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Lung proteome alterations in a mouse model for nonallergic asthma

A mouse model for nonatopic asthma was employed to study the alterations of the lung proteome to gain insight into the underlying molecular mechanisms of disease pathophysiology post-challenge. Lung samples from asthmatic and control mice were used to generate 24 high quality two-dimensional electrophoresis gels wherein 2115 proteins were examined for disease relevance. In total, 23 proteins were significantly up- or down-regulated following hapten-challenge of dinitro-fluorobenzene-hypersensitive mice. Twenty proteins were identified by mass spectrometry, of which 18 could be linked to asthma related symptoms, such as stress and inflammation, lung detoxification, plasma exudation and/or tissue remodeling. As such, proteomics was clearly vindicated as a means of studying this complex disease phenomenon. The proteins found in this study may not necessarily play a role in the immunological mechanisms and/or pathophysiology of asthma development. However, they may prove useful as surrogate biomarkers for quantitatively monitoring disease state progression or response to therapy. The mathematics of achieving statistical confidence from low numbers of gel replicates containing large numbers of independent variables stress the need for high numbers of replicates to better sample the population of proteins revealed by two-dimensional gel electrophoresis.

Keywords: Delayed-type hypersensitivity / Dinitro-fluorobenzene / Surrogate biomarkers / Two-dimensional gel electrophoresis
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1 Introduction

Asthma is a chronic inflammatory and immunological airway disease, characterized by infiltration of the lungs by inflammatory cells, formation of reactive oxygen species (ROS), plasma exudation due to vascular leakage, mucus production, bronchoconstriction and bronchial hyper-responsiveness [1–4]. Treatments currently used for asthma are aiming for bronchodilation by short- and long-acting β_2 -agonists or inhibition of inflammation by corticosteroids [5–8]. These treatments provide only temporal symptomatic relief but do not prevent the irreversible pathology characterized by airway tissue remodeling, including hyperplasia of airway smooth muscle (ASM), and subsequent impaired airway function often observed in asthma patients [1, 9, 10]. Treatment aimed at the intervention of fundamental disease genesis is thus needed, but requires a better insight into the molecular aspects of asthma.

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Abbreviations: ASM, airway smooth muscle; DNFB, 2,4-dinitro-fluorobenzene; DNS, dinitrobenzene sulfonic acid; DTH, delayed-type hypersensitivity; ROS, reactive oxygen species

Interactions between environmental factors and host-dependent genetically determined background lead to the asthmatic phenotype [11, 12]. Phenotypes are determined by proteins [13], and therefore, to better understand the molecular basis of this pathology, we have adopted a proteomics approach, *i.e.* analysis of the total complement of proteins, including post-translational modifications, expressed by a genome [14, 15].

At present, 2-DE is still the method of choice when it comes to the highest resolving power for the separation of complex protein mixtures [16–18]. On the other hand, it suffers from high variation since the intensity of spots that represent the expression level of the same protein can vary significantly on different gels. This variation is introduced during sample preparation and protein loading and by the complex nature of the silver staining procedure used to visualize separated proteins [19, 20]. Moreover this technique-inherent variation is further exacerbated by biological variation such as tissue and cell heterogeneity. It is therefore necessary to produce a large number of replicate gels from the same sample or treatment group, in order to obtain statistically significant changes in protein levels between different groups, *e.g.*, asthma *versus* control.

In this study, a mouse model for nonatopic asthma was used. The latter involves a nasal hapten challenge at five days after contact sensitization with the hapten 2,4-dini-

tro-fluorobenzene (DNFB), which provokes delayed-type hypersensitivity (DTH) in the lungs and is characterized by all clinical features described for asthma. Although established as a model for nonatopic asthma, previous data have shown that sensitization induced an antigen-specific humoral factor, namely immunoglobulin light-chain [21]; and that clinical phenomena provoked by the allergen were mast cell-dependent [22]. This suggests that the development of the pathology of atopic and nonatopic asthma may have a similar base. Lung protein samples were analyzed by 2-DE to find changes in the lung proteome caused by a hapten-specific airway challenge. A synthetic protein map of total lung tissue for asthmatic and control mice was constructed and proteins with statistically significant alterations in expression level were subjected to characterization by mass spectrometry.

2 Materials and methods

2.1 Animals

Male BALB/c mice (6–8 week, Central Animal Laboratory, Utrecht University, Utrecht, The Netherlands) were used. The experiments were approved by the Animal Care Committee, Utrecht University.

2.2 Induction of DTH

DTH in mice was established as described previously [22]: BALB/c mice were sensitized by application of DNFB (50 μ L, 0.5% in acetone:olive oil (4:1)) onto the abdomen (50 μ L) and paws (50 μ L) at day 0, boosted by abdominal application of DNFB (50 μ L) at day 1 and challenged intranasally with dinitrobenzene sulfonic acid, the water-soluble antigen-equivalent of DNFB (DNS, 50 μ L, 0.6% in PBS) at day 5 (treatment: D/D). Control mice were sensitized (V/D) or challenged (D/V) or both (V/V) with vehicle (acetone:olive oil (4:1)) instead of DNFB/DNS.

2.3 Ear swelling response, isolation of lungs

At day 6, mice were challenged by application of DNFB (0.2%) on one ear and vehicle on the other. Two hours after the challenge, mice were sacrificed with an overdose of pentobarbital (60 mg/mL, 200 μ L intraperitoneally; Sanofi, Maassluis, The Netherlands). Ear thickness was measured with an engineer's micrometer (Mitutoyo, Japan) at three different places of the ear. Results were expressed as the difference between the DNFB ear

and vehicle ear (ear swelling, μ m). Additionally, lungs were isolated, frozen in liquid nitrogen and stored at -80°C .

2.4 Sample preparation

For all buffers, high quality, freshly dispensed MilliQ purified water was used (Millipore, Etten-Leur, The Netherlands). Lung tissue was placed in a clean mortar containing liquid nitrogen and roughly crushed. Next, 1 mL of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM DTT, trace of bromophenol blue) was added and samples were transferred to a 1.5 mL Eppendorf vial. Additionally, 0.8 g of Zirconia beads (0.7 mm diameter; Biospec Products, Bartlesville, OA, USA) was added and samples were processed for 25 cycles at 4°C in a Bioflot (C. H. Barman, Sydney, Australia). Samples were rested at room temperature for 30 min; supernatants (sup1) were transferred to 10 mL polycarbonate round bottom tubes. Beads were washed three times with sample buffer and supernatants were pooled with sup1. Samples were centrifuged for 30 min at $75\,600\times g$ in an Avanti J25I centrifuge using rotor JA-25.50 (Beckman Coulter, Mijdrecht, The Netherlands). Supernatants were transferred to clean vials, protein concentrations were quantified using Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands) and stored at -80°C in aliquots of 400 g. Each lung was processed and stored separately.

2.5 Protein separation and visualization

Protein (400 μ g) was applied to an 18 cm Immobiline Dry-Strip pH 4–7 (Amersham Biosciences, Roosendaal, The Netherlands). Strips were rehydrated for 12 h at 30 V, followed by focusing 1 h at 500 V, 2 h at 1500 V, and subsequently at 8000 V to 80 kVh on an IPGPhor (Amersham Biosciences) and stored at -80°C . Focused IPG strips were mounted on 20×20 cm SDS-PAGE gels (15% T + 0.2% C; WITA, Berlin, Germany) followed by electrophoresis, eight gels in parallel, using an ISO-DALT (Amersham Biosciences) at 25 mA/gel. Separated proteins were visualized by silver staining as described previously [23], sealed in plastic with MilliQ and stored at 4°C .

2.6 Image analysis

Gels were scanned on a GS-710 densitometer (Bio-Rad). Images (.tif) of scanned gels were analyzed using PDQuest software V6.2.1 (Bio-Rad).

2.7 Protein identification

Target spots were excised from the gel, gel pieces were destained and proteins digested with bovine trypsin (Roche, Almere, The Netherlands) for identification using mass spectrometry as described previously [23]. For MALDI-TOF, reactions were stopped with TFA (final concentration: 10%), peptides were concentrated and desalted using ZipTip_μ-C18 (Millipore, Etten-Leur, The Netherlands), eluted directly on the MALDI-target with 1 μL of a saturated solution of α -cyano-hydroxycinnamic acid in 50% acetonitrile. Peptides were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) in reflectron mode at 20 kV accelerating voltage. Mass fingerprints were submitted to ProFound database search interface <http://prowl.rockefeller.edu/cgi-bin/ProFound>.

Microcapillary LC-MS/MS was performed by interfacing an Ultimate HPLC pump (LC Packings, Amsterdam, The Netherlands) with an ESI quadrupole-time of flight instrument (Q-TOF; Micromass, Manchester, UK) operating in positive ion mode and equipped with a Z-spray nano-ESI source. Microcapillary columns and connections were used as described previously [24]. Briefly, samples were introduced by using a Famos autosampler (LC Packings), which first transferred the peptide extracts onto a 200 μm id trapping column packed with 2 cm of AquaTM C18 resin (5 μm; Phenomenex, Torrance, CA, USA). A switching valve was then used to transfer the eluted peptides *via* micro-Tee onto the analytical column (25 cm × 50 μm id), packed with PepMap C18 resin (LC Packings). The HPLC column eluent (flow rate of 100–150 nL/min) was sprayed directly into the ESI source of the mass spectrometer *via* a butt-connected nano-ESI emitter (spraying tip opening of 5 μm), which was prepared as described [24]. The peptide separation protocol consisted of an initial wash step with buffer A (100 mM acetic acid in MilliQ water) for 10 min, and subsequent elution of peptides with a linear gradient from 0–60% buffer B (100 mM acetic acid in acetonitrile) over 30 min. Fragmentation of eluting peptides was performed in data dependent mode, and mass spectra were acquired in full-scan mode. For protein identification, MASCOT software (www.matrixscience.com) was used for database searches both for peptide mass fingerprinting and peptide sequence tagging.

2.8 Statistical analysis

The asthma and control group proteins were analyzed for changes in levels of expression using the Wilcoxon two-sample test, a nonparametric statistic employed ideally for number sets less than 20 and greater than 5. Statistical data analysis was performed using SPSS (Chicago, IL, USA) for Windows version 10.0.05.

3 Results

3.1 Systemic hypersensitivity to DNFB

The protocol (Fig. 1A) resulted in four treatment groups (Table 1). To confirm systemic sensitization, mice were challenged with DNFB or vehicle on either ear at day 6. After 2 h, ear thickness was measured and the difference in ear thickness between left and right ears was calculated (Fig. 1B). DNFB-sensitized mice (D/V, D/D), showed significant ear swelling upon DNFB challenge, which was absent in vehicle-sensitized mice (V/V, V/D). Because the site of challenge in this experiment (ear), is different from the site of sensitization (footpad and abdomen) we concluded that systemic hypersensitivity to DNFB had been established in these mice.

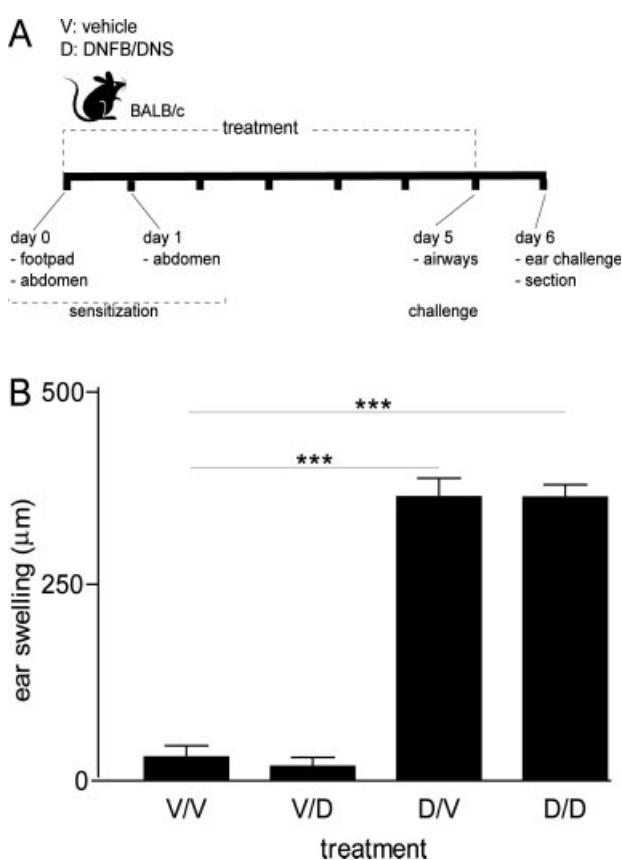


Figure 1. A, Schematic presentation of the applied DNFB protocol. BALB/c mice were sensitized by application of dinitro-fluorobenzene onto the abdomen and paws at day 0, boosted by abdominal application of DNFB at day 1 and challenged intranasally with DNS at day 5 (D/D). Control mice were sensitized (V/D) or challenged (D/V) or both (V/V) with vehicle instead of DNFB/DNS. At day 6, ear swelling experiments were performed. B, Results are expressed as the difference in thickness between the DNFB- and vehicle-challenged ear (ear swelling, μm, ***: $p < 0.0001$ by Student's *t*-test).

Table 1. Overview of experimental setting and results *per* treatment group

	V/V	V/D	D/V	D/D
Number of mice	4	4	4	4
Sensitization	vehicle	vehicle	DNFB	DNFB
Challenge	vehicle	DNS	vehicle	DNS
Ear swelling	—	—	+ ^{a)}	+ ^{a)}
Number of gels	8 ^{b)}	4	4	8 ^{b)}
Gel analysis group	control	control	control	asthma
Min/max spot number	1434/2567	2254/2453	2181/2642	1430/2630
Average spot number	2032	2356	2307	2059

a) $p < 0.0001$ compared to control (V/V) by Student's *t*-test

b) Duplicate *per* mouse

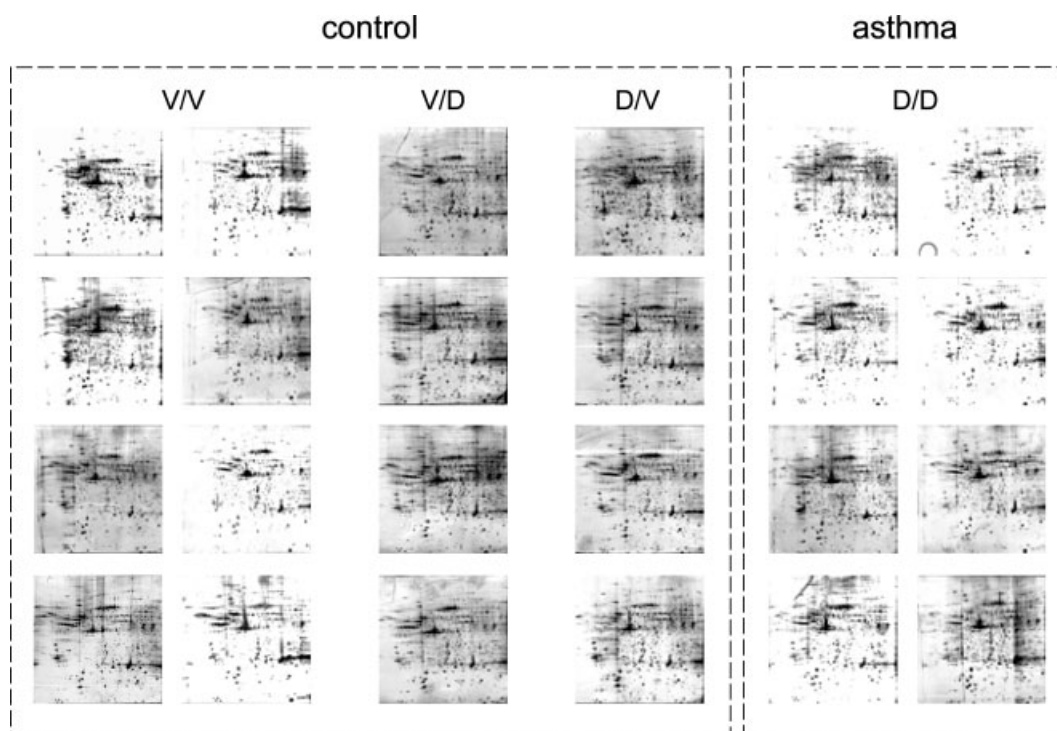
3.2 2-DE and spot detection

At day 6, one day after the airway challenge, lungs were isolated. Protein samples were prepared and separated by 2-DE (pH 4–7, 15% PAGE). Two gels *per* mouse of

samples derived from both extreme treatment groups (V/V and D/D) and one gel *per* mouse from both intermediate treatment groups (V/D and D/V). Thus, the resulting gel series numbered 24 gels ($8 \times V/V$, $4 \times D/V$, $4 \times V/D$ and $8 \times D/D$, Fig. 2 and Table 1). Proteins were visualized by silver staining, gels were scanned and images were subjected to semiautomated analysis for spot content using PDQuest software. Despite the intra-group variation in spot number *per* gel, no significant differences in average spot number between different treatment groups (Wilcoxon two-sample test) could be observed.

3.3 Spot matching across all gels, generation of synthetic protein map

Intra-group gel-to-gel variation reinforced the need for multiple gels during the construction of the reference dataset (*cf.* Fig. 3). Matching of each additional gel to the initial reference gel resulted in fewer spots being present in all matched gels. Indeed, only 210 spots were seen in all 24 gels, 412 in the 8 asthma gels and 244 in the 16 control gels (Fig. 3A). Alternatively, Fig. 3B shows a set of proteins selected by the criterion of “being present in at least six gels”, chosen because six replicates is the minimum number necessary to achieve significance by the

**Figure 2.** Total lung tissue protein samples were separated by 2-DE (pH 4–7, 15% PAGE) and visualized by silver staining.

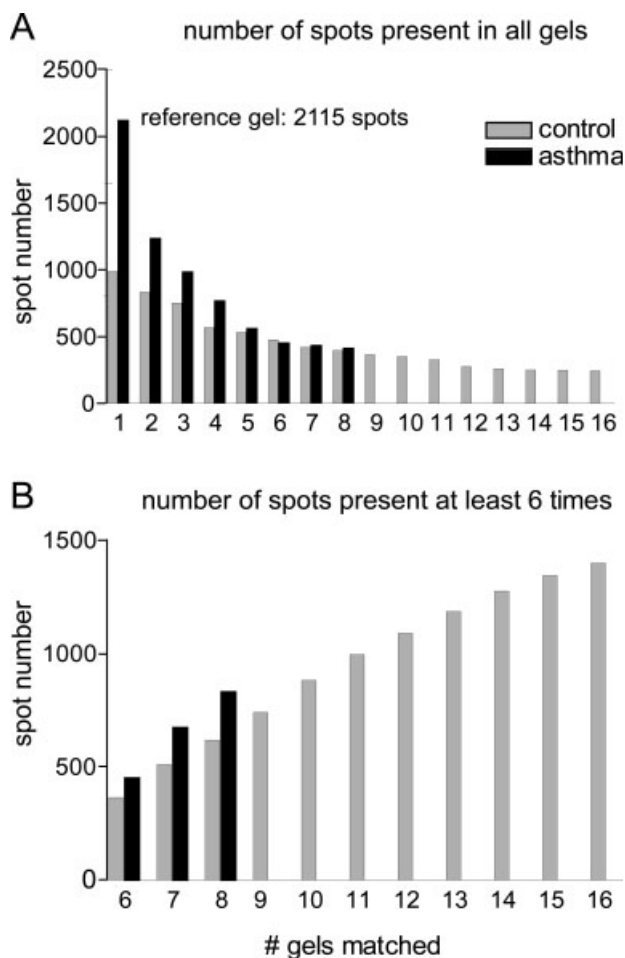


Figure 3. Spot matching across all gels, resulting in a reference library consisting of 2115 spots. Library spot numbers derived from control (gray bars) and asthma (black bars) are shown. A, 'Spot must be present in all gels' used as selection criterion. B, 'Spot must be present at least six times' used as selection criterion.

Wilcoxon two-sample test ($p < 0.05$). This set grows when additional gels are matched to the reference dataset. Thus, by matching more gels to the reference dataset, both the number of spots and the statistical significance of comparisons made on the library members increases.

3.4 Differential expression of proteins

Of each gel, spot intensities were normalized using a set of proteins ($n = 11$) that were expressed in a comparable manner across all gels. Protein expression levels from the asthma group, 8 D/D gels, were compared to those from the control group, including 8 V/V, 4 V/D and 4 D/V gels. The latter two groups served to correct for potential effects caused directly by treatment with DNFB or DNS but are not related to the DTH effects caused by the challenge with

this hapten. Statistically significant changes in protein expression were determined by the Wilcoxon two-sample test. Enhanced expression was found in 13 proteins while the expression of 10 others was decreased (Fig. 4).

3.5 Identification

The 23 differentially expressed proteins were excised from the gels and identified using trypsin digestion combined with MALDI-TOF or LC-MS/MS and database searching. Results are summarized in Table 2. Proteins 13, 20 and 23 could not be identified, probably due to the limited amount of protein in these spots. Furthermore, MS spectra derived from spots 10, 15 and 19 revealed the presence of at least two different proteins in these spots, which prevented conclusive identification of the protein causing the altered spot intensity. Spots 1, 4, 8, 9, 10, 16 and 21 were all found to represent pre-albumin. The location on the gel, below the theoretical 65.2 kDa, of the majority of these proteins suggests that these are albumin fragments. MS spectra indicated talin in both spots 2 and 6. Assigned peptides of spot 6 were all located at the N-terminal part of talin when compared the ones found in spot 2. This suggests that these are proteolytic fragments derived from the same native talin molecule. Analysis of spot 17 revealed actin, however our data did not allow for conclusive identification of the isoform (alpha, beta or gamma) of this protein.

4 Discussion

4.1 Strategy

In this study we have taken a proteomics approach to study altered lung protein levels correlated with nonatopic asthma in mice. In previous studies we have shown that intranasal hapten challenge of DNFB-sensitized mice leads to acute bronchoconstriction, increased vascular permeability, mucosal exudation and infiltration of neutrophils in bronchial alveolar lavage fluid [22]. Here, we found 23 proteins with significantly altered levels of expression, of which 20 were identified by MS. Because of the intrinsic variability of 2-DE based proteomics, a large dataset was generated to obtain statistical confidence in the observed correlation between altered protein levels and altered phenotype [19, 25]. Our data show that increase in the number of replicate gels enables construction of larger high-quality protein maps or libraries, e.g. consisting of proteins with statistically significant expression.

4.2 Plasma extravasation

Increased levels of serum albumin fragments probably reflect plasma leakage from pulmonary blood vessels into lung tissue. Serum albumin is proteolytically cleaved

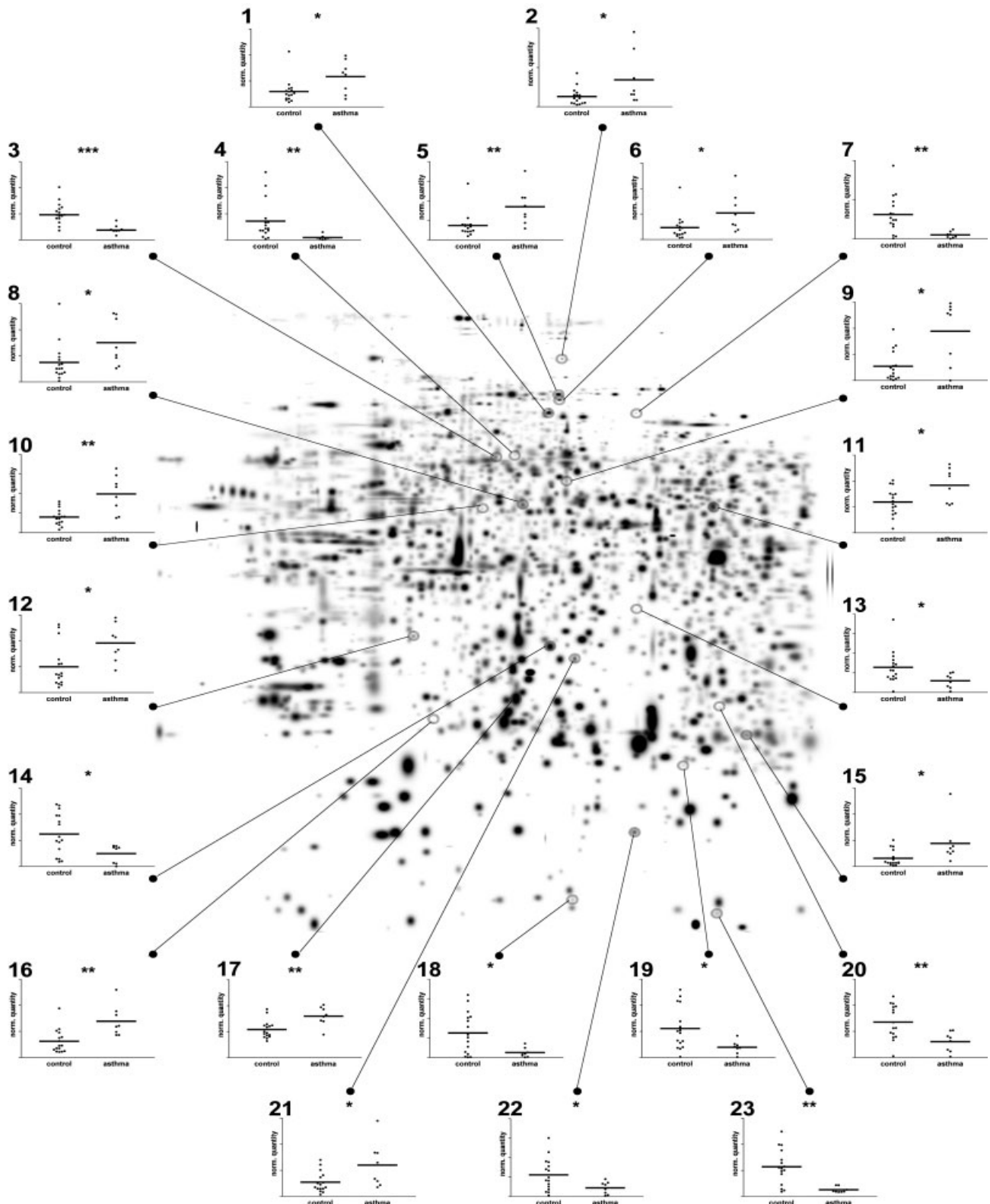


Figure 4. Differentially expressed proteins correlated with DNFB treatment. Synthetic protein reference map composed of dataset of 24 gels. Normalized spot intensities of the asthma (8 D/D gels) versus control (8 V/V, 4 V/D and 4 D/V gels) group were compared. Mean intensity and spot intensity of each individual gels are shown, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Wilcoxon two-sample test).

Table 2. Identification of proteins with altered expression levels in asthmatic *versus* control mouse lung

No.	Protein name	Expr. ^{a)}	SWISS-PROT acc. no.	Identif. method ^{b)}	MS results ^{c)}	Theoret. mass (kDa)/pI ^{d)}	Observed mass (kDa)/pI ^{e)}
<i>Vasodilatation/plasma extravasation</i>							
1	Serum Albumin	↑	P07724	MS/MS	6 p	68.6/5.8	94.4/5.8
4	Serum Albumin (fragment)	↓	P07724	PMF	33%	65.2/5.6	69.2/5.6
8	Serum Albumin (fragment)	↑	P07724	PMF	33%	65.2/5.6	54.9/5.6
9	Serum Albumin (fragment)	↑	P07724	MS/MS	2	65.2/5.6	61.9/5.8
10	Serum Albumin (fragment)	↑	P07724	MS/MS	7 p	65.2/5.6	53.9/5.5
16	Serum Albumin (fragment)	↑	P07724	PMF	21%	65.2/5.6	34.5/5.8
21	Serum Albumin (fragment)	↑	P07724	PMF	30%	65.2/5.6	33.2/5.9
<i>Tissue remodelling</i>							
2	Talin (fragment)	↑	P26039	MS/MS	3 p	269.7/5.8	121.1/5.8
5	Vinculin	↑	Q64727	MS/MS	17 p	117.0/6.0	108.7/5.8
6	Talin (fragment)	↑	P26039	MS/MS	4 p	269.7/5.8	105.0/5.8
7	Gelsolin	↓	P13020	MS/MS	10 p	80.7/5.5	94.0/6.1
17	actin	↑	P02571	MS/MS	13 p ^{f)}	41.0/5.6 ^{f)}	29.1/5.6
<i>Detoxification, cell responses to stress and inflammation</i>							
10	HSP60	↑	P19226	MS/MS	7 p	57.9/5.4	53.9/5.5
12	HSP90	↑	P11499	MS/MS	8 p	83.2/5.0	35.6/5.2
15	Lung carbonyl reductase 2	↑	P08074	MS/MS	5 p	26.0/9.1	24.7/6.6
19	Glutathione S-transferase μ	↑	P10649	MS/MS	3 p	25.8/8.1	22.7/6.4
	Glutathione S-transferase μ		P10649		3 p	25.8/8.1	
	Proteasome subunit alpha type 2		P49722		4 p	25.8/8.4	
22	T-DSP11	↓	Q9D7X3	MS/MS	5 p	20.5/6.1	18.7/6.2
<i>Various</i>							
3	Annexin VI	↓	P14824	PMF	43%	75.9/5.3	69.4/5.5
11	Coronin 1A	↑	O89053	MS/MS	10 p	51.0/6.0	54.6/6.5
18	Protein kinase C inhibitor protein 1	↓	Q9CQV8	MS/MS	5 p	28.0/4.8	15.4/5.9
<i>Unknown function</i>							
13	Not identified	↓	Q9D0A3	MS/MS	11 p	25.2/5.1	38.5/6.1
14	2610034B18Rik protein	↓					26.0/5.3
20	Not identified	↓					27.8/6.5
23	Not identified	↓					14.9/6.5

a) Protein expression asthma/control

b) PMF, peptide mass fingerprinting; MS/MS, LC-ESI-Q-TOF

c) PMF, % protein coverage; MS/MS, number of assigned peptides

d) Calculated from the corresponding SWISS-PROT entry. Signal sequences documented by SWISS-PROT were removed and theoretical M_r/pI values were recalculated

e) Estimated from the position of the protein spot on the 2-D gel (PDQuest software)

f) Based on SWISS-PROT acc. no. P02571, gamma-actin

into smaller fragments by proteases that are abundantly present in the lungs [26]. It has been suggested that altered airway permeability and plasma extravasation into the lung interstitial cavity and lumen during inflammation acts as a first line of defense [27–29]. Increased

albumin levels have been reported in bronchoalveolar lavage samples of human asthmatics [30, 31], and were used as a parameter for plasma extravasation in the DNFB model [22, 32] and various other animal models [33–35].

4.3 Tissue remodeling

The actin cytoskeleton plays an important role in cell morphology and motility. From our MS data we were not able to determine which actin isoform is up-regulated in our model. Alpha- and beta-actin are present in most cells, however gamma-actin is mainly expressed in muscle cells where it plays a role in contraction. Enhanced contractility of ASM found in asthma [36] and the DNFB model [22], could be the result of increased gamma-actin levels, although it remains unclear whether airway hyper-responsiveness is caused by changes in ASM phenotype [37, 38].

Talin, vinculin and gelsolin are expressed in a wide variety of cells and each play a role in the regulation of actin cytoskeleton dynamics. Gelsolin plays a role in building of the actin cytoskeleton itself by filament severing and capping [39, 40], while talin and vinculin are involved in linking the actin cytoskeleton to adherence junctions, namely structures in which cells make contacts with neighboring cells or to extracellular matrix [41–43]. *In vitro* stimulation of lung microvascular endothelial monolayers with various inflammatory agents induces intracellular translocation of vinculin and talin to cell-matrix contacts. This leads to enhancement of these structures and is accompanied by a decrease of endothelial barrier function [44]. This suggests that the observed alterations in talin and vinculin levels are linked to vasodilatation as a result of DNFB-induced inflammation. Additionally, functional analysis of the human vinculin gene has indicated the presence of a serum response element [45]. The latter is involved in transmigration of inflammatory cells [46, 47]. Enhanced vinculin levels in this study could therefore also reflect infiltration of the lungs by inflammatory cells. Alternatively, although MS analysis did not reveal any sequence of the 68 amino acid metavinculin-specific insert, our data does not exclude the option that the protein we found here is actually metavinculin, exclusively expressed in ASM. In the case of metavinculin, enhanced levels could be linked to remodeling of ASM responsible for altered airway function [9].

Factors that regulate gelsolin synthesis have not been identified, however loss of gelsolin is one of the most frequently occurring alterations in mammary cancers [48, 49]. In these tumors, gelsolin expression is down-regulated through promoter silencing by ATF-1 [50], a member of the ATF/cAMP response element binding protein family [51]. Enhanced ATF-1 activity has been reported in inflammatory processes [52, 53] and hypoxia [54, 55], which provides a possible explanation for the decreased gelsolin level observed in this study. Alternatively, our MS spectra revealed sequences of gelsolin beyond amino acid 376, which is a site for proteolysis during apoptosis of

neutrophils [56]. Reduced levels of full-length gelsolin found here could therefore also imply enhanced caspase 3 mediated cleavage and subsequent apoptosis of neutrophil, a prominent effector cell in this asthma model.

Coronin is exclusively expressed in hematopoietic cells and associates with the actin cytoskeleton. At present, little is known about its biological function although possible roles in phagocytosis and chemotaxis have been suggested [57]. Up-regulation of coronin gene expression in interleukin-4 stimulated B-cells has been reported [58]. IL-4 stimulation of B-cells promotes IgE production, an important event in the development of allergy.

4.4 Detoxification, cell responses to stress and inflammation

Carbonyl reductase and class μ GST, both found in lung tissue of various species [59–62], are critical in the protection of cells from the damage by ROS [63]. GST gene polymorphism has been linked to susceptibility for the development of atopic [64–66] and as well as nonatopic [67] asthma in humans previously.

Expression of heat shock proteins (HSPs), such as HSP60 and 90 found here, is up-regulated in cells undergoing stress, such as inflammation, and thereby playing a vital role in their survival [68–70]. Previous studies have shown that self-HSP-peptides expressed by stressed host cells are recognized by $\gamma\delta$ T-cells. It has been proposed that these cells represent a scavenger system for cells suffering from inflammation [71–73]. Asthma is a chronic inflammatory disease and an increase of $\gamma\delta$ T-cells in the bronchoalveolar lavage and peripheral blood of asthmatics has been observed [74–78]. A role for these cells and HSPs in asthma has therefore been suggested previously. Additionally, HSP90 is part of the intracellular glucocorticoid receptor complex [79], which can down-regulate the production of cytokines that mediate the inflammatory response in the airways during asthma [80].

Proteasomes are protein complexes in eukaryotic cells that direct the degradation of ubiquitin-tagged proteins. Proteolysis of ubiquitinated proteins is a feature of many cellular processes and these proteins are also involved in cellular events that do not require proteolysis [81]. A role for proteasomes in asthma was demonstrated previously as inhibition of proteasome activity attenuated DNFB-induced cellular influx and ear swelling in mice [82], and prevented chemotactic activity of allergen-stimulated human airway mucosa [83] and human ASM hyperplasia [84] *in vitro*.

4.5 Various functions

Annexin VI belongs to a family of proteins which bind both calcium and phospholipids, and form voltage-dependent calcium channels within planar lipid bilayers [85, 86]. Although its physiological role remains largely unknown, it seems to be involved in a 'switching' mechanism that regulates secretion in various cells [87]. Secretion of inflammatory mediators by mast cells is an important event leading to the clinical characteristics of asthma in the model employed here [21, 22]. Using a subtraction library, up-regulation of annexin VI mRNA expression in antigenically stimulated mast cells has been demonstrated previously [88].

Protein kinase C inhibitor protein-1 is a member of the 14-3-3 protein family, key regulators in various signaling pathways involving serine/threonine phosphorylation [89]. Although also expressed in the lungs [90] its physiological role or link with asthma remains unknown.

T-DSP11 is a member of the dual specificity protein phosphatase family that are able to dephosphorylate both threonine and tyrosine residues. These enzymes can regulate the activity of mitogen- and stress-activated protein kinases, which are activated during inflammation such as asthma and promote various activities such as cell proliferation, differentiation and survival and cytokine synthesis [91, 92].

4.6 Unknown function

The 2610034B18Rik protein was derived from a cDNA library constructed from mouse embryo [93]. Functional motifs or domains of this protein have not been identified nor is any physiological function known.

4.7 Biological relevance

Changes in protein levels do not directly imply altered protein translation, but may also occur because of post-translational modification, extended half-life *in vivo*, or altered turnover and translocation to or from a 'compartment' that is less available for protein extraction during sample preparation. Any role for the proteins found in this study with respect to the pathophysiology of asthma remains speculative until validation has been performed by bioassays. However, apart from a causal role, these proteins may prove useful as surrogate markers of the disease state.

4.8 Significance

For more than a quarter of a century, 2-DE has remained unsurpassed in its ability to resolve complex protein mixtures. Like both cDNA and protein biochips, 2-DE gels are capable of parallel analysis of large numbers of independent variables. Unfortunately, the number of experimental replicates employed to reach conclusions with any of the above techniques is not always as high as one would wish in order to obtain the highest levels of statistical confidence. This is due to a combination of the inherent cost measured in both time and financial expenditure, and the difficulty in obtaining numerous high quality results. In this study, 23 proteins behaved significantly ($p < 0.05$) differently between the asthma mouse model and controls among 2115 protein spots followed in 24 different 2-DE gels. In other words, amongst any 20 such observations, one apparently significant observation can be expected to occur by chance alone. Thus, the question arises as to whether or not at this level of significance, one should not expect 106 occurrences (1/20) from within 2115 to behave differently by chance alone. Whenever a large number of variables is being examined, the number of replicates needs to be increased accordingly to afford better statistical confidence so as to avoid this dilemma. In so doing, the number of observations about which meaningful conclusions can be reached also increases. In the current experimental set-up, only 830 of the 2115 spots included in our reference dataset were seen often enough (6 or more times), *i.e.* at the limit of a possible statistically valid conclusion. Thus, one must conclude further experimental validation is required. However, the fact that most of the up- or down-regulated proteins identified (18/20) could be linked to known asthma symptomatology would suggest that the phenomena being reported should be given credence – the likelihood of such occurring by chance alone being infinitesimally small.

5 Conclusion

In conclusion, we have shown that comparison of protein maps of lung tissue derived from control mice and mice with DNFB-induced asthma revealed changes in protein expression levels correlated with asthma. In more recent studies on the same experimental samples, up to 8000 different proteins have been resolved using higher resolution zoom gels. Thus, the experimental procedures employed here are likely to be capable of resolving still more asthma-related proteins. Noticeably, no immunologically-relevant proteins were observed among the differentially expressed proteins reported here. Elsewhere, differences in cytokine levels and presence of inflammatory

cells for example in bronchoalveolar lavage fluids have been reported [1, 22, 30, 94, 95]. These and other cytokines and chemokines known to be significantly changed during asthma have been reviewed and summarized recently [96]. These immuno-regulators are very potent biomolecules but do not necessarily occur at high abundance *in vivo*. Nonetheless, differentially expressed proteins recorded here were able to be linked to stress and inflammation, lung detoxification, plasma exudation or tissue remodeling. Altered expression of these proteins is probably caused by immunological agents provoked by the hypersensitivity response to the DNFB challenge. Yet, these same proteins are not likely to be involved in the immunological mechanisms leading to asthma. However, they may prove invaluable markers for monitoring disease state and/or disease progression during alternate therapies. An increase in the number of protein spots resolved and the number of experimental replicates employed should overcome these shortcomings, namely detection of immuno-regulators and molecules linked to the pathophysiology of asthma. In addition, as the proteome is a highly dynamic entity varying temporarily and with respect to cell and tissue type, future analyses should also address the time course post-exposure to the allergenic hapten DNFB in, for example, lung lavage and lymph nodes.

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