these difficulties, there is a great need for identifying proper targets for the management of this disease.

In this study, we have investigated the changes in protein expression patterns that occur in lung tissue and airspaces after allergen challenge. We used a mouse model for allergic lung inflammation where airway hypersensitivity was induced by sensitization and challenge with ovalbumin (OVA). This model is widely used as a model for human allergic airway disease because the pathology observed in the model shares many features with the human disease, for example, activation of T-helper 2 cells leading to expression of IL-4, IL-5, IL-13, and a systemic IgE response, resulting in eosinophilic airway inflammation and airway hyperactivity. However, it should be emphasized that despite these similarities between the OVA-induced airway inflammation and human allergic asthma, it is controversial whether other asthma-associated symptoms such as plasma exudation and eosinophil degranulation are present in the experimental animal models (4, 5).

To investigate the changes in protein patterns that are accompanying an airway inflammation, we used an approach based on proteomic screening of protein levels. Samples from lung tissue and from bronchoalveolar lavage fluid (BALF) were separated by two-dimensional gel electrophoresis, and protein spots that showed a marked difference in intensity between sensitized and challenged versus control animals were identified by mass spectrometry. Several proteins were identified for which there was a clear increase during the allergen-induced airway inflammation. Interestingly, many of the proteins that were increased are known to be associated with hypoxia and cell stress, indicating a link between allergen-induced airway inflammation and increases of hypoxia-related gene products. Furthermore, a dramatic increase was seen for Ym2, a protein of unknown function that has previously been reported to be dependent on CD4⁺ T cells and IL-4 or IL-13 signaling for expression (6).

METHODS

Animals (9- to 13-week-old female C57BL/6 mice) were sensitized intraperitoneally with 200- μ l OVA/aluminum hydroxide gel (1:3) on Days 0 and 14 (for additional details, see online data supplement). On Days 30, 33, and 35, mice were challenged in the lungs by inhalation of aerosolized OVA for 30 minutes as previously described (7). Three different control groups were used: mice receiving either no treatment, only OVA-aerosol challenge (as mentioned previously here), or sensitization as mentioned previously without subsequent challenge. Mice were killed after the last aerosol challenge, and BAL was performed. The BALF was centrifuged, and the supernatants were collected for the two-dimensional electrophoresis analysis. The cells were resuspended, and total leukocytes were counted. For determination of BALF eosinophils, cytopsin slides were prepared and stained with May-Grünwald Giemsa. The percentage of eosinophils was determined by differential counts of 300 cells per slide using standard morphologic criteria. For histologic assessment of lung tissue eosinophils, the lungs were inflated with 0.3 ml of TissueTek OCT and diluted 1:3 in phosphate-buffered saline before excision, and the left lobe was dissected and covered with TissueTek OCT before freezing in liquid petroleum gas. Frozen tissue was thereafter serially sectioned and mounted on superfrost slides. Eosinophils were stained using the phenol red assay as described by Ain and colleagues (8). Duplicate sections per animal were examined from each group of five mice.

(Received in original form February 10, 2004; accepted in final form May 18, 2004)

Supported by grants from the Swedish Medical Research Council, Vårdalstiftelsen, King Gustaf V's 80th anniversary Fund, and the Swedish Ministry of Defense.

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This article has an online supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Am J Respir Crit Care Med Vol 170, pp 477–484, 2004

Originally Published in Press as DOI: 10.1164/rccm.200402-1780C on May 19, 2004

Internet address: www.atsjournals.org

One-milliliter aliquots of BALF supernatants corresponding to five different animals (five control subjects or five sensitized and challenged animals) were pooled, concentrated, and stored at -80°C . Immediately after the BAL, lungs were frozen in liquid nitrogen and stored at -80°C . For extraction of proteins, the entire lungs were homogenized at 4°C in 350 μL of lysis buffer (50-mM Tris/HCl [pH 7.4] containing 1% Triton-X-100, 0.1% sodium dodecyl sulfate, and 1 mM ethylenediamine-tetraacetic acid). Homogenates were centrifuged, and supernatants were recovered and stored at -80°C . Four hundred micrograms (BALF samples) or 750 μg (lung extract samples) of protein were applied to nonlinear pH 3–10 immobilized pH gradient strips. Isoelectric focusing was performed, and strips were then equilibrated at room temperature for 15 minutes in sodium dodecyl sulfate-equilibration buffer and for another 15 minutes with sodium dodecyl sulfate-equilibration buffer supplemented with 2.5% (wt/vol) iodoacetamide. After equilibration, strips were applied to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Electrophoresis was performed at 2.5 W per gel during the first 30 minutes followed by 17 W per gel until complete. Gels were stained using the colloidal Coomassie procedure (9) and were stored at 4°C in 25% ammonium sulfate. For gel-image analysis, gels were scanned at high resolution, and the PDQuest software was used for detection of qualitative and quantitative alterations in protein spots. Spots of interest were analyzed by mass spectrometry using either a Reflex IV MALDI-ToF or an Ultraflex MALDI-ToF/ToF mass spectrometer. Protein identification was achieved by peptide mass fingerprinting of the spectral data. Digestion of proteins in the spots, mass spectrometry, and peptide mass fingerprinting searches were performed by the Proteomics Resource Center, Uppsala University (Uppsala, Sweden). Gelatin zymography assays were performed as previously described (10).

RESULTS

Characteristics of the Allergic Airway Inflammation

C57BL/6 mice were sensitized by intraperitoneal injections of 10- μg OVA adsorbed to aluminum hydroxide gel adjuvant and were thereafter challenged repeatedly with an aerosol of 10-mg/ml OVA. Eighteen hours after the last aerosol challenge, BAL was performed, and lungs were isolated and frozen, followed by counting and morphologic analysis of cells in BALF and lung tissue. In control mice, receiving no other treatment than OVA aerosol, the number of total recovered leukocytes in BALF was less than 300,000, with a predominance of alveolar macrophages ($>95\%$) and only a few eosinophils (Figure 1). We have previously shown that the cellular composition in BALF taken from the other two control groups (nontreated or only sensitized) is similar to the composition in control subjects that were only challenged (7).

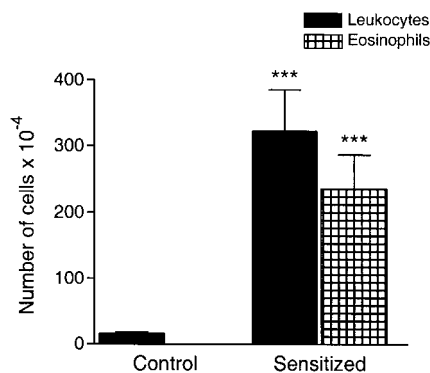


Figure 1. Numbers of eosinophils and total leukocytes in bronchoalveolar lavage fluid (BALF) from control (only challenged) and sensitized and challenged C57BL/6 mice 18 hours after the last of three repeated exposures of aerosolized ovalbumin (OVA). Control mice received no other treatment than aerosolized OVA. Mean values and standard deviations are shown ($n = 5$). *** $p < 0.001$, Student's t test (two-tailed).

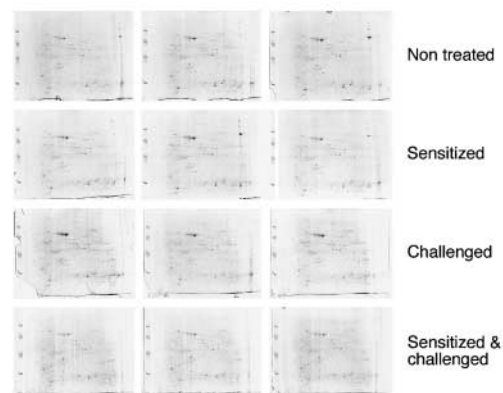
In contrast, BALF from sensitized and OVA-challenged mice showed a 10-fold increase in total number of leukocytes and was composed of up to 80% eosinophils.

For histologic assessment of infiltrating eosinophils into the lung tissue, serial sections were made and stained with phenol red. In lung tissue sections from sensitized and OVA-challenged mice, infiltrating eosinophils were concentrated in the perivascular regions, and a smaller number was found in the interstitial compartment of the peripheral alveoli. In control mice, only few eosinophils could be detected in the lung tissue (Figure E1 in the online supplement).

Proteomic Analysis

Proteins were extracted from lung tissues and were separated by two-dimensional gel electrophoresis. Samples from three different control (either nontreated, only sensitized, or challenged only) and sensitized and challenged animals, respectively, were analyzed. The three different samples from each group of animals produced virtually identical protein patterns after two-dimensional gel electrophoresis (Figure 2). Furthermore, the protein patterns were essentially identical in control animals that were either nontreated, only sensitized, or only challenged (Figure 2). Representative enlarged gels are shown in Figure 3 (as control is shown a sample from a mouse that had been

Lung tissue (A)



BALF (B)

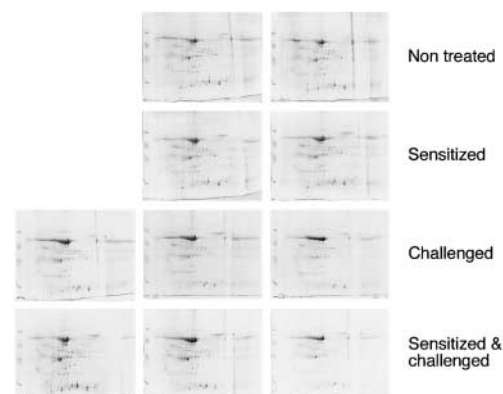


Figure 2. A general overview of the two-dimensional gels used in the proteomic analysis. Samples from lung tissue (A) or BALF (B), prepared as described in METHODS, were separated by two-dimensional electrophoresis (pH 3–10; 10% polyacrylamide gel electrophoresis), and proteins were stained with colloidal Coomassie.

challenged only). A number of protein spots exhibited a marked change in intensity as a result of the induced lung inflammation and were selected for identification by mass spectrometry. In many cases, protein spots were clearly detectable in samples from sensitized and challenged animals but were undetectable in samples from all three groups of control animals (indicated by “+” in Table 1). In other cases, spots were detectable in samples from both control and sensitized and challenged animals but showed a significantly altered intensity (indicated by “↑” for spots that are increased in inflamed tissue and “↓” for spots that are decreased in inflamed tissue; the degree of increase/decrease when comparing sensitized and challenged versus only challenged is indicated). In several cases, proteins were identified by a fragment thereof, and in these cases, it is thus possible that the allergic conditions results in degradation of the respective protein rather than upregulation of its expression.

From Table 1 it is apparent that several of the proteins that were increased in inflammatory lung tissue belong to the glycolytic

pathway: aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and enolase. We also observed an increase in the levels of transketolase, an enzyme that is involved in glucose metabolism, although not being a part of the glycolytic pathway. There was also a clear increase in proteins that are related to the metabolism of reactive oxygen species: selenium-binding protein 1/2 and peroxiredoxin. Furthermore, the β subunit of prolyl-4-hydroxylase, a protein that is involved in collagen synthesis, was markedly elevated. Several of the spots that were increased in intensity in inflamed tissue corresponded to the molecular chaperones glucose-regulated protein 78 kD and glucose-regulated protein 58 kD. We also detected increases in fragments of intracellular structural proteins: tubulin, actin, and myosin. A number of the spots that were markedly increased in lung samples from sensitized and challenged animals were identified as arginase, an enzyme for which upregulated transcription has previously been associated with allergic airway inflammation (11). Furthermore, the mature form of cathepsin S was

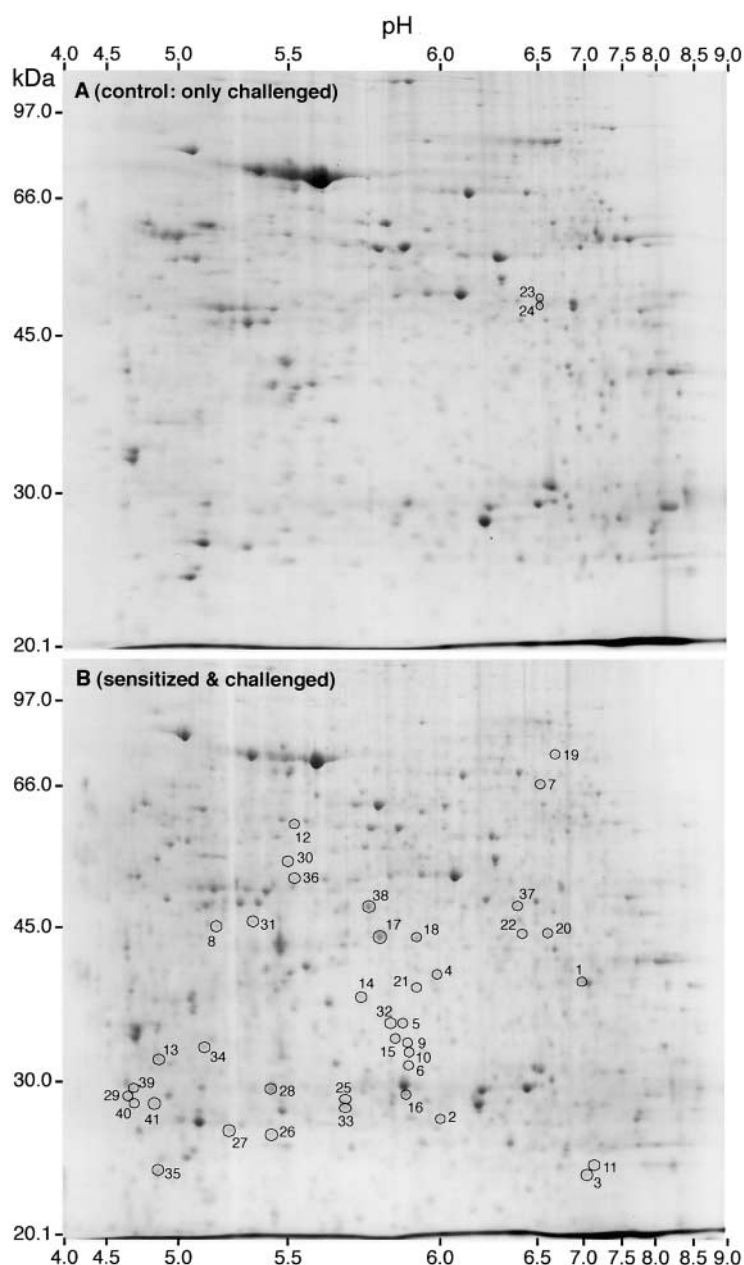


Figure 3. Representative gels for lung tissue samples corresponding to control (only challenged; A) and sensitized and challenged (B) animals. Spots of interest are enclosed in circles and are numbered for their identification in Table 1. The position of the molecular weight standards is indicated at the left of each gel; the pH range is indicated at the top and bottom, respectively.

TABLE 1. PROTEINS WITH ALTERED EXPRESSION LEVELS IN SENSITIZED AND CHALLENGED VERSUS CONTROL MOUSE LUNG

No.	Protein Name	Protein Expression [§]	SwissProt/TrEMBL Access No.	Peptide Match [†]	Theoretic Mass (kD/pI)	Observed Mass ^{**} (kD/pI)
1	Fructose biphosphate aldolase A (muscle-type aldolase)	+	P05064	8 p	39.2/8.4	39.7/7.1
2	Triose phosphate isomerase	+	P17751	8 p	26.6/7.1	25.9/6.0
3	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	↑ (10.24; p < 0.01)	P16858	3 p	35.7/8.4	21.8/7.1
4	Enolase 1 (nonneuronal)	+	P17182	6 p	47.0/6.4	40.5/6.0
5	Aldehyde dehydrogenase 2, mitochondrial (fragment)	+	P47738	12 p	54.4/6.0	34.8/5.9
6	Aldehyde dehydrogenase 2, mitochondrial (fragment)	+	P47738	10 p	54.4/6.0	30.5/5.9
7	Transketolase	↑ (4.07; p < 0.01)	P40142	12 p	67.6/7.2	72.9/6.5
8	Prolyl 4-hydroxylase β subunit	+	P09103	5 p	55.2/4.7	46.7/5.2
9	Selenium binding protein 1 (fragment)*	+	P17563	7 p	52.3/6.0	32.7/5.9
	Selenium binding protein 2 (fragment)		Q63836	7 p	52.6/5.8	
10	Selenium binding protein 1 (fragment)*	+	P17563	2 p (sc 36)	52.3/6.0	31.7/5.9
	Selenium binding protein 2 (fragment)		Q63836	2 p	52.6/5.8	
11	Peroxiredoxin 1 (macrophage 23-kDa stress protein)	↑ (7.77; p < 0.01)	P35700	4 p	22.2/8.3	22.5/7.2
12	Glucose-regulated protein 78 kDa	+	P20029	7 p	70.5/5.0	63.9/5.6
13	Glucose-regulated protein 78 kDa (fragment)	+	P20029	5 p (sc 38)	70.5/5.0	31.1/4.9
14	Glucose-regulated protein 58 kDa (fragment)	+	Q99LF6	13 p	56.7/5.9	37.7/5.7
15	Glucose-regulated protein 58 kDa (fragment)	+	Q99LF6	9 p	56.7/5.9	33.0/5.9
16	Glucose-regulated protein 58 kDa (fragment)	+	Q99LF6	9 p	56.7/5.9	27.8/5.9
17	Ym2 (Chitinase 3-like 4)	+	Q91Z98	10 p	42.8/5.7	45.3/5.8
18	Ym2 (Chitinase 3-like 4)	+	Q91Z98	8 p	42.8/5.7	45.3/5.9
19	Aconitase 2, mitochondrial (fragment)	+	Q99K10	8 p	85.5/8.1	80.0/6.8
20	Arginase 1 (liver type)	+	Q61176	8 p	34.8/6.5	45.7/6.6
21	Arginase 1 (liver type)	+	Q61176	3 p (sc 50)	34.8/6.5	38.9/5.9
22	Arginase 1 (liver type)	+	Q61176	3 p (sc 44)	34.8/6.5	46.2/6.4
23	Advanced glycosylation end-product-specific receptor	↓ (2.30; p < 0.05)	Q62151	6 p (sc 52)	40.5/5.7	52.4/6.5
24	Advanced glycosylation end-product-specific receptor	↓ (2.75; p < 0.01)	Q62151	4 p (sc 49)	40.5/5.7	51.4/6.5
25	Cathepsin S (mature form) [†]	+	O70370	4 p	24.9/6.1	27.3/5.7
26	β-Actin (fragment)	+	P02570	5 p	41.6/5.3	24.6/5.4
27	β-Actin (fragment)*	+	P02570	5 p	41.6/5.3	24.9/5.3
	γ-Actin (fragment)		P02571	5 p	41.7/5.3	
	A-X actin (fragment)		Q61276	5 p	41.7/5.2	
28	β-Actin (fragment)*	↑ (5.11; p < 0.01)	P02570	11 p	41.6/5.3	28.1/5.5
	γ-Actin (fragment)		P02571	11 p	41.7/5.3	
	A-X actin (fragment)		Q61276	11 p	41.7/5.2	
29	Tubulin β-5 chain	+	P05218	4 p (sc 51)	49.7/4.8	27.5/4.7
30	Nonmuscle heavy-chain myosin II-A (fragment)	↑ (6.17; p < 0.01)	Q8VDD5	10 p	226.4/5.5	56.9/5.5
31	Nonmuscle heavy-chain myosin II-A (fragment)	+	Q8VDD5	9 p	226.4/5.5	47.6/5.4
32	Serum albumin (fragment)	+	P07724	4 p	65.9/5.5	34.8/5.8
33	Serum albumin (fragment)	+	P07724	7 p	65.9/5.5	26.7/5.7
34	Serum albumin (fragment)	+	P07724	6 p	65.9/5.5	32.1/5.2
35	Serum albumin (fragment)	+	P07724	5 p	65.9/5.5	22.0/4.9
36	Serum albumin (fragment) [‡]	+	P07724	9 p	65.9/5.5	54.3/5.5
	Transferrin (fragment)		Q92111	8 p	74.9/6.8	
37	Heterogeneous nuclear ribonucleoprotein A/B	↑ (4.64; p < 0.05)	Q99020	6 p	30.8/7.7	50.1/6.4
38	Not identified	↑ (5.91; p < 0.01)				49.6/5.7
39	Not identified	+				28.4/4.7
40	Not identified	+				27.2/4.7
41	Not identified	+				27.0/4.9

* Because of the high homology of the different isoforms, all possible sequence entries in Swiss-Prot are indicated as well as their corresponding calculated *Mr/pI* values.

[†] *Mr* and *pI* values calculated for the mature form, that is, without the signal and activation peptides.

[‡] A mixture of proteins was identified.

[§] The "+" signs indicate the presence of the corresponding protein spot in sensitized and challenged versus control (nontreated, only sensitized, only challenged) mouse lung. Arrows (↑/↓) indicate significant upregulation/downregulation of the corresponding protein spot in sensitized and challenged versus control mouse lung. Quantitative changes, either increases or decreases, are indicated in parenthesis as well as the level of significance according to a Student's *t* test (fold; *p* value).

[†] The number of assigned peptides. Unless indicated in parenthesis, the score obtained in the MASCOT-PMF searches was above significance (> 62).

^{||} Calculated from the corresponding Swiss-Prot/TrEMBL entry. Signal and propeptide sequences documented by Swiss-Prot were removed, and theoretical *Mr/pI* values were recalculated.

^{**} Estimated from the position of the protein spot on the two-dimensional gel. *Mr* values were calculated according to the migration of *Mr* standards. *pI* values were determined by plotting each protein spot in the percentage of gel length versus pH graphs supplied by Amersham Biosciences (Uppsala, Sweden).

detected in inflamed but not control tissue. Out of the identified proteins, Ym2 was most dramatically increased as a consequence of induced airway inflammation. Ym2 was undetectable in control lung tissue while being one of the dominating proteins in lungs taken from sensitized and challenged animals. We also noted an increase in the level of various fragments of serum albumin in inflamed tissue.

Analysis of the BALFs revealed that the protein content was enhanced approximately threefold as a consequence of airway inflammation. The increase in protein content of the BALF could be due to either increased plasma extravasation into alveoli or could be a result of protein secretion by the cells that are recruited to the inflammatory site. The BALFs were subjected to two-dimensional gel electrophoresis (Figure 4), followed by

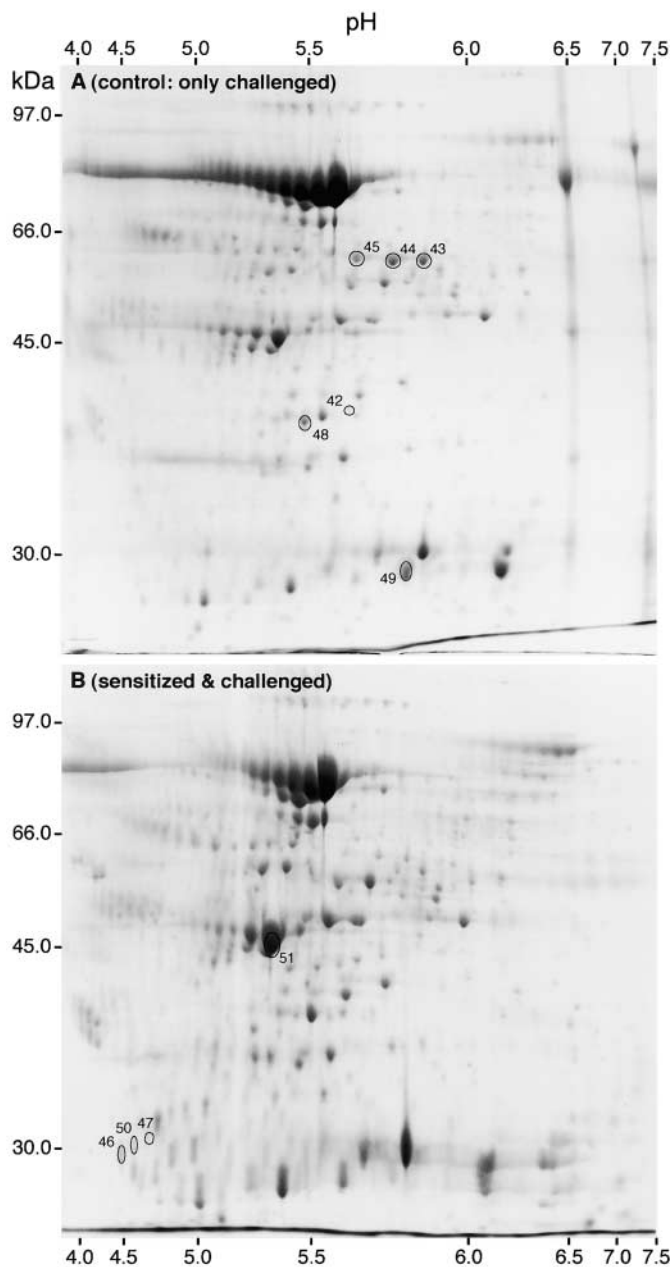


Figure 4. Representative gels for BALF samples corresponding to control (A) and sensitized and challenged (B) animals. Spots of interest are enclosed in circles and numbered for their identification in Table 2. The position of the molecular weight standards is indicated at the left of each gel, and the pH range is indicated at the top and bottom, respectively.

analysis as described for lung tissue samples. Two of the protein spots that were increased in intensity in BALF from sensitized and challenged animals corresponded to different fragments of serum albumin, indicating increased proteolytic degradation of extravasated plasma proteins (Table 2). Thus, to investigate whether the induction of airway inflammation was accompanied by enhanced levels of proteolytic activity, BALF samples were analyzed by gelatin zymography. Indeed, the level of gelatin-degrading proteases, in particular a protease with a molecular weight matching that of matrix metalloprotease 2, was considerably higher in inflammatory than in control (only challenged) BALF (Figure 5). It is noteworthy that the observed increase

in the levels of matrix metalloproteases is consistent with a previous report in which increases in both matrix metalloprotease-2 and matrix metalloprotease-9 were observed during airway inflammation (12).

BALF from inflammatory conditions contained Ym1 (absent in control subjects), although the score for identification of Ym1 was slightly below the threshold for significance. Furthermore, reduced levels of selenium binding protein 1/2, annexin A3, and peroxiredoxin 6 were observed in BALF recovered from the sensitized and challenged mice as compared with control subjects (Table 2).

DISCUSSION

This study was undertaken to identify alterations in the lung proteome that are caused by hypersensitivity responses in airways. Using an unbiased approach based on proteomic analysis, we have identified a number of proteins that were markedly increased during the airway inflammation. Interestingly, many of these proteins (*see later here*) have previously been linked to hypoxia, thus raising the possibility that the allergic lung inflammation induced may be accompanied by induction of hypoxia-sensitive genes. Hypoxia is known to induce large changes in gene expression (13), and these changes are thought to be mainly regulated by hypoxia-inducible factor 1, which during hypoxia is activated and binds to hypoxia-responsive elements in target genes (14–16). We may therefore hypothesize that hypoxia-inducible factor 1 activation accompanies allergic lung inflammation, although further studies will be required to establish a definitive connection between this type of disease, hypoxia, and hypoxia-inducible factor 1-mediated gene expression.

If there is a limited supply of oxygen, the cell is dependent on the glycolytic pathway for anaerobic ATP production, and consequently, many of the glycolytic enzymes are upregulated during hypoxia (17–20). It is apparent from Table 1 that the levels of a number of glycolytic enzymes are increased during the airway inflammation: enolase, aldolase, triose phosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase. Indeed, hypoxia-inducible factor-binding sequences have previously been identified in the genes for these enzymes (17–20), and it is thus possible that the increased levels of these gene products are a result of hypoxia-inducible factor 1 activation that may accompany the inflammatory reaction. We also observed that the allergic inflammation results in increases in transketolase, an enzyme that previously has been suggested to be upregulated by hypoxia (21).

In inflamed lung tissue, we noted increased levels of the β -subunit of prolyl-4-hydroxylase, an enzyme involved in collagen synthesis. Connective tissue remodeling is a typical feature of asthma, a condition that often is associated with increased collagen synthesis resulting in thickening and increased density of the subepithelial basement membrane (22). It is thus possible that the deposit of interstitial collagen that occurs during allergic asthma may be associated with an upregulated expression of prolyl-4-hydroxylase. It is also of interest to note that an induced expression of the α subunit of prolyl-4-hydroxylase (I) has been described under hypoxic conditions (23).

If oxygen is scarce, electrons that are normally donated to the electron transport chain may instead be donated directly to oxygen, forming reactive oxygen species. In line with this notion, we identified reactive oxygen species-metabolizing proteins in inflamed lung tissue. One of the major enzymes involved in the scavenging of reactive oxygen species is glutathione peroxidase, a selenium-dependent enzyme. Possibly, the changes in the levels of selenium binding protein 1/2, both in lung tissue and in BALF,

TABLE 2. PROTEINS WITH ALTERED EXPRESSION LEVELS IN SENSITIZED AND CHALLENGED VERSUS CONTROL MOUSE BRONCHOALVEOLAR LAVAGE FLUID

No.	Protein Name	Protein Expression [†]	SwissProt/TrEMBL Access No.	Peptide Match [‡]	Theoretic Mass [§] (kD/pl)	Observed Mass [§] (kD/pl)
42	Lactate dehydrogenase B	—	P16125	8 p	36.4/5.7	41.6/5.6
43	Selenium binding protein 1*	↓ (2.88; p < 0.01)	P17563	16 p	52.3/6.0	61.6/5.9
	Selenium binding protein 2		Q63836	15 p	52.6/5.8	
44	Selenium binding protein 1*	↓ (3.03; p < 0.01)	P17563	16 p	52.3/6.0	61.6/5.8
	Selenium binding protein 2		Q63836	15 p	52.6/5.8	
45	Selenium binding protein 1*	↓ (3.89; p < 0.05)	P17563	13 p	52.3/6.0	62.1/5.6
	Selenium binding protein 2		Q63836	12 p	52.6/5.8	
46	Ym1 (chitinase 3-like 3; fragment)	+	O35744	3 p (sc 46)	42.3/5.3	27.3/4.4
47	Ym1 (chitinase 3-like 3; fragment)	+	O35744	3 p (sc 43)	42.3/5.3	28.4/4.6
48	Annexin A3	↓ (2.44; p < 0.01)	Q8C1X9	13 p	36.4/5.5	39.9/5.5
49	Peroxiredoxin 6	↓ (3.74; p < 0.01)	O08709	10 p	24.7/5.7	25.9/5.8
50	Serum albumin (fragment)	+	P07724	6 p (sc 49)	65.9/5.5	28.1/4.5
51	Serum albumin (fragment)	↑ (1.79; p < 0.05)	P07724	12 p	65.9/5.5	48.3/5.4

* Because of the high homology of the different isoforms, all possible sequence entries in Swiss-Prot are indicated as well as their corresponding calculated *Mr/pl* values.

[†] The “+/-” signs indicate the presence/absence of the corresponding protein spot in sensitized and challenged versus control (nontreated, only sensitized, and only challenged) mouse BALF. Arrows (↑/↓) indicate significant upregulation/downregulation of the corresponding protein spot in sensitized and challenged versus control mouse BALF. Quantitative changes, either increases or decreases, are indicated in parenthesis as well as the level of significance according to a Student's *t* test (fold; p).

[‡] Number of assigned peptides. Unless indicated in parenthesis, the score obtained in the MASCOT-PMF searches was above significance (> 62).

[§] Calculated from the corresponding Swiss-Prot/TrEMBL entry. Signal and propeptide sequences documented by Swiss-Prot were removed, and theoretic *Mr/pl* values were recalculated.

[¶] Estimated from the position of the protein spot on the two-dimensional gel. *Mr* values were calculated according to the migration of *Mr* standards. *pl* values were determined by plotting each protein spot in the percentage of gel length versus pH graphs supplied by Amersham Biosciences.

may thus be related to an altered reactive oxygen species metabolism during the airway inflammation. Furthermore, peroxiredoxin 1, an enzyme that detoxifies hydrogen peroxides, was markedly increased in inflammatory lung tissue. Notably, a link between peroxiredoxin and hypoxic conditions has been suggested previously (24). Many of the identified protein spots that were increased in intensity on induction of airway inflammation corresponded to glucose-regulated protein 78 kD/glucose-regulated protein 58 kD. Glucose-regulated protein 78 kD/glucose-regulated

protein 58 kD are molecular chaperones that show upregulations under different conditions associated with stress, for example, hypoxia (25, 26).

A marked increase in Ym2 was observed because of the allergic airway inflammation. The biological function of this protein is unknown, although it has been suggested that Ym2 may play a role in the airway wall thickening that is associated with the pathology of allergic lung disease (6). Previous studies have shown that Ym1, a closely related protein, has heparin-binding properties (27), but it is not certain whether binding to heparin or similar glycosaminoglycans is relevant for its function *in vivo*. Ym1 is known to be expressed by macrophages, in a T helper 2-dependent manner (28), and it has been shown that Ym1 displays a marked tendency to form crystals in tissues (27, 29). Induced expression of Ym1/2 during allergic airway inflammation is also supported by a previous study (6).

Advanced glycosylation end product-specific receptor was markedly reduced in inflammatory lung tissue. Advanced glycosylation end product-specific receptor is a receptor for the glycosylated proteins that are formed at high blood glucose levels (30). The reason for its decrease during airway inflammation is not clear. However, we may hypothesize that an altered glucose metabolism affects plasma protein glycation and that this, in turn, may affect the receptor for this group of proteins. Furthermore, it has recently been demonstrated that the expression of advanced glycosylation end product-specific receptor is influenced by hypoxia (31). Nitric oxide is currently widely accepted as a major marker for steroid sensitive asthma. The production of nitric oxide from L-arginine is regulated by arginase, and arginase has recently gained a large interest in the context of airway disease because of the finding that arginase transcription is markedly upregulated in mouse models for asthma (11). The findings reported here thus provide further support for an induced expression of arginase during allergic airway inflammation. Interestingly, arginase expression in macrophages has been found to be upregulated by hypoxia (32).

The increase in cathepsin S observed in allergen-challenged animals may be related to the actual immune response. Cathepsin

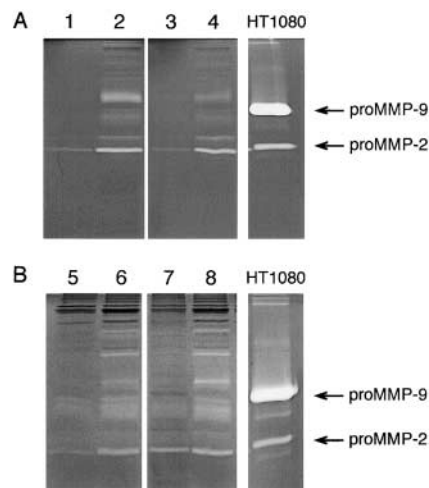


Figure 5. Gelatin zymography of BALF samples recovered from control (only challenged; lanes 1, 3, 5, and 7) and sensitized and challenged (lanes 2, 4, 6, and 8) animals. (A) Equal volumes (30 μ l) of BALF samples were loaded in each lane. (B) Samples were concentrated and equal amounts of protein (12 μ g) were loaded in each lane. A 10- μ l sample of HT1080-conditioned medium was also included as a source of pro-matrix metalloprotease-2 (proMMP-2) and pro-matrix metalloprotease-9 (proMMP-9).

S is a lysosomal cysteine protease that is involved in antigen presentation (33). In fact, it has been shown that inhibition of cathepsin S results in an impaired immune response to the specific allergen used in this study (i.e., OVA) (34). Several spots corresponding to fragments of actin proteins, tubulin β -5 chain, and nonmuscle heavy chain myosin were identified in allergen-sensitized and -challenged tissue. Using a similar approach to ours, Houtman and colleagues recently reported that actin and other cytoskeletal proteins were upregulated in nonallergic asthma (35). It thus appears that cytoskeletal changes occur in both mouse models of IgE-mediated and nonatopic asthma, suggesting that the type of sensitization of the immune system is not a major determinant for this effect. Instead, it is more likely that the induction of these proteins indicates alterations in endothelial barrier function or, alternatively, changes of cell morphology and motility associated with smooth muscle contraction.

An important issue regards the cellular source of the proteins that are increased because of the allergic lung inflammation. Clearly, some of the proteins identified, for example, Ym2 and cathepsin S, may arise from inflammatory cells infiltrating the lung tissue (e.g., eosinophils; see Figure E1 in the online supplement), whereas others, such as the glycolytic enzymes, may be derived from resident cells. In the latter case, the protein expression pattern may have been influenced by the inflammatory conditions. Another important aspect is the apparent lack of identified spots for classic markers of allergic inflammation such as the cytokines, IL-5, IL-4, and IL-13. Most likely, however, the failure to detect such cytokines lies within the limited sensitivity of the method employed, combined with low expression levels.

In summary, this study identifies a number of marker proteins for allergic airway inflammation. Some of the identified proteins, for example, arginase and Ym2, have previously been linked to this type of disease, although the nature of their contribution to disease development is not fully understood. In addition, we here identify several marker proteins for which a link to allergic airway inflammation has, to our knowledge, not been recognized previously. An important and obvious question is whether any of these marker proteins could constitute potential drug targets. Probably, interfering with prolyl-4-hydroxylase or cathepsin S could have beneficial effects by reducing collagen deposition and by interfering with antigen presentation, respectively. However, it is clear that future work is required to determine the contribution to the disease pathogenesis by each of the different proteins that were increased in airway inflammation.

Conflict of Interest Statement: I.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; L.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this article; G.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this article.

Acknowledgment: The mass spectrometry analyses were performed by the Proteomics Resource Center at Uppsala University, sponsored by The Wallenberg Consortium North. The authors thank Jens Forsberg (Proteomics Resource Center) for helpful discussions.

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