

# Comparative Proteomics on Resting and Activated Human Basophilic Leukemic KU812 upon Stimulation with PMA plus A23187

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## ABSTRACT

Basophil activation has been implicated in allergy reaction and immune modulation. Signaling transduction, mediator release and morphology change of basophils have been largely deciphered. This study examined the feasibility of using proteomic strategy to investigate the whole cell proteomic changes in KU812 basophils following activation with phorbol myristate acetate (PMA) plus ionophore (A23187). The two-dimensional electrophoresis (2D) coupled with matrix-assisted laser desorption/ionization-quadrupole/time-of-flight (MALDI-Q-TOF) was selected for studying comparative proteome differences; and cytokine mRNA expressions were monitored. The differentially displayed proteins were further validated by 1D- and 2D-Western blotting for protein expressions. IL-4, IL-5, and IL-13 mRNAs in KU812 were significantly induced; a phenomenon similar to the allergy reaction. The protein expressions of heterogeneous nuclear ribonucleoprotein K (HNRPK) and  $\alpha$ -enolase (ENO1) were shown a good correlation according to the results from the 2D, and 2D-Western blots. Since the majority of the identified proteins were associated with immunological, inflammatory disorders and tumor genesis, this study in defining the proteomic changes in basophilic cells that occur in response to treatment with PMA plus A23187 can serve as biomarkers for characterization of *in vitro* immuno-stimulation.

Key words: basophil, proteomics, immuno-stimulants, Th2 cytokine

## INTRODUCTION

Due to growing prevalence of allergic diseases worldwide, the exact molecular mechanisms of allergy reaction for prevention and/or treatment of allergy diseases are becoming increasingly important. Basophils and mast cells are major effector cells in allergic progression through their highly expressed Fc $\epsilon$ RI on the cell surface<sup>(1)</sup>. These events trigger both preformed and newly synthetic chemical mediator secretions. Among them, IL-4, IL-5 and IL-13 are major cytokines for IgE production, facilitating Th2 differentiation and allergic responses<sup>(2)</sup>. Basophils could rapidly secrete large

amount of IL-4 and IL-13, and constitutively express CD40L and CCR3 on the surface. Basophils have been detected in particular organs affected by allergic reactions, such as bronchial and airway biopsies from asthma patients, nasal lavage fluids from allergic rhinitis patients, as well as skin biopsies from atopic and contact dermatitis. Therefore, human basophils play a crucial role as a high priority source of IL-4, IL-13, histamine, and leukotriene-C<sub>4</sub> (LTC<sub>4</sub>) that polarize the early type 2 immune response leading to allergy and asthma<sup>(3-6)</sup>.

With the advent of proteomics and allergen sequence, biomarker study of immunological molecular mechanisms enables more rapid advances in elucidating allergic inflammatory pathways through proteomic methodologies coupled with bioinformatic techniques. Proteomics

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characterizes cellular proteins, its abundance, modification state, protein complexes and interactions. The global changes in cellular protein expression can be visualized by two-dimensional gel electrophoresis (2D) and identified by mass spectrometry analysis.

Since IL-4 and IL-13 are central in allergic inflammation and share several biological properties, it is crucial to elucidate how human basophils synthesize and release such cytokines upon stimulation. The phorbol 12-myristate 13-acetate (PMA) induction of human basophils and KU812 cells brings about IL-13, not IL-4 secretion<sup>(4,7)</sup>. The ionomycin only initiates IL-4 not IL-13 production in KU812 cells, but it induces both IL-4 and IL-13 secretions in human basophils<sup>(4,8)</sup>. Regarding the release of histamine and LTC<sub>4</sub>, PMA results in histamine slowly released at the maximum level within 1 hr, but no LTC<sub>4</sub> production<sup>(9)</sup>. The calcium ionophore, A23187, induces LTC<sub>4</sub> generation, an event which precedes histamine release<sup>(10)</sup>. Recently, combinational use of PMA and A23187/ionomycin for human immune cells such as mast cell, basophil, T cell, B cell and neutrophil seems to be a representative immuno-stimulant in allergic molecular mechanism, immune cell differentiation, signaling pathway, transcriptional factor activation, and protective lead compound screening<sup>(11-13)</sup>.

The 2D couples with tandem mass spectrometry and validation assays were utilized to evaluate changes in comparative proteomes of human basophilic cells following treatment with PMA and A23187. The combination of PMA and A23187 induced IL-4, IL-5 and IL-13 expressions in human basophilic KU812 cells. The significantly regulated proteins are further classified based on metabolism, macromolecule binding, catalytic activity, association with inflammatory immunological diseases, tumorigenesis, and cell movement. Meanwhile, the selected regulatory proteins as HNRPK and ENO1 were verified by 1D- and 2D-Western blotting. This study is the first of its kind to combine the use of immuno-stimulating treatment with proteomic platform and bioactivity validation assays to elucidate KU812 basophil activation induced by PMA plus A23187.

## MATERIALS AND METHODS

### I. Cell Culture

Human basophilic leukemia KU812 cells were seeded at an initial concentration of  $5 \times 10^5$  cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum and maintained at 37°C in an incubator with controlled humidified atmosphere containing 5% CO<sub>2</sub>.

### II. RT-PCR Analysis of Cytokine mRNA Expressions

The KU812 cells ( $5 \times 10^5$  cells/mL) were treated by PMA (10 ng/mL) plus A23187 (1  $\mu$ mol/L) or an

equivalent amount of diluted dimethyl sulfoxide (DMSO) and incubated for 3 hours. After washing, total RNA was extracted, mixed with the reverse transcriptase (RT) mixture according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and incubated at 42°C for 50 minutes, followed by 95°C for 5 minutes. After each RT, PCR amplification was performed with the following primers. IL-4-sense, 5' ATG-GGT-CTCAC-TCC-CAA-CTG-CT 3'; antisense, 5' GTT-TTC-CAA-CGT-ACT-CTG-GTT-GGC 3'. IL-5-sense, 5' GCT-TCT-GCA- TTT-GAG-TTT-GCT-AGC-T 3'; antisense, 5' TGG-CCG-TCA-ATG-TAT-TTC-TTT-ATT-AAG 3'. IL-6-sense, 5' ATG-AAC-TCC-TTC-TCC-ACA-AGC-GC 3'; antisense, 5' GAA-GAG- CCC-TCA-GGC-TGG-ACT-G 3'. IL-8-sense, 5' ATG-ACT-TCC-AAG-CTG-GCC-GTG-GCT 3'; antisense, 5' TCT-CAG-CCC-TCT-TCA-AAA-ACT-TCT-C 3'. IL-13-sense, 5' CCA-CGG- TCA-TTG-CTC-TCA-CTT-GCC 3'; antisense, 5' CCT-TGT-GCG-GGC-AGA-ATC-CGC-TCA 3'. IL-1 $\beta$ -sense, 5' ATG-GCA-GAA-GTA-CCT-AAG-CTC-GC 3'; antisense, 5' ACA-CAA- ATT-GCA-TGG-TGA-AGT-CAG-TT 3'. IFN- $\gamma$ -sense, 5' TGT-TAC-TGC-CAG-GAC-CCA- TAT-GTA-AAA 3'; antisense, 5' CAT-CAC-TTG-GAT-GAG-TTC-ATG-TAT-TGC 3'.  $\beta$ -Actin-specific primers were as follows: sense, 5' GTG-GGG-CGC-CCC-AGG-CAC-CA 3'; antisense, 5' GTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC 3'. The PCR process was performed with a Thermo Px2Thermal Cycler (San Jose, CA, USA). The products were electrophoresed in 2% agarose gel, stained with ethidium bromide, photographed, and quantitated by computer program.

### III. Two Dimensional (2D) Electrophoresis

KU812 cells were resuspended in lysis buffer at 350  $\mu$ L, as described previously<sup>(14)</sup>. The protein solutions (150  $\mu$ g protein) were applied onto IPG strips (18 cm, pH 3-10NL)(GE Healthcare Life Sciences, Pittsburgh, PA, USA). After rehydration, isoelectric focusing (IEF) was conducted at 8000 V for 66000 Vh. The equilibrated strips were then transferred onto 12.5% homogenous polyacrylamide slab gel (18  $\times$  18 cm), and separated proteins were then visualized with SYPRO<sup>®</sup> Ruby dye (Bio-Rad, Hercules, CA, USA). The 2D gel images were scanned using a Typhoon 9200 laser scanner (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and exported to the image analysis software program using PDQuest software package version 7.1.1 (Bio-Rad, Hercules, CA, USA).

### IV. In-gel Digestion

The in-gel digestion procedure was processed according to our previous study<sup>(14)</sup>. Briefly, the gel pieces were washed and rehydrated with trypsin (Promega, Madison, WI, USA) at 37°C for at least 16h. The extracted peptides were finally eluted with 5  $\mu$ L of 75%

acetonitrile/0.1% formic acid, and directly spotted on the sample plate of a MALDI-Q-TOF mass spectrometry.

#### V. MALDI-TOF and MALDI-Q-TOF Mass Spectrometry.

The trypsinized samples were premixed in a ratio of 1:1 (v/v) with the matrix solution (5 mg/mL  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% v/v trifluoroacetic acid and 2% w/v ammonium citrate) and spotted onto the 96-well MALDI sample stage. The samples were analyzed by the Q-TOF Ultima<sup>TM</sup> MALDI instrument (MALDI<sup>TM</sup>; Micromass, Manchester, UK), as described previously<sup>(9)</sup>. The MASCOT search engine (<http://www.matrixscience.com>) was used for MS/MS ions search using a MALDI-Q-TOF-MS hybrid quadrupole/orthogonal acceleration TOF spectrometer (MALDI<sup>TM</sup>, Micromass, Manchester, UK). The product ion spectra generated by Q-TOF MS/MS were searched against NCBI<sup>nr</sup> and Swiss-PROT databases for exact matches using the Mascot search program.

#### VI. 1D- and 2D-Western Blotting Verification of HNRPK and ENO1 Protein Level Expressions

After 1D and 2D procedures, proteins were transferred onto PVDF membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Primary antibodies including HNRPK and ENO1 were purchased from Abcam (Abcam, Cambridge, UK). After incubation with secondary antibodies, the immunoblots were visualized with the ECL detection kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA) then exposed to X-ray film, or Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

#### VII. Statistical Analysis

Analysis of proteins regulation changed upon PMA plus A23187 treatment was done on three independent experiments. Each set of 2D gels was compared separately by an image comparison software. Different proteins that were found in all sets of experiment were selected for identification.

#### VIII. Bioinformatics Tools for Protein Searches

The protein search programs used the following sequential order of databases: NCBI (<http://www.ncbi.nlm.nih.gov>), Swiss-Prot/TrEMBL (<http://www.expasy.ch/sprot>) and Proteome (<http://www.proteom.com/databases/HumanPD/reports>). In addition, the functional protein association networks or protein interactions were searched by STRING database (<http://string.embl.de/>). The combining pathway databases of BioCarta (<http://www.biocarta.com>), KEGG (<http://www.genome.ad.jp/kegg/pathways.html>) and the PubMed literature (<http://www.ncbi.nlm.nih.gov/PubMed>) were also used to search

the correlated regulatory pathways of cellular response in KU812 and other immune cells. The two bioinformatic tools including our previous work as Bulk Gene Search System for Java (BGSSJ)<sup>(15)</sup>. The Ingenuity Pathway Analysis (IPA) was applied in thoroughly compiling protein functional classification and interaction networks.

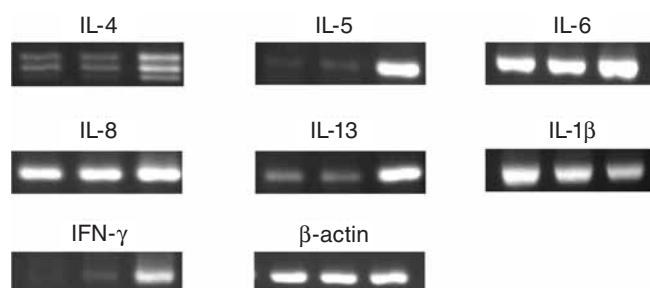
## RESULTS

#### I. Cytokine Expressions of KU812 after PMA plus A23187 Treatment

We treated human basophil cells KU812 with PMA (10 ng/mL) and A23187 (1  $\mu$ mol/L). The PMA structure was analogous to diacylglycerol that activates protein kinase C. The A23187 was a calcimycin that greatly increases the ability of  $\text{Ca}^{2+}$  crossing biological membranes. In RT-PCR, mRNA expressions of the cytokine genes were normalized to each sample by using  $\beta$ -actin gene as internal controls. The up-regulation of IL-4, IL-5, IL-6, IL-8, IL-13, and IFN- $\gamma$  (no IL-1 $\beta$ ), was detected after PMA plus A23187 stimulation at 3 hours. The typical Th2 cytokines consisting of IL-4, IL-5 and IL-13 were apparently enhanced more than 2 fold changes (Figure 1). The IL-5 elevated to the highest level at 5 fold changes among all test cytokines. The combination treatment with PMA and A23187 stimulated KU812 cells elevation of Th2 type cytokine mRNA expression levels.

#### II. Expression Changes of 17 Identified Proteins in KU812 after PMA plus A23187 Treatment

The time-course experiments were conducted at least three times and showed good reproducibility among different cell batches and 2D gels (Supplementary Figure 1). The 2D patterns revealed that the largest number of significantly-regulated proteins occurred at 6h treatments using the PDQuest analysis. We identified



**Figure 1.** RT-PCR patterns for cytokines of human basophil cell line KU812. Blank (left): no treatment; Control (middle): treated only with an equivalent amount of diluted DMSO; Treated (right): stimulated with PMA plus A23187 for 3h.

**Table 1.** Summary of significantly regulated proteins in total cellular lysates of KU812 cells in a time course treatment study with PMA plus A23187 using the MALDI-Q-TOF analysis

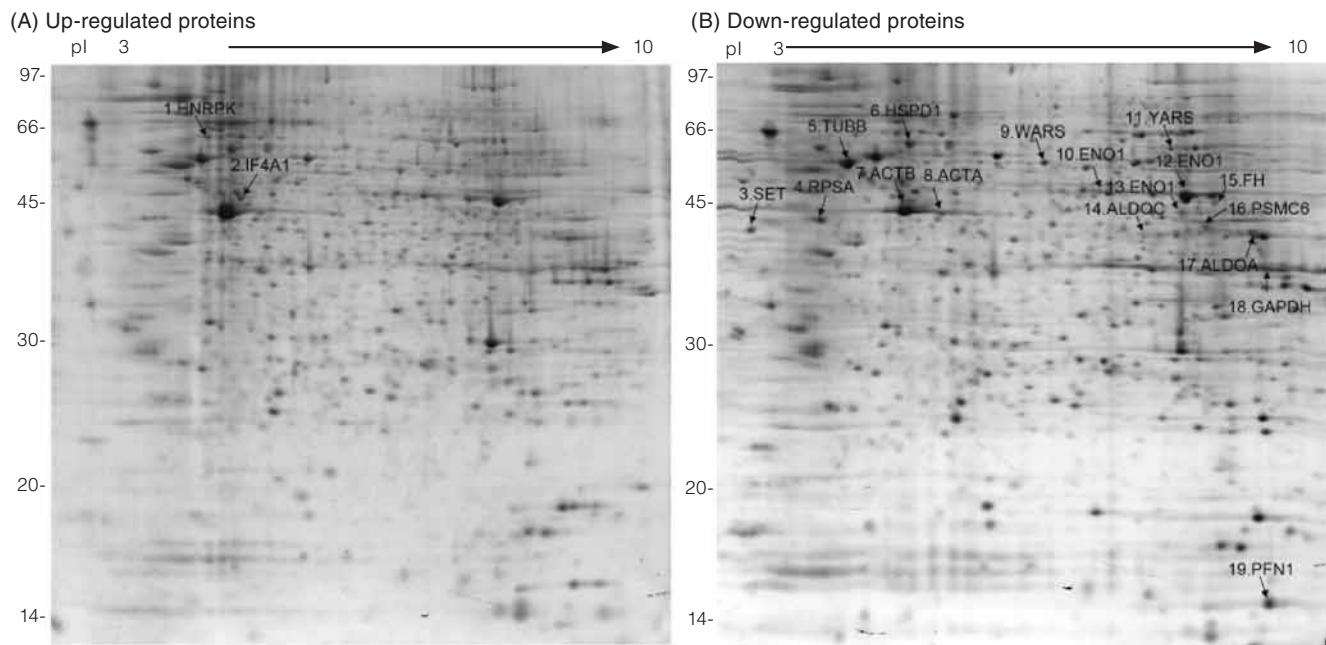
| Spot                  | Change<br>Con 3h 6h 12h | Accession<br>Sprot <sup>a</sup> | Protein name                                      | Score | Theoretic |      | SC <sup>b</sup> | Matched sequences   |
|-----------------------|-------------------------|---------------------------------|---|-------|-----------|------|-----------------|---|
|                       |                         |                                 |   |       | Mr (kD)   | pI   |                 |   |
| <b>Up-regulated</b>   |                         |                                 |   |       |           |      |                 |   |
| 1                     |                         | P61978                          | Heterogeneous nuclear ribonucleoprotein K (HNRPK) | 37    | 50.9      | 5.39 | 2               | NLPLPPPPPR  |
| 2                     |                         | P60842                          | Eukaryotic initiation factor 4A-1 (EIF4A1)        | 126   | 46.1      | 5.32 | 8               | GIYAYGFEKPSAIQQR<br>AEVQKLQMEAPHIIVGTPGR  |
| <b>Down-regulated</b> |                         |                                 |   |       |           |      |                 |   |
| 3                     |                         | Q01105                          | Protein SET (SET)                                 | 55    | 33.5      | 4.23 | 8               | LRQPFFQK<br>SGYRIDFYFDENPYFENK  |
| 4                     |                         | P46783                          | 40S ribosomal protein SA (RPSA)                   | 85    | 32.8      | 4.79 | 11              | AIVAIENPADVSVISSL<br>FTP GTFTNQIAAFREPR   |
| 5                     |                         | P07437                          | Tubulin $\beta$ -5 chain (TUBB)                   | 211   | 49.6      | 4.78 | 10              | ISVYYNEATGGKYVPR<br>LHFFMPGFAPLTSR<br>MAVTFIGNSTAIQELFKR  |
| 6                     |                         | P10809                          | Heat shock protein 60 (HSPD1)                     | 214   | 61.0      | 5.70 | 6               | TLNDELEHEGMKFDR<br>KISSIQSIVPALEIANAHR<br>ISSIQSIVPALEIANAHR  |
| 7                     |                         | P60709                          | Actin $\beta$ -chain (ACTB)                       | 275   | 41.7      | 5.29 | 23              | AVFPSIVGRPR<br>VAPEEHPVLLTEAPLNPK<br>LCYVALDFEQEMATAASSSSLEK<br>SYELPDGQVITIGNER<br>DLYANTVLSGGTTMYPGIADR |
| 8                     |                         | P62736                          | Actin $\alpha$ -chain (ACTA)                      | 57    | 42.0      | 5.23 | 4               | SYELPDGQVITIGNER  |

**Table 1.** Continued.

| Spot | Change | Accession | Protein name                                     | Score | Theoretic |      | SC <sup>b</sup> | Matched sequences |  |
|------|--------|-----------|--|-------|-----------|------|-----------------|-------------------|--|
|      |        |           |  |       | Con       | 3h   | 6h              | 12h               | Sprot <sup>a</sup>   |
| 9    |        | P23381    | Tryptophanyl-tRNA synthetase, cytoplasmic (WARS) | 126   |           | 53.1 | 5.83            | 7                 | ISFPAIQAAAPSFSNSFPQIFR<br>ALIEVLQPLIAEHQAR                       |
| 10   |        | P06733    | $\alpha$ -enolase (ENO1)                         | 70    |           | 47.1 | 7.01            | 3                 | LAMQEFMILPVGAANFR  |
| 11   |        | P54577    | Tyrosyl-tRNA synthetase, cytoplasmic (YARS)      | 51    |           | 59.1 | 6.61            | 3                 | TVVSGLVQFVPKEELQDR   |
| 12   |        | P06733    | $\alpha$ -enolase (ENO1)                         | 258   |           | 47.1 | 7.01            | 12                | EIFDSRGNPTVEVDLFTSK<br>LAMQEFMILPVGAANFR<br>AGYTDKVVIGMDVAASEFFR |
| 13   |        | P06733    | $\alpha$ -enolase (ENO1)                         | 67    |           | 47.1 | 7.01            | 8                 | LAMQEFMILPVGAANFR<br>AGYTDKVVIGMDVAASEFFR                        |
| 14   |        | Q6FH94    | Fructose-bisphosphate aldolase C (ALDOC)         | 75    |           | 39.4 | 6.41            | 4                 | TPSALAILENANVLAR   |
| 15   |        | P07954    | Furmarate hydratase (FH)                         | 33    |           | 54.6 | 8.85            | 4                 | THTQDAVPLTLGQEFSGYVQQVK  |
| 16   |        | P62333    | 26S protease regulatory subunit (PSMC6)          | 63    |           | 44.1 | 7.10            | 3                 | EVIELPLTNPELFQR  |
| 17   |        | P04075    | Fructose-bisphosphate aldolase A (ALDOA)         | 51    |           | 39.4 | 8.30            | 5                 | IGEHTPSALAIMENANVLAR   |

**Table 1.** Continued.

| Spot | Change<br>Con 3h 6h 12h | Accession<br>Sprot <sup>a</sup> | Protein name                                     | Score | Theoretic |      | SC <sup>b</sup> | Matched sequences                      |
|------|-------------------------|---------------------------------|--|-------|-----------|------|-----------------|--|
|      |                         |                                 |  |       | Mr (kD)   | pI   |                 |  |
| 18   |                         | P04406                          | Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | 128   | 36.0      | 8.57 | 11              | VIHDNFGIVEGLMTTVHAITATQKLISWYDNEFGYSNR |
| 19   |                         | P07737                          | Profilin-1 (PFN1)                                | 126   | 15.0      | 8.44 | 12              | TFVNITPAEVGVLVGKTFVNITPAEVGVLVGKDR     |

<sup>a</sup>Accession number from UniProtKB/Swiss-Prot.<sup>b</sup>SC stands for Sequence Coverage from Mascot search result.**Figure 2.** The representative 2DE gel for 6 hr PMA plus A23187 treatment.

Protein spots present in the pI range of 3-10 and molecular weight range of 14-97 kD. (A) The up-regulated proteins are shown in the gel of 6h treatment group. (B) The down-regulated proteins are shown in the gel of control group. The 2D experiments for each group were conducted at least three times with good reproducibility among different cell batches and 2D gels (Supplementary Figure 1). Statistically significant variations were defined as a minimum 2 fold changes in expression level and  $P<0.05$  at 6 h compared to protein expression level in control group.

2 up-regulated proteins (heterogenous nuclear ribonucleoprotein K (HNRPK) and eukaryotic initiation factor 4A-1 (EIF4A1), and 15 down-regulated protein expressions including protein set (SET), 40S ribosomal protein SA (RPSA), tubulin  $\beta$ -5 chain (TUBB), heat shock protein 60 (HSPD1), actin  $\beta$ -chain (ACTB), actin  $\alpha$ -chain

(ACTA), tryptophanyl-tRNA synthetase, cytoplasmic (WARS),  $\alpha$ -enolase (ENO1), tyrosyl-tRNA synthetase, cytoplasmic (YARS), fructose-bisphosphate aldolase C (ALDOC), furmarate hydratase (FH), 26S protease regulatory subunit (PSMC6), fructose-bisphosphate aldolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase

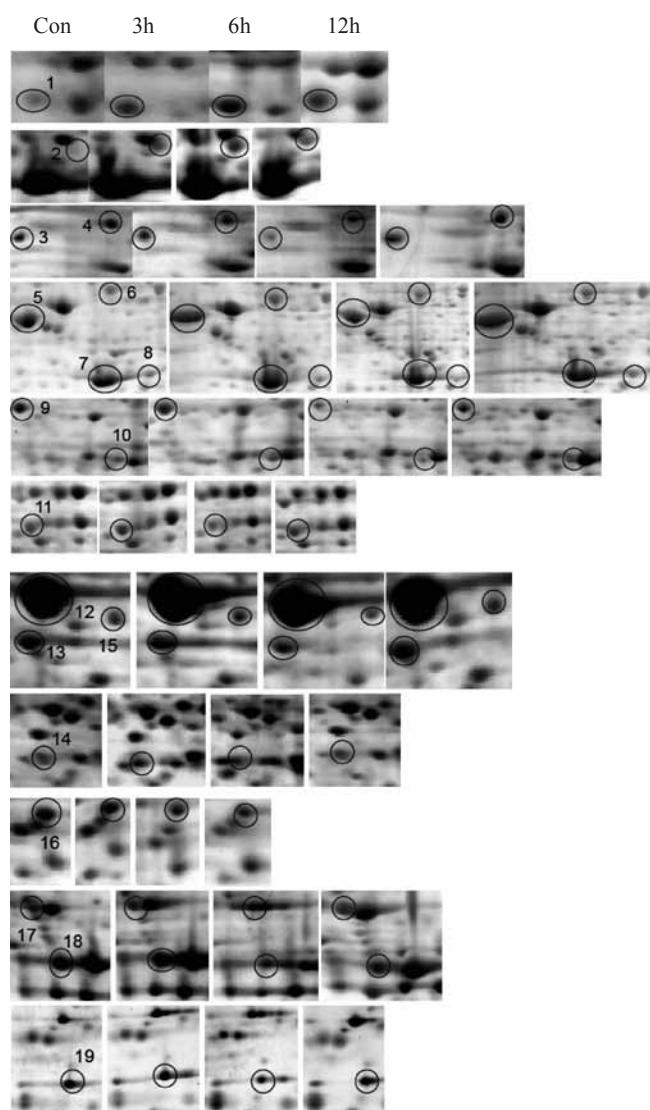
(GAPDH) and profilin-1 (PFN1) at 6h treatment (Table 1). Statistically significant variations were defined as a minimum 2-fold changes and  $p < 0.05$  when comparing to protein expression levels among control and treatment groups.

The 2 up-regulated protein spots were shown in a representative 6h-treatment gel, and 15 down-regulated protein spots were revealed in control group (Figure 2). The computerized close-up vision for each spot was collected (Figure 3), and optical density of identified spots from three independent experiments were analyzed and piled up into histograms (Table 1). To identify protein spot individually, the 3D diagram utility provided by PDQuest was subjected to careful examination of each spot. The HNRPK and EIF4A1 levels had 2-fold elevation with time increases at 6h. In contrast, the SET, RPSA, ALDOA levels were markedly reduced by 4-fold

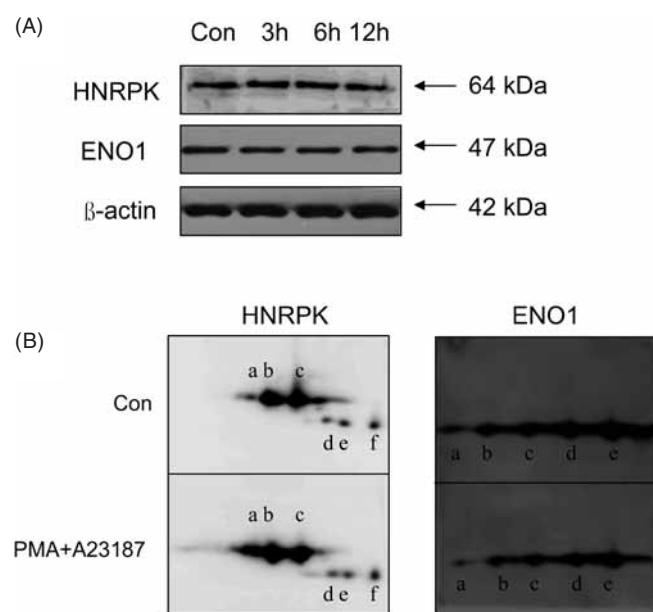
at 6h time point but gradually increased at 12h. The remaining down-regulated proteins reached a peak at 3h and declined at 6h down to 30-50% levels comparable to untreated cells at 3h. Furthermore, we identified specific isoforms and several spots for one protein identity for HNRPK (up-regulated) and ENO1 (down-regulated). The five protein spots of HNRPK revealed 3 up-regulated and 2 down-regulated isoforms (data not shown); however, only one up-regulated spot was shown due to statistical significance. On the other hand, the three ENO1 spots showed significant reductions at 6h compared to control group. These spots with varied mobility indicated possible alternative splicing, post-transcriptional or post-translational modifications for ENO1 and HNRPK.

### III. Verification of Significantly-Regulated Proteins by 1D- & 2D-Western Blotting

From the time-course 1D-Western blotting analyses (Figure 4A), the 6h treated cells seemed to have a slightly higher HNRPK expression than the untreated KU812 cells. In contrast, ENO1 exhibited a slightly elevated level in the untreated KU812 cells. We further conducted 2D-Western blotting analysis to better understand the expressions of isoforms for HNRPK and ENO1 (Figure 4B). The acidic isoform (indicated by a) of HNRPK in 6h PMA plus A23187-stimulated KU812 cells was significantly higher than that in the untreated KU812 cells. This spot might result from inducible phosphorylation of HNRPK by PMA plus A23187 treatment. Other HNRPK isoforms did not show significant variances. On



**Figure 3.** Close-up view of PMA plus A23187-dependent expression of 17 identified proteins. Gel sections (1-19) represent varied expression of the indicated protein spots at different time periods.



**Figure 4.** HNRPK and ENO1 validations were achieved by 1D- and 2D-Western blotting. (A) 1D-Western blotting of KU812 cells at differential time points. (B) 2D-Western blotting of KU812 cells at 6h PMA plus A23187 treatment.

the contrary, almost all ENO1 isoforms (indicated by a to e) exhibited down-regulation after 6h PMA plus A23187 treatment. These 2D and 2D-Western blotting results showed a good correlation.

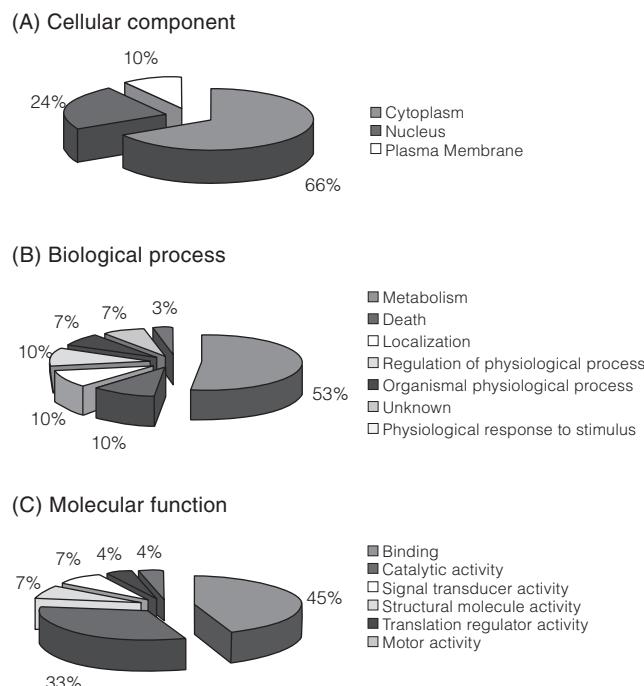
#### IV. Subcellular and Functional Classification

We used 2 bioinformatic tools including our previous work as Bulk Gene Search System for Java (BGSSJ) shown in Figure 6, and Ingenuity Pathway Analysis (IPA) shown in Table 2 to thoroughly compile protein functional classification and interrelationships. The PMA plus A23187-stimulated proteins correlate primarily to immunological disorder (ACTA, ACTB, ALDOA, ENO1, GAPDH, RPSA, TUBB), and tumorigenesis (ACTB, ALDOA, ALDOC, ENO1, FH, PFN1, TUBB) (Table 2). From subcellular localization analysis, the identified proteins were mainly distributed in the cytoplasm, followed by nucleus and plasma membrane (Figure 5A). The proteins in the nucleus consist of HNRPK, SET, RPSA, PSMC6, and PFN1 according to BGSSJ and IPA databanks. Primary metabolism on carbohydrate, protein, and nucleic acid plays an important role in the physiological process (Figure 5B). From molecular function classification, the identified proteins abundantly bind to protein, nucleotides, nucleic acid, and metal ion (Figure 6C).

## DISCUSSION

Basophils comprise less than 1% of peripheral blood leukocytes and serve as primary Th2 type effector cells during allergic inflammation and parasite infection<sup>(5,16)</sup>. Activated basophils produce rapid cytokine release due to sufficiently preformed IL-4 and IL-13 mRNA transcripts in basophil ontology<sup>(17)</sup>. In addition to IL-4 and IL-13, IL-5 and IFN- $\gamma$  were greatly elevated in PMA plus A23187-stimulated KU812 cells within 3h in this study (Figure 1). The phenomenon is similar in chronic urticaria patients that PMA plus ionomycin heavily enhanced both IL-4 and IFN- $\gamma$  productions in CD4+ lymphocyte<sup>(18)</sup>. Human basophils are a more effective source of IL-4 than mast cells or eosinophils. Human basophils release equivalent IL-4 amount as Fc $\epsilon$ RI-activated Th2 cells. The activated basophils provided early IL-4 source which might drive naïve CD4 T cells to IL-4-producing Th2 phenotypes *in vitro* and *in vivo*<sup>(19,20)</sup>. Even IL-4 deficient basophils still could inhibit naïve CD4 T cells differentiate into Th1 cells, although they did not help in Th2 differentiation<sup>(21)</sup>. Basophils might serve as a potential connection between innate and adaptive immunity. Basophils serve as an initiator and a good therapeutic target in IgE-mediated chronic allergic inflammation from basophil-depleting mAb studies<sup>(22)</sup>.

PMA plus A23187 induces histamine<sup>(12)</sup>, IL-4, IL-5, IL-6, IL-8 and IL-13 protein secretions in activated



**Figure 5.** Functional classification assay conducted by the Bulk Gene Search System for Java (BGSSJ). (A) Localization, (B) Biological process, and (C) Molecular function.

basophil cells<sup>(11)</sup>. They also enhance CD40 ligand<sup>(13)</sup>, and phosphorylation of cytoplasmic p38 MAPK, p44/42 MAPK, p54/46 SAPK/JNK, as well as nuclear c-Jun phosphorylation both on ser63 and ser73<sup>(23)</sup>. PMA and A23187 increased DNA-binding activity of phosphorylated c-Jun, JunB, JunD, c-Fos, and FosB nuclear extracts of KU812 cells<sup>(23)</sup>.

Heterogeneous nuclear ribonucleoproteins (hnRNP) are predominantly nuclear RNA-binding proteins that involve in a range of RNA life including splicing, processing, export of the mature RNAs and translation, and signal transduction that influence gene expression<sup>(24)</sup>. The hnRNP family was recently regarded as an important target of the autoimmune response in rheumatic diseases. The hnRNP-K (HNRPK) was associated to rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and mixed connective tissue disease<sup>(25)</sup>. The HNRPK was significantly up-regulated by 6h PMA plus A23187 stimulated KU812 cells. From 2D-western blot of HNRPK, a spot (indicated by a) appeared at the more acidic side. HNRPK were phosphorylated at multiple sites, especially on tyrosine residues, in response to various signals. HNRPK was assumed to be a target of SH2-containing protein tyrosine kinases such as Src, Lck, Lyn and Fyn<sup>(24)</sup>. B cell receptor (BCR) activation did not change HNRPK concentration in 1D immunoprecipitated protein, but greatly enhanced 1D and 2D immunoblotting with anti-phosphotyrosine antibodies, which cause inducible phosphorylation of HNRPK<sup>(26)</sup>.

**Table 2.** Functional classification of significantly regulated proteins by Ingenuity Pathway Analysis (IPA) Software

| Function  | Protein symbol                                    |
|---|---|
| Immunological Disease   |   |
| Acute allergic pulmonary eosinophilia   | ACTB <sup>a</sup> , ALDOA, ENO1, GAPDH, TUBB      |
| Immunological disorder  | ACTA, ACTB, ALDOA, ENO1, GAPDH, RPSA, TUBB        |
| Rheumatoid arthritis and autoimmune disease   | ACTA, ALDOA, ENO1, RPSA                           |
| Cancer  |   |
| Tumorigenesis and neoplasia   | ACTB, ALDOA, ALDOC, ENO1, FH, PFN1, TUBB          |
| Carcinoma, primary and malignant tumor  | ALDOA, ALDOC, ENO1, PFN1, TUBB                    |
| Lung cancer, lung tumor, lung adenocarcinoma, lung carcinoma                            | ALDOA, ALDOC, ENO1                                |
| Growth and development of breast cancer cell lines                                      | HNRPK, PFN1                                       |
| Attachment and invasion of melanoma cell lines  | RPSA  |
| Migration of fibrosarcoma cell lines  | HNRPK   |
| A apoptosis of tumor cells  | GAPDH, ENO1                                       |
| Cellular assembly and organization  |   |
| Formation of filaments  | ACTA, PFN1, TUBB                                  |
| Formation of microspikes and actin  | PFN1  |
| Formation of mitotic spindle and spindle fibers   | TUBB  |
| Formation of myofilaments   | ACTA  |
| Formation of nucleosomes  | SET   |
| Binding of microsomal membrane  | GAPDH   |
| Binding of ribosome   | EIF4A1  |
| Inflammatory disease  |   |
| Inflammatory disorder   | ACTA, ALDOA, ENO1, GAPDH, HSPD1, RPSA             |
| Rheumatic arthritis disease   | ACTA, ALDOA, ENO1, RPSA                           |
| Iridocyclitis, and uveitis  | HSPD1   |
| Acute respiratory distress syndrome   | GAPDH   |
| Cellular growth and proliferation   |   |
| Growth of cells   | ACTB, EIF4A1, ENO1, HNRPK, HSPD1, PFN1, RPSA, SET |
| Growth of breast cancer cell lines  | HNRPK, PFN1                                       |
| Cellular movement   |   |
| Cell movement   | ACTB, HNRPK, HSPD1, TUBB, WARS, YARS              |
| Cell movement of fibrosarcoma cell lines  | HNRPK   |
| Chemotaxis and homing of mononuclear leukocytes, blood cells and lymphatic system cells | HSPD1, YARS                                       |
| Migration of eukaryotic cells   | HNRPK, HSPD1, WARS, YARS                          |
| Invasion of melanoma cell lines   | RPSA  |

**Table 2.** Continued.

| Function                                | Protein symbol          |
|---|-------------------------|
| Carbohydrate metabolism                 |                         |
| Glycolysis                              | ALDOA, ENO1             |
| Metabolism of fumaric acid              | FH                      |
| Quantity of UDP-N-acetylglucosamine     | GAPDH                   |
| Binding of lipopolysaccharide           | RPSA                    |
| Small molecular biochemistry            |                         |
| Aminoacylation                          | WARS, YARS              |
| Recovery of ATP                         | HSPD1                   |
| Production of nric oxide                | HSPD1, PFN1             |
| Gene expression                         |                         |
| Activation of synthetic promoter        | ACTB, GAPDH, HNRPK, SET |
| Recruitment and binding of mRNA         | EIF4A1                  |
| Transactivation of protein binding site | ENO1                    |
| Transactivation of synthetic promoter   | GAPDH, HNRPK            |

<sup>a</sup>Refer to Table 1 for protein abbreviations.

Our immunoblotting results agreed with the phenomena of BCR activation, indicating that PMA plus A23187 might induce phosphorylation of HNRPK in KU812 cells (Figure 4B). Regarding cancer biology, HNRPK increased transcriptional activation of the *c-myc* gene and oncogene *c-Src* related to cancer development, survival, and migration. The HNRPK was identified as the potential targeted protein of cancer metastasis from loss-of-function screening by randomized intracellular antibodies. Furthermore, the cytoplasmic accumulation of HNRPK played an important role in metastasis<sup>(27)</sup> and colorectal cancer<sup>(28)</sup>. The HNRPK protein elevated at 6h PMA plus A23187 treatment might enhance DNA and RNA binding activity, transcriptional and translational modulating activity. The PTM study of HNRPK on KU812 cells upon treatment with PMA and A23187 is a multifunctional protein abundantly expressed in the cytoplasm and serves as Myc-binding protein (MBP-1) in the nucleus. Enolase is a plasminogen receptor on the surface of the hematopoietic, epithelial and endothelial cells. Auto-antibodies to airway epithelial cells (A549) were examined in sera from patients with severe asthma<sup>(29)</sup>. Anti-enolase antibodies have been incriminated in various inflammatory and autoimmune diseases including SLE, RA, mixed cryoglobulinemia (MC), systemic sclerosis (SSc), Behcet's disease, multiple sclerosis, Hashimoto's encephalopathy (HE), and paraneoplastic retinopathy<sup>(30)</sup>. Anti-enolase antibodies

could serve as prognostic indicators for autoimmune or neoplastic disorders but not diagnostic biomarkers due to broad distribution in numerous diseases. ENO1 was also a highly conserved fungal allergen and its IgE-binding epitopes were elucidated. PMA treated hematopoietic cells such as B cells, T cells, monocytes, neutrophils showed greater amounts of ENO1 on their surface. The translocation of ENO1 seemed to be associated with its hydrophobic domain, posttranslational acylation or phosphorylation. The isoform of ENO1 detected by our 2D-western blot at 6h PMA plus A23187 treatment were significantly decreased (Figure 4B). Such drastic change of ENO1 expressions in the treated-KU812 cells with PMA plus A23187 might be associated with the ENO1 location or its posttranslational modifications. This area needs to be further investigated.

The tubulin beta-5 chain (TUBB) is a major constituent of microtubules responsible for cell mobility. It belongs to MHC I binding protein and is associated to NK-mediated cytotoxicity. The TUBB was identified as autoantigen in allergic rhinitis, and 52% of allergic rhinitis patients possessed anti-TUBB autoantibodies<sup>(31)</sup>. Pharmacological and morphological data revealed that the cytoskeleton as tubulins and actin associated to histamine secretion in basophil and mast cells. Furthermore, IL-3 and anti-IgE alone did not promote tubulins synthesis, but combinational treatment can induce both histamine release and tubulins up-regulation<sup>(32)</sup>.

The 60 kDa heat shock protein (HSPD1) resulted in mitochondrial protein transportation and macromolecule assembly. The HSPD1 were proven to possess dominant immuno-modulatory capability in innate, adaptive immune systems, autoimmune, and tumor immunology<sup>(33)</sup>. Although the dichotomous immunoregulatory ability is still not completely elucidated, the intracellular (self) HSPD1 is generally regarded as chaperonin and anti-inflammatory. On the other hand, extracellular (from pathogenic organisms) HSPD1 is associated to pro-inflammatory<sup>(34)</sup>. Due to admirable immunogenicity of HSP family, HSP have been designed for their adjunct use in vaccines<sup>(34)</sup>. HSPD1 down-regulation in KU812 cells upon PMA and A23187 stimulation (Table 1) might point toward immunological inflammatory pathway.

The actin family was mainly involved in cell motility and ubiquitously expressed in eukaryotic cells. In rat basophilic leukemia (RBL-2H3), both F-actin and myosin were remolded into membrane ruffles and aggregations, and followed by parallel stress fibers located on the ventral membrane originally from plasma membrane during secretion of histamine after PMA or A23187 treatment<sup>(35)</sup>.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ENO1 and ALDOA are key enzymes in glycolysis. Fumarate hydratase (FH), a major enzyme in citric acid cycle, function as interconversion of malate and fumarate. PMA plus A23187 synergistically activate H<sub>2</sub>O<sub>2</sub> generation in cultured porcine thyroid cells through activating protein kinase C and increasing cytoplasmic free calcium, respectively<sup>(36)</sup>. Oxidizing agents as H<sub>2</sub>O<sub>2</sub> were shown to carbonylate and induce polypeptide degradation in glycolytic enzymes including ENO1, pyruvate kinase, and GAPDH<sup>(37)</sup>. ENO1 was a primary target of H<sub>2</sub>O<sub>2</sub>- or O<sub>2</sub><sup>-</sup>-mediated carbonylation in *Escherichia coli* (38). Posttranslational modification of glycolytic enzymes was recognized as an intracellular sensor of oxidative stress<sup>(39)</sup>. We detected down-regulation of glycolytic enzymes including ALDOA, ALDOC, FH, ENO1 and GAPDH in PMA plus A23187 treated KU812 basophils. Therefore, the carbohydrate catabolism was reduced possibly through the production of oxidizing agents induced by the PMA plus A23187 treatment.

The inconsistency between 1D- and 2D-Western blots might be caused by the following reasons: (1) the alternative splicing transcript variants of HNRPK and ENO1 could not be recognized by 1D-western blots; (2) phosphorylated HNRPK was not revealed by 1D-Western blots, but the more acidic isoform of HNRPK up-regulated in the PMA plus A23187 treated KU812 cells (shown in the 2D-Western blots). This finding agreed with the inducible phosphorylation of HNRPK in the BCR activation<sup>(26)</sup>, and in response to the oxidative stress that was possibly induced by PMA plus A23187 treatment<sup>(36)</sup>; (3) the isoforms of ENO1 largely shifted in the pI and Mw range of the 2D gel<sup>(40)</sup> so that the selected 2D region for Western blots could include the major (not

all) isoforms of ENO1.

In summary, numerous differentially expressed proteins were identified in PMA plus A23187-activated KU812 cells. Most of these proteins were the key factors involved in metabolism, macromolecule binding, and catalytic activity, and were associated with inflammatory immunological diseases, tumorigenesis, and cell movement. HNRPK and ENO1 variations were consistently validated by protein level examinations. However, further functional study of these proteins may lead to better understanding of the activation mechanisms for PMA plus A23187 treatment.

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