BIOMEDICAL AND ENVIRONMENTAL SCIENCES **18**, 164-168 (2005)

Effects of Formaldehyde Inhalation on Lung of Rats[1]

YU-HUA YANG, ZHU-GE XI, FU-HUAN CHAO, AND DAN-FENG YANG

Institute of Health and Environmental Medicine, Tianjin 300050, China

Objective To analyze protein changes in the lung of Wistar rats exposed to gaseous formaldehyde (FA) at 32-37 mg/m^3 for 4 h/day for 15 days using proteomics technique. **Methods** Lung samples were solubilized and separated by two-dimensional electrophoresis (2-DE), and gel patterns were scanned and analyzed for detection of differently expressed protein spots. These protein spots were identified by MALDI-TOF-MS and NCBInr protein database searching. **Results** Four proteins were altered significantly in 32-37 mg/m^3 FA group, with 3 proteins up-regulated, 1 protein down-regulated. The 4 proteins were identified as aldose reductase, LIM protein, glyceraldehyde-3-phosphate dehydrogenase, and chloride intracellular channel 3. **Conclusion** The four proteins are related to cell proliferation induced by FA and defense reaction of anti-oxidation. Proteomics is a powerful tool in research of environmental health, and has prospects in search for protein markers for disease diagnosis and monitoring.

Key words: Proteomics; Formaldehyde; Rat; Lung; Toxicity; Two-dimensional electrophoresis; Mass spectrometry

INTRODUCTION

Formaldehyde (FA) is a ubiquitous chemical found in garments, food, indoor air, gasoline and diesel exhausts, etc. It belongs to volatile organic chemicals with a boiling point at -19℃ and is easy to volatilize at indoor temperature. Therefore gaseous FA is more dangerous to health compared with liquid FA. Its critical effects on health include sensory irritation, allergenic reaction and a variety of genotoxic actions[1-3]. Long-term inhalation experiments in rats have confirmed that FA is a nasal carcinogen[4]. FA is classified as a "probable human carcinogen". The US Environmental Protection Agency (EPA) identifies it as a class 2A carcinogen. At present, the toxic effects of FA are clear, however, the underlying mechanism is little understood. We speculated that protein molecules were involved in harmful effects of FA, and used proteomics technique to explore the toxicity mechanism of FA and to find indicative protein markers for its toxicity.

Along with the successful completion of the human genome project (HGP) on April 14, 2003[5] and the comimg of post genomic era, proteomics has become an important area of research in life science. Coined by Wilkins and Williams in 1995, the word "proteome" indicates "the entire proteins expressed by a genome"[6]. The concept of "functional proteome" is a basis of proteome study[7]. At present, researches concerning FA are mainly focused on environment monitoring, epidemiological investigation and DNA damage at molecular level, while studies at proteome level have not been reported.

Respiratory system is the major target of FA. It was reported that after rats inhale FA, the volume of FA is higher in the lung than in the blood, brain, liver and kidney[8]. Much attention to the effects of FA is paid on the upper airway, in particular the nose, much less concern is focused on the pulmonary toxicity. Ohtsuka *et al.*[9] reported that after F344 rats inhale 20-27 mg/m^3 FA for 3 h per day for 10 days, changes such as degeneration, necrosis, stratification, and squamous metaplasia are observed in bronchi of the lungs. Roemer *et al.*[10] reported that exposures to FA at 2.7, 8.0, 26.8 mg/m^3 by inhalation for 6 h per day for one or three consecutive days could induce lung cell proliferation of rats, which has a carcinogenic potential. The aim of this study was to apply the method of proteomics to analyze protein changes of lung of rats in response to FA inhalation. Firstly, the 2-DE patterns of lung tissues were obtained by the 2-DE technology. Secondly, protein spots expressed differently in FA treatment group and control group

[1]The research was supported by brainstorm protect and public good fund from the National Science and Technology Ministry of China. (2001BA704B01&2001DIA10001)

Biographical note of the first author: Yu-Hua YANG, female, born in 1970, Ph. D. candidate, assistant researcher, focus on a study of environmental health. Tel: 86-22-84655424. E-mail: anniyang001@sohu.com

0895-3988/2005
CN 11-2816/Q

were examined and statistically analyzed. Finally, these protein spots were isolated and identified by matrix-assisted laser-desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and database searching. Through these studies, we could get insights into the mechanism and function of protein alteration in rat lung induced by FA exposure and find protein markers indicative of early disease, and pave the way for proteome study of environmental pollutions.

MATERIALS AND METHODS

Reagents

Ultrapure electrophoretic reagents were obtained from Amersham Pharmacia. CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), DTT (dithiothreitol) and trypsin were supplied by Amresco. PMSF (phenylmethyl-sulfonylfluorid) was purchased from Sigma. ACN (acetonitrile) and TFA (trifluoroacetic acid) were provided by Fluka. All other chemicals used were of analytical grade.

Animals and Gaseous FA Exposure

Six female Wistar rats weighing 100±5 g were obtained from the 2nd class animal house of Institute of Health and Environmental Medicine. Rats were randomly divided into exposure group (FA exposure) and control group (double distilled water exposure). Room temperature was controlled at 24±1℃. After liquid FA was volatilized (or double distilled water was vaporized spontaneously) in the bottle, the electron-time controlled gas-sample-pumper pumped the gas into the glass chamber (216 L). There was a micro-fan to mix the gas on top of the chamber. Z-300 detective instrument was used to determine the concentration of FA in the chamber. Inhalation exposure was carried through in dynamic mode. In exposure group gaseous FA concentration was controlled at 32-37 mg/m^3. Rats were exposed to gaseous FA or water vapor for 4 h/day for 15 days. After 15 days experimental rats were fasted for 24 h and then sacrificed. Upper lobe of left lung was obtained immediately and preserved at -80℃.

Sample Preparation

Lung tissue was homogenized in 2 mL lysis buffer containing 8 mol·L urea, 4% CHAPS, 40 mmol·L Tris, 65 mmol·L DTT and 1 mmol·L PMSF. The sample was centrifuged for 20 min at 12 000 rpm after being kept for 1 h at room temperature. Finally samples were stored in aliquots at -80℃. Protein determination was carried out according to Bradford.

Two-dimensional Electrophoresis

First dimension-isoelectric focusing of immobilized pH gradients One milligram of protein sample, 7 μL of DTT(1 mol·L) and 1.75 μL of IPG buffer (20 mol·L) were solubilised in a rehydration solution containing 8 mol·L urea, 2% CHAPS, trace of bromophenol blue and the total volume was 350 μL. This protein solution was pipetted into each strip holder. The 18 cm of IPG strip with a linear pH gradient between 3 and 10 was placed with the gel side down. The cover was placed on the strip holder after IPG cover fluid was applied. The strip holder was positioned on the IPGphor^{TM} isoelectric focusing system (Amersham Pharmacia). Rehydration and isoelectric focusing (IEF) were carried out at 20℃. The current limit of per IPG strip was 50 μA. IEF was stopped up to a total amount of 70 100 V per h.

Second dimension—SDS-PAGE of vertical slab After IEF, the strips were equilibrated in the equilibration solution containing 50 mmol·L Tris-HCl, 6 mol·L urea，30% glycerol, 2% SDS and 1% DTT for 15 min, and then in the same solution with 2.5% iodoacetamide instead of 1% DTT for an additional 15 min.

After equilibration, vertical second dimension separation was performed on 180 mm×180 mm×1 mm and 13% homogeneous SDS-polyacrylamide gels. Therefore, the strips were placed on top of the gels and low molecular weight protein markers were next to one end of the IPG strips. The strips and markers were sealed using 0.5% agarose sealing solution. Electrophoresis buffer contained 25 mmol·L Tris base, 192 mmol·L glycine and 0.1% SDS. The temperature of circulation water was 16℃. Electrophoresis parameter of a strip was 20 mA×40 min+30 mA×5 h. Electrophoresis was stopped when the bromphenol blue front was 1 mm from bottom of the gel. Coomassie brilliant blue R-250 staining was adopted for the gels.

Image Analysis

Stained gels were scanned using an imagescanner, and images were processed using an ImageMaster2D Elite (Version 3.01) software. After spot detection and boundary average background subtraction, the gels were matched. For comparison, volumes of the protein spots were standardized. Differential analysis was performed according to the change of spot volume. Student's *t*-test was used to analyse all the spots that differed significantly between the control and exposure groups (P<0.05).

In-gel Digestion and Protein Identification by MALDI-TOF-MS

Protein spots were excised from 2-D gels, cut into 1 mm^3 and destained by washing in 100 μL solution containing 50% ACN and 25 mmol·L ammonium bicarbonate. The samples were then dried in a centrifugal evaporator for 20 min. Five microliters of trypsin solution (0.01 μg/μL containing 25 mmol·L ammonium bicarbonate) was added to the gel pieces and placed for 20 min at 4℃ before incubation overnight at 37℃. Peptides were extracted by the addition of 40 μL of 5% TFA and then by the addition of 40 μL of 2.5% TFA and 50% ACN. The two extraction volumes were incorporated and MALDI-TOF-MS was performed.

Database Searching

Peptide mass fingerprinting from MALDI-TOF-MS was used to search the NCBInr protein database using Mascot searching tool on MOWSE[11]. Searching was performed using missed cleavage site of 1 and peptide mass tolerance of at most ±0.5 Da. Variable modifications were considered carbamidomethyl and/or oxidation. Taxonomy was rattus and protein scores greater than 57 were significant(P<0.05).

RESULTS

After separation of 1 mg protein sample, the 2-D patterns obtained are shown in Fig.1. About 700 protein spots could be visualized in the range of pH 3-10 and Mr 14400-94000, and their normalization volumes were compared statistically. Of these proteins, 4 were significantly altered (P<0.05) and distribution of these spots in the gel is shown in Fig. 1b.

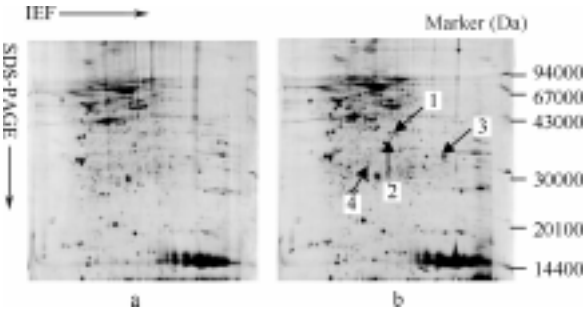

FIG. 1. Comparison of the 2-DE protein patterns of control group (a) and FA-treatment group of 32-37 mg/m^3 (b). IEF was carried out in the range of pH 3-10 (linear) as the first dimension, and the second dimensional vertical SDS-PAGE concentration was 13% constant. Distribution of the spots altered significantly is indicated.

The spot volumes of altered proteins are shown in Fig. 2. In comparison to control group, spots 1, 2, and 3 were up-regulated significantly, and only spot 4 was down-regulated significantly in 32-37 mg/m^3 group (both P<0.05).

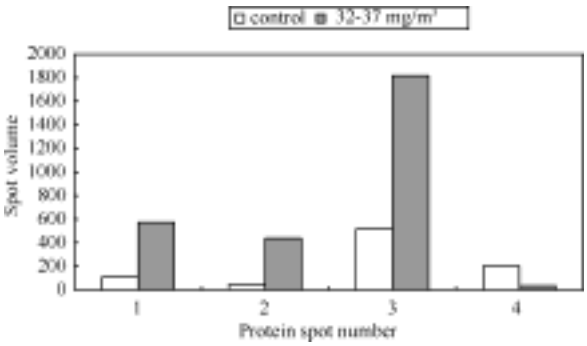

FIG. 2. Difference in spot volumes between control group and FA-treated group. Bars, mean spot volume (n=3, P<0.05).

Peptide mass fingerprints (PMFs) of the significantly altered proteins were obtained by MALDI-TOF-MS (Fig. 3, only protein spot 1 is shown as an example). By searching the NCBInr protein database, in conjunction with estimates of protein Mr and pI from the gel image, these protein spots were identified successfully (Table 1). Protein spot 1 was identified as aldose reductase, protein spot 2 as LIM protein, and protein spot 3 as glyceraldehyde-3-phosphate dehydrogenase. These three proteins were significantly up-regulated at FA concentration of 32-37 mg/m^3. Protein spot 4 significantly down-regulated in 32-37 mg/m^3 FA group was recognized by the computer software as chloride intracellular channel 3 (CLIC3).

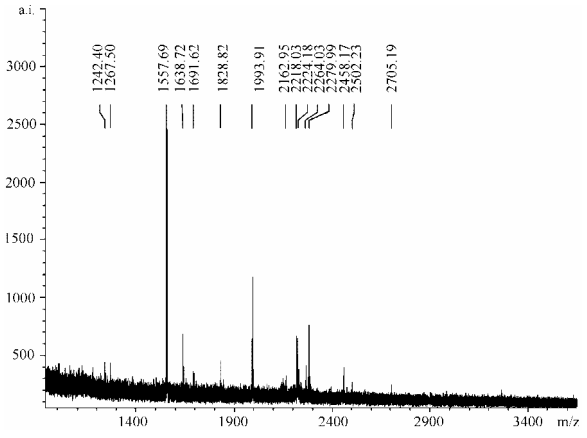

FIG. 3. Peptide mass fingerprinting of protein spot 1 significantly up-regulated in 32-37 mg/m^3 FA group (P<0.05).

TABLE 1

Searching Results of Protein Spots

Number	Score	Mr	pI	Protein
1	66	35774	6.26	Aldose Reductase
2	67	35503	6.56	LIM Protein
3	66	35805	8.14	Glyceraldehyde-3-phosphate Dehydrogenase
4	134	26760	5.61	Similar to Chloride Intracellular Channel 3

DISCUSSION

The expression of four proteins in rat lung tissue was altered after FA inhalation exposure. These proteins include a protein related to free radicals, a domain protein, a glycolytic protein and an ion channel protein. They could be implicated in the process of lung damage induced by FA or might have protected lung cells against damage.

Aldose reductase (AR) is a member of the aldo-keto reductase superfamily. Srivastava *et al*.[12] found that AR could deoxidize 4-hydroxynonenal (HNE) and the glutathione adduct of HNE (HNE-GS), thus they considered that HNE and HNE-GS are the best natural substrates of aldose reductase known so far. HNE is one of the most abundant and toxic lipid aldehydes generated during the process of degradation of lipid peroxides. Rittener *et al*.[13] demonstrated that inhibition of AR increases 2-fold of HNE-GS adducts and 3-fold of the number of apoptotic cells, suggesting that AR is one of the important members involved in oxidative defense mechanism and has a tissue-protective function by eliminating lipid peroxidation products. Rats exposed to FA could produce oxidative stress because of the formation of reactive oxygen species (ROS) and the reduction of anti-oxidative ability[14-15]. Oxidative stress can initiate damage of lipid peroxidation. Therefore we consider that AR significantly up-regulated at the FA-concentration of 32-37 mg/m^3 can reduce oxidative damage of rat lung by oxidative stress.

LIM (Lin-11, Isl-1 and Mec-3 gene) protein contains one or more LIM domains which are evolutionarily conserved double-zinc finger motifs. LIM protein is capable of interacting with many types of proteins including structural protein, kinase, and several classes of transcription factors. These interactions play important roles in a variety of fundamental biological processes including cell differentiation[16], organ development[17], oncogenesis[18-19] and cytoskeleton organization[20-21], *etc*. Because FA could cause cell proliferation in rat lung[10], and LIM protein exerts effect on oncogenesis, we consider the effect of LIM protein significantly up-regulated in 32-37 mg/m^3 FA group might be associated with cell proliferation.

Glyceraldehyde-3-phosphate dehydrogenase (G-APDH) is a classical glycolytic protein and closely related to energy production. It is also used as a model protein for analysis of protein structure and enzyme mechanisms. Recent evidence indicates that GAPDH has new, intriguing function including its role in DNA replication, DNA repair and endocytosis[22-23]. Some other investigations suggest that GAPDH is involved in apoptosis[24-25]. FA inhaled into rat lung is oxidized to formate mostly by formaldehyde dehydrogenase and excessive FA is oxidized to O_2^- and formate by cytosolic xanthine oxidase. Under normal conditions cytosolic xanthine oxidase exists in the form of xanthine dehydrogenase. Under particular conditions, e.g., the existence of a great deal of aldehydes, xanthine dehydrogenase can be changed into xanthine oxidase and oxidize aldehydes, producing. O_2^- There is a subsequent dismutation of O_2^- to H_2O_2, and the latter, in combination with Fe^{2+}, can produce highly reactive hydroxyl radicals，resulting in oxidative damage to rat lung. Because GAPDH is significantly up-regulated in rat lung exposed to 32-37 mg/m^3 FA, we speculate that GAPDH not only can improve energy supply but also may reduce xanthine oxidase from xanthine dehydrogenase, lower the production of ROS, alleviate oxidative damage of ROS to rat lung. Thereby we suggest that GAPDH can serve as an antioxidant defensive factor.

Chloride intracellular channel 3 (CLIC3) has been found to interact with extracellular signal-regulated kinase 7(ERK7)[26]. CLIC3 is a novel protein that has significant homology to human CLIC1 (NCC27/CLIC1), CLIC2, and bovine kidney chloride channel p64. CLIC3 is localized primarily in nuclei and stimulates chloride ion channel activity. ERK7 is the newest member of the mitogen-activated protein (MAP) kinase family and can function as a negative regulator of cell growth. The specific association of CLIC3 with ERK7 suggests that CLIC3 may play a role in the regulation of cell growth. Therefore in our experiment after rats were exposed to 32-37 mg/m^3 FA, significantly down-regulated CLIC3 could promote cell growth, which is consistent with the report that FA could lead to cell proliferation in rat lung[10].

Using a proteomics approach we separated and identified 4 distinct protein markers indicative of toxicity of FA in lung, and analyzed the functions of these proteins through literature review. However, to understand the precise toxic actions and the

underlying mechanisms of FA's toxicity in lung, further studies on the structure and function of the proteins are needed. The potential toxicity of FA to humans is implied by these proteomics data but must be definitively evaluated by searching for protein markers in urine, plasma, *etc*. Equally important, results of this study show that proteomics is a powerful tool in the study of the toxic mechanisms of environmental pollutants at the level of whole protein, finding protein markers of toxicity effects of pollutants and application to human monitor, searching for diagnostic markers and therapy target in clinical setting. Although proteomics technique is imperfect, especially in resolution and reproducibility of 2-DE, its development is very rapid. With the startup of the International Human Proteome Project on December 15, 2003, this technique is deemed to attract much more attention and get improvement in the future.

REFERENCES

1. Rumchev, K. B., Spickett, J. T., Bulsara, M. K., Phillips, M. R., and Stick, S. M. (2002). Domestic exposure to formaldehyde significantly increases the risk of asthma in young child. *Eur. Respir. J.* **20**(2), 403-408.
2. Speit, G., Schutz, P., and Merk, O. (2000). Induction and repair of formaldehyde-induced DNA-protein crosslinks in repair-deficient human cell lines. *Mutagenesis* **15**(1), 85-90.
3. Bagchi, M., Bagchi, D., and Hassoun, E. A. (1998). Subchronic effects of smokeless tobacco extract (STE) on hepatic lipid peroxidation, DNA Damage, and Excretion of Urinarymetabolites in rats. *Toxicology* **127**(1-3), 29-38.
4. Swenberg, J. A., Barrow, C. S., Boreiko, C. J., Heck, H. D., Levine, R. J., Morgan, K. T., and Starr, T. B. (1983) . Non-linear biological responses to formaldehyde and their implications for carcinogenic risk assessment. *Carcinogenesis* **4**, 945-952.
5. Pennisi, E. (2003). Human genome. Reaching their goal early, sequencing labs celebrate. *Science* **300**(5618), 409.
6. Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., and Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis* **16**, 1090-1094.
7. Li, B. L. (1998). Functional proteomics. *Chemistry of Life* **18**(6), 1-4.
8. Cui, X. L., Lei, L., Han, G., Li, Y. P., Liu, Y. P., and Wang, Y. J. (1996). Distribution and influence to GSH of inhaled formaldehyde in the rat. *Chin. J. Prev. Med.* **30**(3), 186.
9. Ohtsuka, R., Shuto, Y., Fujie, H., Takeda, M., Harada, T., and Itagaki, S. (1997). Response of respiratory epithelium of BN and F344 rats to formaldehyde inhalation. *Exp. Anim.* **46**(4), 279-286.
10. Roemer, E., Anton, H. J., and Kindt, R. (1993). Cell proliferation in the respiratory tract of the rat after acute inhalation of formaldehyde or acrolein. *J. Appl. Toxicol.* **13**(2), 103-107.
11. Perkins, D. N., Pappin, D. J. C., Creasy, D. M., and Cottrell, J. S. (1999). Probability-based protein identification by searching sequence database using mass spectrometry data. *Electrophoresis* **20**, 3551-3567.
12. Srivastava, S., Chandra, A., Bhatnagar, A., Srivastava, S. K., and Ansari, N. H. (1995). Lipid peroxidation product, 4-hydroxynonenal and its conjugate with GSH are excellent substrates of bovine lens aldose reductase. *Biochem. Biophys. Res. Comm.* **217**(3), 741-746.
13. Rittner, H. L., Hafner, V., Klimiuk, P. A., Szweda, L.I., Goronzy, J. J., and Weyand, C. M. (1999). Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. *J. Clin. Invest.* **103**(7), 1007-1013.
14. Adams, J. D. and Klaidman, L. K. (1993). Acrolein-induced oxygen radical formation. *Free radic Biol. Med.* **15**, 193-197.
15. Liu, J., Liu, H. L., Wang, G. X., Yan, Y., Li, R., and Yang, X. (2003). Suppression effect of gasous formaldehyde on superoxide dismutase (SOD) of various organs of mice. *J. Environ Health* **20**(3), 81-83.
16. Pinto, do. O. P., Richter, K., and Carlsson, L. (2002). Hematopoietic progenitor/stem cells immortalized by Lhx2 generate functional hematopoietic cells *in vivo*. *Blood* **99**(11), 3939-3946.
17. Birk, O. S., Casiano, D. E., Wassif, C. A., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang. S., Kreidberg, J. A., Parker, K. L., Porter, F. D., and Westphal, H. (2000). The LIM homeobox gene Lhx9 is essential for mouse gonad formation. *Nature* **403**(6772), 909-913.
18. Visvader, J. E., Venter, D., Hahm, K., Santamaria, M., Sum, E. Y., O'Reilly, L., White, D., Williams, R., Armes, J., and Lindeman, G. J. (2001). The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells *in vitro* and is overexpressed in breast cancer. *Proc. Natl. Acad. Sci. USA* **98**(25), 14452-14457.
19. Sum, E. Y., Peng, B., Yu, X., Chen, J., Byrne, J., Lindeman, G. J., and Visvader, J. E. (2002). The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 and inhibits BRCA1 activity. *J. Biol. Chem.* **277**, 7849-7856.
20. Matsui, S., Matsumoto, S., Adachi, R., Kusui, K., Hirayama, A., Watanabe, H., Ohashi, K., Mizuno, K., Yamaguchi, T., Kasahara, T., and Suzuki, K. (2002). LIM kinase 1 modulates opsonized zymosan-triggered activation of macrophage-like U937 cells. Possible involvement of phosphorylation of cofilin and reorganization of actin cytoskeleton. *J. Biol. Chem.* **277**(1), 544-549.
21. Amano, T., Tanabe, K., Eto, T., Narumiya, S., and Mizuno, K. (2001). LIM-kinase 2 induces formation of stress fibres,focal adhesions and membrane blebs,dependent on its activation by Rho-associated kinase-catalysed phosphorylation at threonine-505. *Biochem. J.* **354**, 149-159.
22. Sirover, M. A. (1996). Emerging new functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. *Life Sci.* **58**(25), 2271-2277.
23. Sirover, M. A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim. Biophys. Acta* **1943**(2), 159-184.
24. Ishitani, R. and Chuang, D. M. (1996). Glyceraldehyde-3-phosphate dehydrogenase antisense oligodeoxynucleotides protect against cytosine arabinonucleoside-induced apoptosis in cultured cerebellar neurons. *Proc. Natl. Acad. Sci. USA* **93**(18), 9937-9941.
25. Tajima, H., Tsuchiya, K., Yamada, M., Kondo, K., Katsube, N., and Ishitani, R. (1999). Over-expression of GAPAD induces apoptosis in COS-7 cells transfected with cloned GAPAD cDNAs. *Neuroreport* **10**(10), 2029-2033.
26. Qian, Z., Okuhara, D., Abe, M. K., and Rosner, M. R. (1999). Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *J. Biol. Chem.* **274**(3), 1621-1627.

(*Received May* 12, 2004 *Accepted November* 26, 2004)