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# Proteomic Analysis for the Anti-Apoptotic Effects of Cystamine on Apoptosis-Prone Macrophage

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## ABSTRACT

Increased macrophage vulnerability is associated with progression of systemic lupus erythematosus. Our previous studies have shown that cystamine, an inhibitor of transglutaminase 2 (TG2), alleviated the apoptosis of hepatocyte and brain cell in lupus-prone mice NZB/W-F1. In present study, we further investigated the effects of cystamine on apoptosis-prone macrophages (APMs) in the lupus mice. Using two-dimensional gel electrophoresis (2-DE) analysis, we found that cystamine induced a differential protein expression pattern of APM as comparing to the PBS control. The protein spots presenting differential level between cystamine and PBS treatment were then identified by peptide-mass fingerprinting (PMF). After bioinformatic analysis, these identified proteins were found involved in mitochondrial apoptotic pathway, oxidative stress, and mitogen-activated protein (MAP) kinase-mediated pathway. Further investigation revealed that cystamine significantly decreased the levels of apoptotic Bax and Apaf-1 and the activity of caspase-3, and increased the levels of anti-apoptotic Bcl-2 in APM. We also found that these apoptotic mediators were up-regulated in a correlation with the progression of lupus severity in NZB/W-F1, which were little affected in BALB/c mice. We also found that the reduced serum glutathione was restored by cystamine in NZB/W-F1. Interestingly, the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in APM and the phagocytic ability was diminished in presence of cystamine. In conclusion, our findings indicate that cystamine significantly inhibited mitochondrial pathway, induced antioxidant proteins, and diminished phosphorylation of extracellular ERK1/2, which may alleviate the apoptosis and the phagocytic ability of APM. J. Cell. Biochem. 110: 660–670, 2010. 

**KEY WORDS:** APOPTOSIS; CYSTAMINE; LUPUS-PRONE MICE; APOPTOSIS-PRONE MACROPHAGES; CASPASE; ERK

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, which is usually characterized by loss of tolerance to self-antigens and production of circulating autoantibodies to nuclear antigens, and consequent immune-mediated tissue injury of multiple organs [Munoz et al., 2005]. Apoptotic cells might provide self-antigens; therefore, persistence of apoptosis and impaired clearance of pro-apoptotic cells, causing the spillage of potential immunogenic macromolecules to the exterior, could enhance the production of auto-reactive T and B cells and then promote pathogenesis of SLE [Wu et al., 2001; Cohen, 2006]. Actually, increase of apoptosis in polymorphonuclear cells and macrophages leading to impaired clearance of pro-apoptotic cells was commonly observed in SLE patients [Ren et al., 2003]. Similarly, the apoptosis of lupus-prone macrophages (APM) in lupus-prone mice was markedly expended that it was proportional to the severity and development of lupus syndrome [Russell et al., 1985].

Shao-Hsuan Kao and Tsai-Ching Hsu contributed equally to this work.

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Recently, transglutaminase 2 (TG2) has been implicated in the pathogenesis of SLE [Szondy et al., 2003; Pitidhammabhorn et al., 2006]. TG2 is known as a multi-functional enzyme that is expressed in the majority of tissues and implicated in numerous biological functions, including remodeling of the extracellular matrix [Aeschlimann et al., 1995], stimulus-secretion coupling [Bungay et al., 1986], receptor-mediated endocytosis [Davies et al., 1980], cell differentiation [Aeschlimann et al., 1993], tumor growth [Johnson et al., 1994], and apoptosis [Fesus et al., 1987]. Recent study reported that TG2-knockout mice developed typical lupus-prone symptoms, such as autoantibodies, immune complex glomerulonephritis, and impaired engulfment of apoptotic cells by macrophages [Falasca et al., 2005]. These evidences strongly suggest that TG2 may contribute to SLE pathogenesis.

Cystamine, the FDA-approved precursor of cysteamine, is known to inhibit the transamidation activity of TG2 [Jeon et al., 2004] and to exhibit anti-oxidant activity. It is also reported that cystamine inactivates protein kinase C-epsilon, gamma-glutamylcysteine synthetase and tissue transglutaminase (tTG) by S-cysteaminylation-triggered mechanisms [Seelig and Meister, 1984; Chu et al., 2005; O'Brian and Chu, 2005]. Our previous study showed that cystamine diminished the activity of matrix metalloproteinase-9, the mRNA expression of tumor necrosis factor-α and transforming growth factor-β in APM, and suppressed the production of anti-cardiolipin autoantibody in lupus-prone mice NZB/W-F1 [Hsu et al., 2007]. Additionally, cystamine also shows neuroprotective effects, prolongs cell survival and alleviates abnormal cell movements in the transgenic model of Huntington disease [Karpuj et al., 2002]. However, the effects of cystamine on lupus-induced apoptosis of APM in NZB/W-F1 are still unclear.

In this study, we used two-dimensional gel electrophoresis (2-DE) to investigate the effects of cystamine on protein expression of APM. The proteins with different expression level regulated by cystamine were further identified by peptide-mass fingerprinting (PMF) and characterized by bioinformatic analysis. The findings revealed that cystamine significantly regulated the expression of the proteins associated with mitochondrial apoptotic signaling, oxidative stress, mitogen-activated protein (MAP) kinase signaling, protein degradation, and energy metabolism, which were further evaluated by immunoblotting. Moreover, the serum reduced glutathione (GSH) and the phagocytic ability of APM in cystamine-treated mice were also determined.

## MATERIALS AND METHODS

### MATERIALS

Female BALB/c mice and NZB/W-F1 mice were purchased from the Animal Center, National Taiwan University, Taiwan and maintained under supervision of the Institutional Animal Care and Use Committee at Chung Shan Medical University. To determine lupus activity in mice, proteinuria was determined biweekly by Albustix test strips (Bayer Diagnostics, Hong Kong, China) as described [Hsu et al., 2007]. Antibodies (Abs) against mouse Bax, Apaf-1, Bcl-2, extracellular signal-regulated kinase (ERK), phospho-ERK (p-ERK), Janus N-terminal kinase (JNK), phospho-JNK (p-JNK), p38 MAP kinase (p38), phosphor-p38 (p-p38), and actin were purchased from Upstates (Charlottesville, VA). Alkaline phosphatase-conjugated secondary Abs were purchased from BioSource International (Camarillo, CA). Chemicals for immunoblotting and phagocytic analysis were purchased from Sigma–Aldrich (St. Louis, MO). Reagents and IPG strips for 2-D gel analysis were purchased from Bio-Rad Laboratory (Hercules, CA).

### CYSTAMINE TREATMENT

NZB/W F1 mouse is a lupus-prone mouse strain and the clinical symptoms appear at the age of 8–12 weeks and reach the peak at the age of 24–28 weeks [Denman et al., 1967; Holborow and Denman, 1967]. Accordingly, 6-month-old NZB/W-F1 mice were used for cystamine treatment. The mice were randomly divided into two groups (12 mice for each group) by body weights. Treatment was performed by daily intraperitoneal injection (100 μl of 10 mM cystamine in normal saline or 100 μl of normal saline as control) for 14 days as described [Karpuj et al., 2002; Hsu et al., 2007]. After the 14-day injection, the mice were sacrificed and the macrophages were obtained and transferred onto a Petri dish for 2 h incubation to allow cell adherence and then the attached cells were collected and pooled for the subsequent analysis [Mühlradt and Frisch, 1994]. Age-matched BALB/c mice were used as control and the treatment were performed as described for NZB/W-F1 mice. Macrophages obtained from BALB/c mice were used as normal macrophages.

### PROTEIN EXTRACTION AND QUANTITATION

Macrophages were homogenized in lysis buffer (50 mM Tris-HCl, containing 1 mM PMSF, $Na_4P_2O_7$ and NaF; and 0.1 mM $Na_3VO_4$). After centrifuging at 20,000*g* for 15 min to remove the cell debris, the supernatant was subjected to 2-D gel electrophoresis and immunoblotting. Protein quantitation was performed by using the modified Bradford method for quantitative 2-D gel analysis as described [Kao et al., 2008]. Each protein sample for 2-DE analysis, immunoblotting and caspase activity assays was obtained from a pool of four mice.

### 2-D GEL ELECTROPHORESIS AND IMAGE ANALYSIS

For 2-DE analysis, 300 μg pooled crude protein was applied for each 2-D gel electrophoresis. To verify the changes of protein level between cystamine-treated and control samples, three independent 2-D gels for each group were performed, and then the density of protein spots on 2-D gels of the two groups were normalized. In first dimension, IEF was performed using the Bio-Rad PROTEAN™ IEF cell (Bio-Rad). The pooled crude proteins were prepared in 2-D sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3-10) and applied to a ReadyStrip™ IPG Strip (pH 3–10, 17 cm; Bio-Rad) through active rehydration method (50 V for 16 h). The rehydrated strips were focused in a three-step procedure, first ramped to 300 V for 1 min, then linearly increased to 3,000 V over 4 h, and finally maintained at 3,000 V until 10,000 V h in total focusing at 20°C. Prior to the second dimension, the focused strips were equilibrated in equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, and 2% dithiothreitol) for 10 min, then reacted with the equilibration buffer supplemented with 2.5% iodoacetamide for 10 min. The equilibrated strips were

positioned on 12.5% polyacrylamide gel and the separation was performed at a constant current (20 mA) using PROTEAN II electrophoresis unit (Bio-Rad). Protein spots on the gel were detected by Coomassie Brilliant Blue R-250 (CBR) staining as previously described [Mortz et al., 2001].

All developed gels were scanned at 400 dpi resolutions in TiFF format by using an optical flatbed scanner with transparency unit (Xlite, Avegene, Taipei, Taiwan). 2-D gel images were analyzed by using Phoretix 2D Elite software (Nonlinear, Durham, NC). The detection stringencies were as follows. Phoretix 2D Elite (operator size 25): sensitivity 9700, noise factor 3, background 2.

### IN-GEL DIGESTION AND PROTEIN IDENTIFICATION

The CBR-stained protein spots on 2-D gel with differential level (>1.5-fold) between cystamine-treated and control samples were excised, destained with 50% acetonitrile (ACN) in 25 mM $NH_4HCO_3$ and then dried in a SpeedVac concentrator as described previously [Moritz et al., 1996]. Protein was digested for 16 h at 37°C with sequencing-grade trypsin (0.1 mg/ml; Promega, Madison, WI) in 25 mM $NH_4HCO_3$. The resulting peptides were extracted twice with 50 μl 1% trifluoroacetic acid (TFA) and 0.1% TFA/ACN respectively. The peptide extracts were lyophilized, resuspended in 10 μl 0.1% TFA and desalted on ZipTip C18-microcolumn (Millipore, Bedford, MA). The desalted peptide extracts were premixed in a ratio of 1:1 with the matrix solution (5 mg/ml (R)-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA), and then spotted onto the 100-well sample plate. The peptide masses were analyzed by a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied Biosystems, Framingham, MA). Protein identification was carried out by peptide mass fingerprint (PMF) using the Mascot software (http://www.matrixscience.com). The search parameters used in PMF were: database, SwissProt (57.2, 466739 sequences); species, *Mus musculus*; enzyme, trypsin; fixed modifications, carbamidomethylation; variable modifications, oxidation (M); peptide tolerance, 100 ppm; and allowance of one missed cleavage. Multiple isoforms of the protein were identified as the isoform which the gel spot location matched the theoretical molecular weight and pI.

### IMMUNOBLOTTING

For immunoblotting, crude protein samples were denatured at 95°C for 10 min in denaturing buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 20% 2-mercaptoethanol and 10% glycerol) and separated by 12.5% SDS-PAGE. After the electrophoresis, proteins were transferred onto a nitrocellulose membrane (Millipore). The blotted membrane was blocked with 5% w/v skimmed milk in Tris-buffered saline (TBS)(50 mM Tris-HCl, 150 mM NaCl, pH 7.2), and then incubated for 1.5 h with 1,000-fold diluted primary antibody (Ab). Bound Ab was detected using 2,000-fold diluted peroxidase-conjugated secondary Abs (BioSource International) and ECL chemiluminescence as the substrate system. The photographic density was quantitated by image analysis system (Alpha Imager 2000, Alpha Innotech Corp., San Leandro, CA). Reacted density of actin was used as internal control for relative quantitation.

### CASPASE 3 ACTIVITY ASSAY

A caspase 3 ELISA kit (BD Pharmingen, San Diego, CA) was used for in vitro determination of caspase-3 enzymatic activity in cell lysates according to the manufacturer's instructions.

### DETERMINATION OF SERUM GSH

Concentrations of reduced glutathione GSH in the serum samples were measured using GSH assay kit (Chemicon, Inc., Temecula, CA) according to the manufacturer's protocol. In brief, 90 μl of protein extracts and 10 μl of prepared monochlorobimane solution were incubated 2 h at 25°C away from light and fluorescence was read at 380/461 nm using a 96-well fluorometric plate reader.

### ASSESSMENT OF PHAGOCYTOSIS

For phagocytosis assessment, the macrophages ($2 \times 10^5$ per well) were overnight cultured in a 16-well Lab-TekRII Chamber Slide (Nunc, Denmark) and following incubated with FITC-labeled Latex beads (1.1 μm in diameter; Sigma) or UV-induced pro-apoptotic HeLa cells. For phagocytosis of Latex beads [Kiama et al., 2001], 10× FITC-labeled Latex beads were suspended in PBS and opsonized by incubation with the overnight-cultured macrophages at 37°C for 1 h. For phagocytosis of pro-apoptotic bodies [Williams et al., 2005], HeLa cells were irradiated with UV (25 J/$m^2$) and incubated in serum-free media for 12 h. Apoptosis of HeLa cells was assessed by DNA fragmentation. The HeLa cells were then collected and stained with a red membrane dye, PKH26, according to the manufacturer's instructions (Sigma). The PKH26-labeled HeLa cells were then cultured with the macrophages as described above for 4 h at 37°C. Phagocytic index was determined by observing engulfed particles or pro-apoptotic HeLa cells. One hundred macrophages in five random fields were counted and the phagocytic index was expressed as the number of phagocytosed particles divided by the total number of macrophage in percentage.

### STATISTICAL ANALYSIS

Results were expressed as mean ± SD of three independent experiments. Statistical analysis was performed using the SigmaStat version 3.5 for Windows (Systat Software, Inc., San Jose, CA). Statistical significance between groups was determined using Student's *t* test. A *P*-value less than 0.05 was considered statistically significant.

## RESULTS

### CYSTAMINE INDUCED A DIFFERENTIAL PROTEIN EXPRESSION OF APM

To investigate the effects of cystamine on protein expression of APM, 2-DE was performed and the protein spots were detected by CBR-staining. The representative 2-D images were shown in Figure 1. Comparing to PBS control, cystamine treatment induced a differential protein profile of APM on 2-D gel. After quantitation and normalization by using three pairs of 2-D gels, protein spots, whose density changing over than 1.5-fold as comparing to the control, were excised and in-gel digested. The resulting peptides were extracted for MALDI-MS analysis and identified by PMF. As

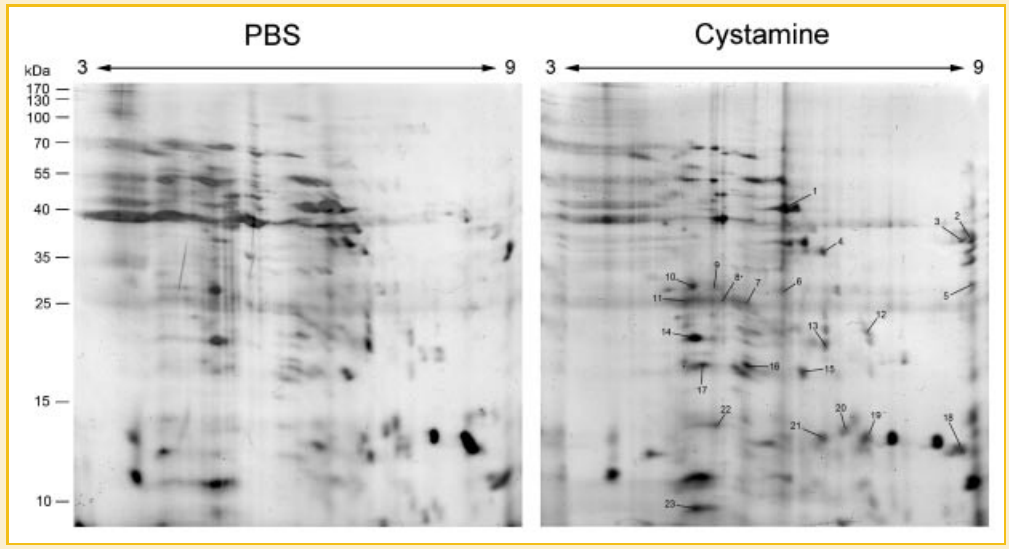


Fig. 1. 2-DE analysis for protein expression in APM. Proteins (300 μg) of APM obtained from NZB/W-F1 mice treated with (A) PBS (control) and (B) cystamine were separated by linear pH gradient 3–10 IPG strip and following by 12.5% SDS-PAGE. Selected protein spots were numbered and collected for identification by MALDI-MS. The molecular weight (kDa) and pI scales are indicated.

listed in Table I, among the 23 PMF-identified proteins, 19 proteins were up-regulated and 4 proteins were down-regulated. By bioinformatic analysis, the 23 identified proteins were found involving in mitochondrial apoptotic signals, oxidative stress responses, protein degradation and energy metabolisms. The apoptosis related proteins included Apaf-1 interacting protein (APIP) (spot 7), phosphatidylethanolamine-binding protein 4 (PEBP4) (spot 11) and caspase-3 precursor. The oxidative stress related proteins included ribose-5-phosphate isomerase (spot 4), glutathione S-transferase P2(GSTP2) (spot 12), superoxide dismutase (SOD) (spot 16 and 17), and peroxiredoxin (spot 21 and 22). The protein degradation related proteins included E3 ubiquitin-protein ligase (spot 8), proteasome (spot 9) and ubiquitin-conjugating protein (spot 15). The energy metabolism related proteins included mitochondrial uncoupling protein-2/3 (spot 2 and 3) and adenylate kinase (spot 13). Furthermore, several signaling proteins were also identified such as MAP kinase-activated protein kinase 5 (spot 1), GTP-binding protein Rit 1 (spot 5), and M-Ras precursor (spot 18).

## CYSTAMINE INHIBITED MITOCHONDRIAL APOPTOTIC PATHWAY

Cystamine treatment was found to up-regulate the level of mitochondrial apoptotic proteins including APIP, PEBP4 and to down-regulate the level of pro-caspase 3 (Table I). Therefore, the effects of cystamine on the important regulators, including Bax, Apaf-1, Bcl-2, and caspase-3, in mitochondrial pathway were further investigated [Hengartner, 2000]. As shown in Figure 2A, the cystamine treatment significantly diminished the level of apoptotic mediators Bax and Apaf-1 as well as increased the level of anti-apoptotic mediator Bcl-2 in APM. Quantitative analysis revealed that the changes of Bax, Apaf-1, and Bcl-2 were 4.9-, 2.8-, and 3.3-fold as comparing to the control (Fig. 2B). Cystamine treatment also significantly reduced the caspase 3 activities in the APM (Fig. 2C). Additionally, cystamine treatment showed no significant effects on the level of Bax, Apaf-1 and Bcl-2 and on the activity of caspase-3 in normal macrophages (Fig. 2A–C). The findings indicate that cystamine treatment inhibit the mitochondrial pathway which may subsequently alleviate the apoptosis of APM.

## MITOCHONDRIAL PATHWAY ASSOCIATED WITH LUPUS PROGRESSION

Since cystamine treatment showed significant inhibitory effects on mitochondrial pathway, the significance of mitochondrial pathway on lupus progression was further investigated. Lupus symptoms of NZB/W F1 mice generally appear at the age of 8–12 weeks and reach the maximum at the age of 24–28 weeks [Denman et al., 1967; Holborow and Denman, 1967]. As shown in Figure 3, the protein levels of apoptotic Bax and Apaf-1 and the activity of caspase-3 in APM were significantly increased in the late stage of lupus development (28-week) as comparing to the early stage (6-week) in NZB/W-F1 mice. There were no significant changes in the three apoptotic mediators in the normal macrophages from 28-week-old BALB/c mice as comparing to that from 6-week-old BALB/c mice. These findings indicate that the increase of mitochondrial apoptotic mediators in APM parallels the lupus severity in NZB/W-F1 mice.

## CYSTAMINE RAISED THE GSH LEVEL IN SERUM

Cystamine was found to up-regulate several antioxidant proteins, including glutathione S-transferase, superoxide dismutase, peroxiredoxin, and thioredoxin, by 2-DE and MS analysis. Therefore, the effects of cystamine on serum GSH were further determined. As shown in Figure 4, cystamine treatment significantly increased the serum GSH in both NZB/W-F1 and BALB/c mice. Additionally, the increase of serum GSH in NZB/W-F1 mice was higher than in BALB/c mice. The serum GSH in BALB/c and NZB/W-F1 mice were increased to 1.25-fold ($P = 0.011$) and 1.62-fold ($P = 0.012$) by

TABLE I. Identification of Proteins Regulated by Cystamine Treatment

| No. | Reg[a] | Theo. MW/pI[b] | Accession no. | Protein name | Peptides matched | Cov.[c] (%) |
|---|---|---|---|---|---|---|
| Signal proteins | | | | | | |
| 1 | ↓ | 54.2/7.3 | O54992 | MAP kinase-activated protein kinase 5 | QVIEEQTLPHEPQ<br>ETSILEEYSINWTQK<br>VSLKPLHSVNNPILR<br>LLIVMEMMEGGELFHR<br>QIALALQHCHLLNIAHR<br>ARLLIVMEMMEGGELFHR<br>DGIYIHDHENGTEDSNVALEK | 24.8 |
| 5 | ↑ | 25.2/9.3 | P70426 | GTP-binding protein Rit1 | EEGLSLAR<br>SAMTMQFISHR<br>YYIDDVFHALVR<br>LVMLGAGGVGK<br>TDDTPVVLVGNK<br>FPEDHDPTIEDAYK | 30.3 |
| 11 | ↑ | 26.9/5.7 | Q9D9G2 | Phosphatidylethanolamine-binding protein 4 precursor | EDVSLCR<br>GCFLPPLPK<br>FLQQYGLR<br>SNPVMKYWR<br>GCFLPPLPKEDVSLCR<br>GNVLSDYSPPTPPPETGVHR | 29.4 |
| 18 | ↑ | 23.9/8.8 | O08989 | M-Ras precursor | ASFEHVDR<br>ESFPMILVANK<br>LVVVGDGGVGK<br>DPPLNVDKTFHDLVR<br>YNIPYIETSAKDPPLNVDK<br>LVVVGDGGVGKSALTIQFFQK | 38.9 |
| 6 | ↑ | 29.0/6.4 | P00756 | Kallikrein 1-related peptidase b3 | NSQPWHVAVYR<br>CEKNSQPWHVAVYR<br>FLEYDYSNDLMLLR<br>LSKPADITDTVKPITLPTEEPK<br>AHIEKVTDAMLCAGEMDGGK | 32.2 |
| Mitochondrial apoptotic pathway | | | | | | |
| 7 | ↑ | 26.9/6.4 | Q9WVQ5 | APAF1-interacting protein | FLIPELCK<br>CTSGGYYR<br>ITHQEMIK<br>EHPRFLIPELCK<br>MGLDPTQLPVGENGIV<br>KMGLDPTQLPVGENGIV<br>MAHAMNEYPDSCAVLVR | 35.9 |
| 2 | ↑ | 16.5/5.9 | P70406 | Mitochondrial uncoupling protein-2 | GSEHAGIGSR<br>ATDVPPTATVK<br>NAIVNCAELVTYDLIK<br>FLGAGTAACIADLITFPLDTAK | 36.7 |
| 3 | ↑ | 33.9/9.6 | P56501 | Mitochondrial uncoupling protein-3 | SPYSGLVAGLHR<br>LQIQGENPGAQSVQYR<br>NAIVNCAEMVTYDIIKEK<br>SPLHCMLKMVAQEGPTAFYK<br>FLGAGTAACFADLLTFPLDTAK | 28.3 |
| 20 | ↓ | 31.5/6.5 | P70677 | Caspase-3 (precursor) | LFIIQACR<br>LTSFFRGDYCR<br>SGTDVDAANLR<br>EDILELMDSVSKEDHSK<br>STGMSSRSGTDVDAANLR<br>MDYPEMGICIIINNKNFHK | 30.2 |
| Oxidative stress | | | | | | |
| 4 | ↑ | 32.5/7.8 | P47968 | Ribose-5-phosphate isomerase | IVAGYASR<br>FIVIADFRK<br>VYFGMQDGSVNVR<br>VKQENLDLICIPTSFQAR<br>GIPIEVIPMAYVPVSRAVAQK | 23.8 |
| 12 | ↑ | 23.5/7.7 | P46425 | Glutathione S-transferase P 2 | YGTMIYR<br>SLGLYGKNQR<br>YGTMIYRNYENGK<br>AFLSSPEHVNRPINGNGK<br>FEDGDLTLYQSNAILRHLGR | 33.6 |
| 16 | ↑ | 15.8/5.7 | P00441 | Superoxide dismutase [Cu–Zn] | ESNGPVK<br>HVGDLGNVTADK<br>GDGPVQGIINFEQK<br>GGNEESTKTGNAGSR<br>GDGPVQGIINFEQKESNGPVK | 45.2 |
| 17 | ↑ | 22.2/6.9 | P04179 | Superoxide dismutase precursor [Mn] mitochondrial | GELLEAIK<br>NVRPDYLK<br>GDVTAQIALQPALK<br>HHAAYVNNLNVTEEK<br>LTAASVGVQGSGWGWLGFNK<br>YQEALAKGDVTAQIALQPALK | 41.9 |
| 21 | ↑ | 17.1/6.7 | P30044 | Peroxiredoxin-5 (mitochondrial) | VNLAELFK | 43.4 |

(*Continued*)

TABLE I. (*Continued*)

| No. | Reg[a] | Theo. MW/pI[b] | Accession no. | Protein name | Peptides matched | Cov.[c] (%) |
|---|---|---|---|---|---|---|
| | | | | | FSMVVQDGIVK<br>THLPGFVEQAEALK<br>ETDLLLDDSLVSIFGNR<br>VGDAIPAVEVFEGEPGNK | |
| 22 | ↑ | 17.0/6.7 | P30044 | Peroxiredoxin-5 (mitochondrial) | AHKAEGK<br>LLADPTGAFGK<br>GVLFGVPGAFTPGCSK<br>ETDLLLDDSLVSIFGNR<br>VGDAIPAVEVFEGEPGNK | 41.5 |
| 23 | ↑ | 11.6/4.8 | P10599 | Thioredoxin | LEATINELV<br>CMPTFQFFK<br>VGEFSGANK<br>GQKVGEFSGANK<br>TAFQEALDAAGDK | 48.4 |
| Protein folding, transportation, and degradation | | | | | | |
| 8 | ↑ | 28.0/6.4 | Q8CJC5 | E3 ubiquitin-protein ligase | AIELLDPK<br>VNAGRPLLLR<br>AGNVVCFWVNR<br>WQIEEVAVVSSLK<br>STFHDGIVFSQRPVWPGER<br>LMPCGHSHFCGSCAWHIFK | 32.3 |
| 9 | ↓ | 29.5/6.0 | Q9R1P4 | Proteasome subunit alpha type-1 | THAVLVALK<br>TQIPTQRYGR<br>IHQIEYAMEAVK<br>AQPSQAAEEPAEK<br>HMSEFMEC*NLDELVK<br>ILHVDNHIGISIAGLTADAR | 30.1 |
| 10 | ↑ | 34.6/4.9 | O89079 | Coatomer subunit epsilon | DSIVLELDR<br>YLSQLKDAHR<br>MFAEYLASENQR<br>WETAEGVLQEALDK<br>NAFYIGSYQQCINEAQR<br>TLHQGDGLECMAMTIQILLK | 27.6 |
| 14 | ↑ | 20.3/4.9 | O55013 | Trafficking protein particle complex 3 | FVQDTLK<br>LIEDFLAR<br>IEDNLPAGEE<br>ETADVIAKVAFK<br>GALEMVQMAVEAK | 27.5 |
| 15 | ↓ | 16.4/7.8 | Q9CZY3 | Ubiquitin-conjugating enzyme E2 | WQNSHSIK<br>YPEAPPSVR<br>WTGMIIGPPR<br>LLEELEEGQK<br>LPQPPEGQCYSN<br>VNMSGVSSSNGVVDPR | 44.3 |
| 19 | ↑ | 17.9/7.8 | P62937 | Peptidyl-prolyl *cis–trans* isomerase A (Cyclophilin A) | TAENFR<br>VSFELFADK<br>ITIADCGQLE<br>FEDENFILK<br>GFGYKGSCFHR<br>EGMNIVEAMER | 36.6 |
| Energy metabolism | | | | | | |
| 13 | ↓ | 25.6/7.0 | Q9WTP6 | Adenylate kinase | LVSDEMVVELIEK<br>SYHEEFNPPKEPMK<br>LDSVIEFSIQDSLLIR<br>LEAYHTQTTPLVEYYR<br>LAENFCVCHLATGDMLR | 35.0 |

[a]Reg., regulation: ratio of spot density on 2-D gel from cystamine treatment/from PBS control.
[b]Theo. MW/pI, theoretical molecular weight and pI obtained from the UniProt Knowledgebase (Swiss-Prot and TrEMBL).
[c]Cov., coverage: ratio of total molecular weight of matched peptides/molecular weight of identified protein.

cystamine as comparing to the PBS controls respectively. The results indicate that cystamine increased the serum GSH, which might result from the up-regulation of antioxidant proteins.

### CYSTAMINE INHIBITED PHOSPHORYLATION OF ERK

The 2-DE and MS analysis revealed that cystamine treatment down-regulated the protein levels of MAP kinase-activated protein kinase 5 and M-Ras precursor which belonged to MAP signal cascades. Therefore, the effects of cystamine on MAP signaling pathway, the activation of three important MAP kinases including ERK, JNK and p38, were further investigated. As shown in Figure 5, the ratio of both ERK/actin and p-ERK/actin in APM were significantly higher than in normal macrophages. By cystamine treatment, the ratio of both ERK/actin and p-ERK/actin were significantly decreased in APM, the findings which were little affected in normal macrophages. Additionally, cystamine treatment showed no significant effects on the ratio of p-JNK/actin, JNK/actin p-p38/actin and p38/actin in APM or in normal macrophages (data not shown). These findings indicate that cystamine might inhibit the phosphorylation of ERK, but not JNK or p38, in APM.

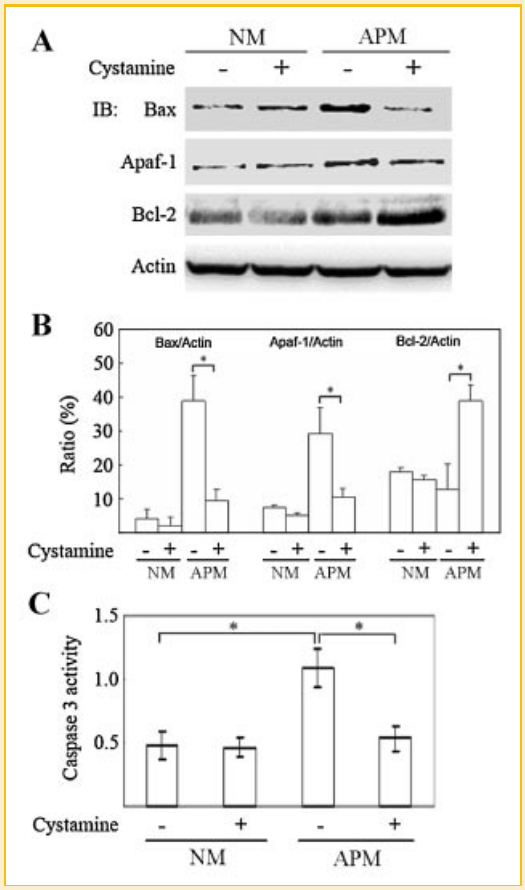


Fig. 2. Effects of cystamine on expression of apoptotic signaling proteins in NM and APM treated with PBS (−) or cystamine (+). A: The expression of Bax, Apaf-1, and Bcl-2. B: Quantitative expression by densitometric analysis. C: Quantitation of caspase 3 activity. Three independent experiments were performed for quantitation and statistics. $^{**}P < 0.01$.

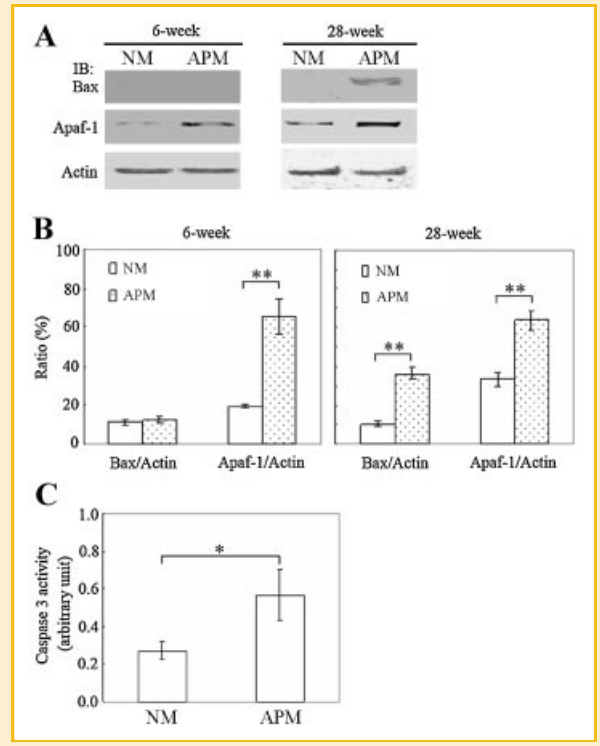


Fig. 3. Effects of lupus progression on expression of apoptotic signaling proteins in APM. A: The levels of Bax, Apaf-1 and actin in NM and APM from 6- and 28-week-old mice. B: Quantitation of protein expression of Bax and Apaf-1 by densitometric analysis. C: Quantitation of caspase 3 activity in NM and APM from 28-week-old mice. Three independent experiments were performed for quantitation and statistics. $^{*}P < 0.05$; $^{**}P < 0.01$.

## CYSTAMINE ATTENUATED PHAGOCYTIC ACTIVITY OF MACROPHAGES

The dysfunction of phagocytes is believed involving in autoimmune progression [Rovere et al., 2000]. Therefore, effects of cystamine on phagocytic activity of APM were investigated. The phagocytosis assessment results revealed that cystamine treatment significantly reduced the non-specific phagocytosis of normal macrophages and APM (Table II, beads). Additionally, the apoptotic body-specific phagocytosis of normal macrophages and APM was also inhibited by cystamine treatment (Table II, apoptotic bodies). In PBS control experiments, the phagocytic activity of APM for pro-apoptotic bodies was decreased to 67.5% of that of normal macrophages. With the cystamine treatment, non-specific phagocytic activity of normal macrophages and APM were decreased to 57.3% and 32.6% of the PBS controls respectively. Additionally, apoptotic body-specific phagocytic activities of normal macrophages and APM by cystamine treatment were decreased to 47.5% and 54.7% of that by PBS respectively. The findings revealed that cystamine significantly diminished the phagocytic activity of normal macrophages and APM.

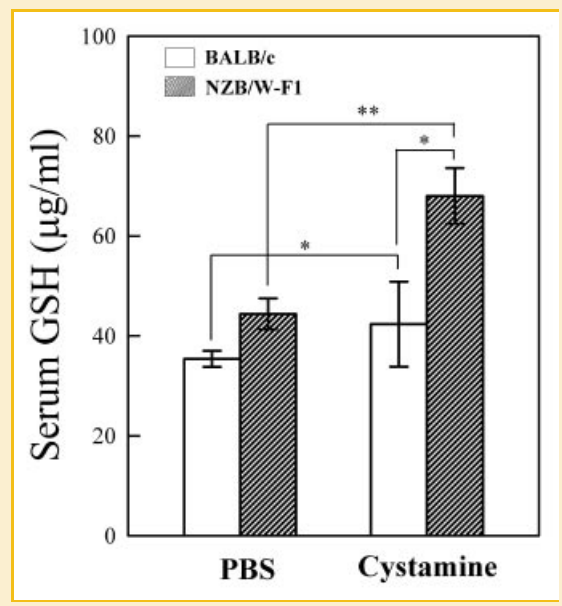


Fig. 4. Effects of cystamine on serum GSH in BALB/c and NZB/W-F1 mice. BALB/c and NZB/W-F1 mice were daily treated with PBS or cystamine for 2 weeks, and their sera were obtained for GSH determination. Three independent experiments were performed for quantitation and statistics. $^{*}P < 0.05$; $^{**}P < 0.01$.

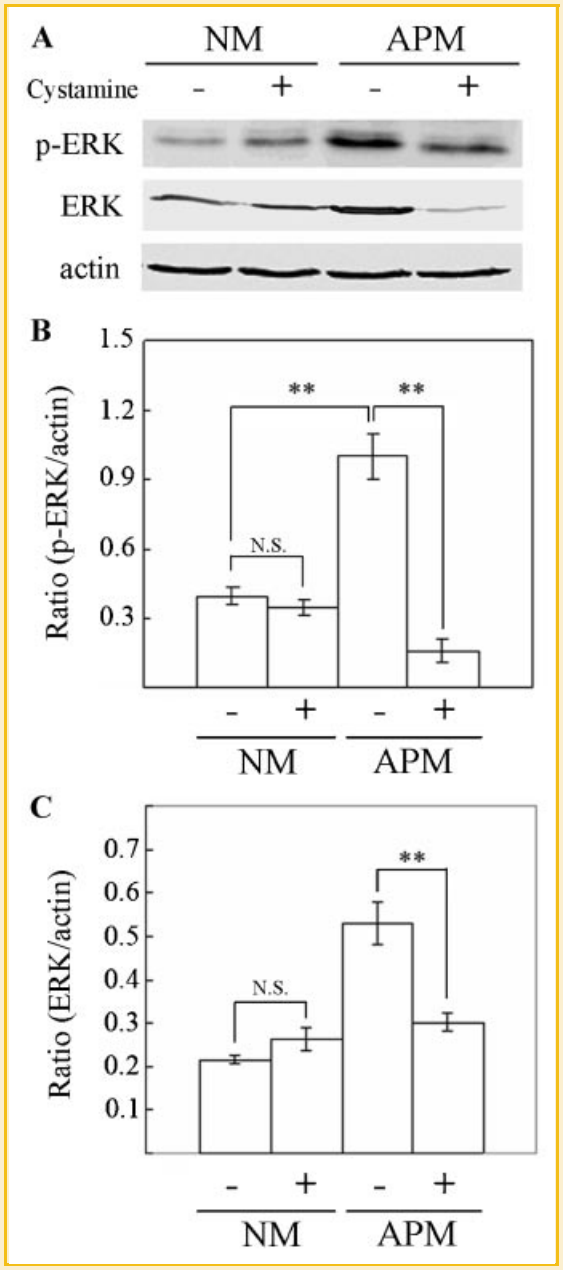


Fig. 5. Effects of cystamine on ERK phosphorylation in NM and APM. A: The level of phosphorylation and protein of ERK. B: Quantitation of p-ERK level by densitometric analysis. C: Quantitation of ERK protein level by densitometric analysis. Three independent experiments were performed for statistics. $^{**}P < 0.01$. NS, no significance.

TABLE II. Cystamine Reduced Phagocytic Activity of Macrophages

| | BALB/c | | NZB/W-F1 | |
|---|---|---|---|---|
| | PBS | Cystamine | PBS | Cystamine |
| Beads | $63.47 \pm 12.99$ | $36.40 \pm 7.47^{*}$ | $47.23 \pm 10.30$ | $15.42 \pm 4.41^{\#}$ |
| Pro-apoptotic bodies | $46.90 \pm 7.88$ | $22.27 \pm 8.18^{*}$ | $31.66 \pm 6.93^{a}$ | $17.27 \pm 7.09^{\#}$ |

Data are presented as mean ± SE (three independent experiments): $^{*}P < 0.05$ compared with cells from BALB/c treated with PBS. $^{\#}P < 0.05$ compared with cells from NZB/W F1 treated with PBS. $^{a}P < 0.05$ compared with cells from BALB/c treated with PBS.

## DISCUSSION

Although cystamine has been demonstrated to alleviate cell death and protect cell from stress-induced damage, the detailed anti-apoptotic mechanisms for APM are still unclear. In this study, the anti-apoptotic effects of cystamine on APM, as well as the significance and the mechanism of the anti-apoptotic effect in lupus progress are investigated. The comparative 2-DE analysis and the following PMF identification reveal that cystamine affects the level of proteins associated with mitochondrial pathway, oxidative stress and MAP kinase cascades, suggesting that cystamine may partially regulate these signaling pathways through altering protein expression.

It is reported that enzyme activity of caspase 3 is inhibited by cystamine through mixed disulfide exchange reactions of sulfhydryl group at the active site [Lesort et al., 2003]. Our findings reveal that both protein expression and enzyme activity of caspase 3 are down-regulated by cystamine. Moreover, the expressions of anti-apoptotic proteins including APIP, PEBP4, and Bcl-2 are simultaneously found up-regulated. APIP, cloning from murine monocyte-macrophage cDNA sequence, is reported to inhibit muscle ischemic damage by binding to Apaf-1 in the Apaf-1/caspase-9 apoptosis pathway [Cho et al., 2004]. Human PEBP4 has been demonstrated to promote the cellular resistance to tumor necrosis factor-induced apoptosis by inhibiting activation of the Raf-1/Mek/ERK pathway, JNK, and PE externalization [Wang et al., 2004]. Our results show that cystamine diminishes the phosphorylation of ERK1/2 in APM, suggesting that cystamine may inhibit the ERK activation partially by upregulation of PEBP4. Taken together, cystamine may alleviate the apoptosis of APM not only by suppressing the Apaf-1/caspase-9 proapoptotic signaling, but also by reducing the phosphorylation of ERK1/2.

The ERK signaling cascade plays important roles in the regulation of various cellular processes, including survival, proliferation, differentiation, development, and apoptosis [Shaul and Seger, 2007]. Previous studies show that cystamine treatment increases ERK phosphorylation either through the blockage of crosslinking of vascular endothelial growth factor receptor 2 and tissue transglutaminase II in endothelial cell line [Dardik and Inbal, 2006], or through the increase of brain-derived neurotrophic factor and following activation of Akt and ERK1/2 [Pillai et al., 2008]. However, the effects of in vivo cystamine treatment on ERK signaling cascade in APM are little known. Our 2-DE analyses reveal that cystamine treatment increases the protein expression of M-Ras and MAPKAPK5, both involving in MAP kinase pathways. M-Ras, also known as R-Ras3 and X-Ras, a novel member of the Ras subfamily of GTPases, is reported to involve in controlling cell proliferation and differentiation [Quilliam et al., 1999]. M-Ras is known to stimulate Akt activation, which has been extensively implicated in mediating cell survival signaling [Kimmelman et al., 2000]. Accordingly, increase of M-Ras by cystamine treatment might enhance the survival of APM through activation of Akt

signaling. In vitro, MAPKAPK5 is reported as a novel substrate of the ERK and p38, which can be phosphorylated by ERK and p38 but not by JNK [Ni et al., 1998]. Similarly, we found that the level of MAPKAPK5 as well as the phosphorylation of ERK was decreased by cystamine treatment. However, the downstream signals mediated by MAPKAPK5 and their roles in the apoptosis of APM need to be further investigated.

Although the pathogenesis of SLE is not completely clear, many factors are reported to implicate in the development or progression of SLE, including altered cytokine levels [Waszczykowska et al., 1999], increased apoptosis [Kaplan, 2004] and elevated levels of oxidative stress. Oxidative stress has been demonstrated to associate with main pathological characteristics of SLE, for example, many of the autoantibodies produced in SLE patients exhibit a preference for the increased oxidized molecules [Cooke et al., 1997; Vaarala, 2000]. Additionally, overexpression of superoxide and reactive oxygen species (ROS) is also reported to induce oxidative stress and subsequently to cause various forms of apoptosis [Simon et al., 2000; Vincent et al., 2002; Kanno et al., 2004]. Our findings reveal that cystamine treatment enhances the protein expression of antioxidant proteins, including Mn-SOD, Cu/Zn-SOD, GSTP2, thioredoxin, and peroxidoxin-5. Cu/Zn-SOD has been reported to localize in mitochondria and to play important roles in removing superoxide in and around mitochondria as well as in protecting cells against mitochondria-derived oxidative damage and apoptosis [Okado-Matsumoto and Fridovich, 2001]. GST superfamily is also known to involve in the detoxification of ROS and the genetic polymorphism of GST may associate with susceptibility to SLE [Kang et al., 2005]. Free cysteamine in mouse tissues is mainly metabolized from pantothenic acid by vanin-1 [Martin et al., 2001]. It is reported that cystamine specifically restores hepatic GST-α3 level without upregulation of its mRNA expression or protein production in vanin-1 knockout mice, suggesting that cystamine may act on protein stability or folding [Di Leandro et al., 2008]. In lupus-prone mice, our findings reveal that exogenous cystamine upregulates the protein expression of GSTP2 in APM. The differences are supposed to be results of diverse cell types and animal models.

Macrophage activation induced by bacterial infection in vivo or by cytokine stimulation in vitro, is associated with enhanced superoxide production and cytolytic activity [Boraschi et al., 1982; Suzuki, 1991]. Previous study using vanin-1 knockout mice provides evidences that strong oxidative stress induced by chemicals or harmful irradiation increases cysteamine/cystamine production, subsequently inhibiting gamma-glutamylcysteine synthetase and diminishing GSH level in thymic tissue [Berruyer et al., 2004]. Although cystamine is reported to inactivate gamma-glutamylcysteine synthetase through S-cysteaminylation, it also has been demonstrated as a potent antioxidant and free radical scavenger [Revesz and Modig, 1965; Skrede and Christophersen, 1966; Stack et al., 2008]. Our previous study showed that cystamine significantly suppressed the expression of TNF-α and TGF-β and decreased the production of anti-cardiolipin autoantibody [Hsu et al., 2007]. The present findings show that cystamine both elevates the serum GSH in normal mice and lupus-prone mice, and the increase of serum GSH by cystamine in lupus-prone mice is higher than in normal mice. Taken together, the elevated serum GSH in lupus-prone mice by cystamine, at least partially, may be attributed to the antioxidant activity and the capability to inhibit inflammatory responses of cystamine. It is postulated that the intervention of macrophage activation in presence of cystamine contributes to attenuated phagocytic activity of both APM and normal macrophages.

In conclusion, the present study demonstrates that cystamine alters the protein expression profile of APM, inhibits the mitochondrial apoptotic pathway and enhances the antioxidant activity and survival signaling. These findings not only provide evidences that cystamine effectively alleviates the apoptosis of APM, but also indicates a possible anti-apoptotic mechanism induced by cystamine.

## ACKNOWLEDGMENTS

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