— **Regular Article** —

Proteomic Analysis of Bronchoalveolar Lavage Fluid Obtained from Rats Exposed to Formaldehyde

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(Received October 12, 2009; Accepted January 13, 2010)

Formaldehyde is an industrial chemical used in the manufacture of building materials and household products, and is one of the major pollutants that cause "sick building syndrome." Formaldehyde causes a wide range of toxic effects such as the formation of DNA-protein cross-links, activation of the immune system, and sensory irritation. In the present study, we investigated the effects of formaldehyde inhalation on protein levels in rat bronchoalveolar lavage fluid with a proteomics approach. Using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight mass spectroscopy, we identified aldo-keto reductase 1B10 as a possible biomarker for formaldehyde inhalation. This study may contribute to a better understanding of the mechanisms involved in the pathogenesis of formaldehyde exposure.

Key words —— formaldehyde, bronchoalveolar lavage fluid, two-dimensional gel electrophoresis, aldo-keto reductase

INTRODUCTION

Formaldehyde is an industrial chemical used in the manufacture of building materials and household products, including textiles, paper products, resins, wood composites, insulating materials, paints, plastics, fabrics, adhesives, and cosmetics.[1,2] It is also found in automobile emissions and tobacco smoke.[3,4] However, formaldehyde emitted from furniture and internal walls affects the eyes, nose and respiratory organs, and causes allergies, and these effects collectively comprise what is commonly known as "sick building syndrome."[5]

In 1995, the International Agency for Research on Cancer (IARC) concluded that formaldehyde is a human carcinogen on the basis of substantial evidence obtained from studies in humans and experimental animals.[6] Formaldehyde is a highly water-soluble gas which, when inhaled, reacts rapidly at the site of contact and is quickly metabolized by enzymes in the respiratory tissue. Inhaled formaldehyde is absorbed in the respiratory tract resulting in formaldehyde-induced toxicity at distant sites.[7] Formaldehyde has cytotoxic, hematotoxic, immunotoxic, and genotoxic properties[8] and is a well-known cross-linking agent, which reacts with cellular macromolecules such as proteins and nucleic acids.[9,10] Moreover, chronic formaldehyde exposure is linked to the incidence of cancer, teratogenicity, and a variety of neurodegenerative and vascular disorders.[11–13]

The collection and analysis of bronchoalveolar lavage fluid (BALF) has become an established technique to study the cellular and soluble components of the lower respiratory tract. The protein pattern of BALF is altered in different lung pathological conditions, and the analysis of BALF proteins is very important in the search for specific disease markers.[14,15] A comparison between serum and BALF proteomes revealed that a certain number of proteins are characterized by their higher prevalence in BALF than in plasma, suggesting that they are specifically produced in the airways. Therefore, the differential expression of these proteins could

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provide useful information for diagnosing lung injuries, and these proteins are also good candidates for lung-specific biomarkers.[16)]

In this study, we investigated changes in rat BALF proteins following exposure to formaldehyde. Using high-resolution two-dimensional gel electrophoresis (2-DE), we identified aldo-keto reductase (AKR) as a possible biomarker for formaldehyde exposure in BALF. To verify this possibility, we used A549 human lung adenocarcinoma epithelial cells as an *in vitro* model. Our results show that formaldehyde induces the release of AKR1B10 (AKR family 1, member B10) in the culture medium of these cells, suggesting that formaldehyde-induced cell death may correlate with AKR1B10 release. Our findings provide useful information to evaluate the exposure of lungs to formaldehyde.

MATERIALS AND METHODS

Materials —— (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl) tetrazolium bromide (MTT), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), formic acid, hydrogen peroxide (H_2O_2), formaldehyde, glutaraldehyde, crotonaldehyde, and 3-[(3-cholamidopropy) dimethyl-ammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All equipment for iso-electric focusing (IEF) and SDS-polyacrylamide gel electrophoresis (PAGE) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Porcine trypsin was obtained from Promega (Promega-Catalys, Wallisellen, Switzerland) and 5-kDa cutoff Centricon filters were from Millipore (Bedford, MA, U.S.A.). Antibodies to AKR1B10 were purchased from Abnova Coporation (Pforzheim, Germany). All other chemicals were of the highest purity available from commercial sources.

Formaldehyde Inhalation —— Specific pathogen-free, male Sprague-Dawley rats were obtained from the Samtaco Animal Breeding Company (Osan, South Korea), and housed under standard laboratory conditions. Formaldehyde exposures were carried out for 2 weeks in 1 m^3 stainless steel and glass inhalation chambers. Formaldehyde exposure groups (2 and 20 ppm) and an unexposed control group were used in this study, with four rats in each group. Rats inhaled formaldehyde for 6 hr a day for two weeks in an inhalation chamber. Formaldehyde concentrations were generated using a Permeater PD-1B gas generator (Gastec, Kanagawa, Japan) and adjusted by altering the humidified airflow in a second mixing chamber. The formaldehyde in the chamber was actively sampled with a 2,4-dinitrophenyl-hydrazone-silica gel cartridge (Sep-Pak XPoSure Aldehyde Sampler, Waters, Milford, MA, U.S.A.). The aldehydes absorbed on the silica gel were extracted and analyzed by high performance liquid chromatography (HPLC, Shimadzu LC-10 AD, Kyoto, Japan). The actual mean formaldehyde concentration achieved over the 2 weeks were 2.05 ± 0.01 and 20.04 ± 0.02 ppm. Rats were sacrificed and BALF samples were collected for proteomic analysis.

Preparation of BALF Samples —— All procedures were performed in accordance with the guidelines for care and use of laboratory animals approved by Chung-Ang University's Institutional Animal Care and Use Committee. Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg). For each animal, the pleural cavity was opened, the tracheas of rats were cannulated with a polyethylene tube (1.14 mm inner diameter; Becton Dickinson, Spark, MD, U.S.A.), and the lungs were washed twice with phosphate-buffered saline (PBS; pH 7.4, 20 ml). The recovered BALFs were centrifuged at 200 × *g* for 10 min, and the resulting supernatant was collected for analysis. BALF proteins were precipitated using 10% trichloroacetic acid (TCA). The protein pellet was dissolved in sample lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (vol/vol) Pharmalyte, and 1 mM benzamidine]. After centrifugation at 15000 × *g* for 1 hr at 15°C, insoluble materials were discarded and the supernatant was recovered as solubilized proteins for 2-DE.

2-DE —— Immobilized pH gradient dry strips were rehydrated for 12 hr in 7 M urea, 2 M thiourea containing 2% CHAPS, 1% DTT, and 1% Pharmalyte. We loaded 200 μg of solubilized protein on each strip. IEF was performed at 20°C using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instruction. Prior to the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS, and 30% glycerol), initially with 1% DTT and then with 2.5% iodoacetamide. The second-dimensional separation (SDS-PAGE) was carried out on 20 cm × 24 cm Tris-glycine gels (10–16%) using the Hoefer

DALT 2D system (Amersham Biosciences). Staining was performed with silver as described by Oakley *et al.*,[17] but we omitted their glutaraldehyde fixing and sensitization step.

Image Analysis —— Quantitative analysis of digitized images was carried out using PDQuest (version 7.0, Bio-Rad, Hercules, CA, U.S.A.) software according to the protocols provided by the manufacturer. The quantity of each spot was normalized by the total valid spot intensity.

Enzymatic Digestion of In-gel Proteins and Matrix-assisted Laser Desorption/Ionization-time-of-flight Spectrometry Analysis —— Protein spots were cut out from silver-stained gels and were enzymatically digested in-gel as previously described using a modified porcine trypsin.[18] After concentration, the peptide mixture was desalted using C18 ZipTips (Millipore), and the peptides were eluted in 1–5 µl acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (97%; Sigma-Aldrich) in 50% aqueous acetonitrile, and 1 µl of the mixture was spotted onto a target plate. Spectra were acquired using an Ettan matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Amersham Biosciences) equipped with delayed ion extraction. The search program ProFound, developed at The Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification by peptide mass fingerprinting.

Cell Culture and Sample Preparation —— A549 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 medium with 10% fetal bovine serum and 100 U/ml each of penicillin and streptomycin (Gibco, Grand Island, NY, U.S.A.). Cultures were maintained in an incubator at 37°C in an atmosphere containing 5% CO_2. Cells were exposed to formaldehyde or other chemicals in serum free RPMI 1640. Cell culture media were precipitated with a final concentration of 10% TCA and the protein pellets were solubilized in Laemmli sample buffer.[19]

Western Blot Analysis —— Cells were washed with PBS and lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitors). Cell debris was removed by centrifugation at $2000 \times g$ for 10 min. Protein concentration was determined by the Bradford assay.[20] A549 cell lysates and TCA precipitates from medium were separated by SDS-PAGE (12% gel) and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated for 6 hr with mouse anti-AKR1B10 monoclonal antibody (1 : 1000). After three washes, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1 : 3000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 2 hr. Proteins were detected with a pre-formulated substrate kit for use with nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) chromogens (1-Step™ NBT/BCIP, Pierce, Rockford, IL, U.S.A.).

Cell Viability Assay —— Cell viability was determined with the MTT assay. At the end of the treatment procedure, MTT stock solution was added to each culture well and the cells were incubated at 37°C for 2 hr. After the medium was carefully removed, the precipitated formazan was dissolved in dimethyl sulfoxide. Cell viability was determined by measuring the absorbance at 570 nm using a Genius Pro EIA plate reader (Tecan Group Ltd., Maennedorf, Switzerland).

RESULTS

2-DE Analysis of BALF Obtained from Rats Exposed to Formaldehyde

To investigate the change in rat BALF proteins associated with formaldehyde inhalation, the animals were exposed to 0, 2, and 20 ppm formaldehyde. Rat BALF was obtained after 2 weeks of exposure. It was concentrated using a 5-kDa cut-off Centricon filter as it contained a low level of proteins, a high salt concentration, and the over-representation of proteins such as albumin and immunoglobulins. Then equal amounts of concentrated samples from the two formaldehyde exposure groups (2 and 20 ppm) and the control group were subjected to 2-DE. Figure 1 shows the 2-DE patterns of BALF from each group exposed to formaldehyde.

After silver staining and computer image analysis, approximately 910 spots were detected. The analysis of BALF protein patterns focused on the protein spots that were up-regulated or down-regulated in the formaldehyde exposure groups as compared to the control group. A total of 11 protein spots—6 up-regulated (Fig. 2) and 5 down-regulated (Fig. 3)—were differentially expressed in the exposed rats. Selected protein spots, indicated by numbers in Fig. 4, were collected and subjected

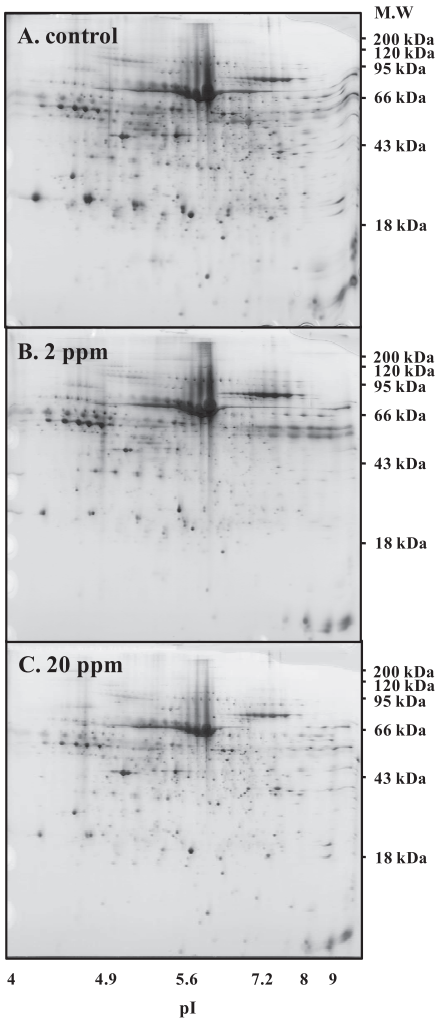

Fig. 1. 2-D Gel Electrophoresis (2-DE) of Pooled BALF from Sprague-Dawley Rats After Formaldehyde Inhalation

The proteins (200 μg) were separated by 2-DE performed on a non-linear pH gradient 4–10 in the first dimension, followed by SDS-PAGE (Tris-glycine 10–16%) in the second dimension. The separated proteins were detected by silver staining. (A) BALF from control, (B) BALF from rats exposed to 2 ppm formaldehyde, (C) BALF from rats exposed to 20 ppm formaldehyde.

to in-gel trypsin digestion and peptide mass fingerprinting by MALDI-TOF. The corresponding protein identities are shown in Table 1.

Formaldehyde-induced Cell Death and the Release of AKR1B10 in A549 Cells

Detoxification of xenobiotic and carcinogenic compounds protect the organism against their toxic or transforming effects. The AKR superfamily is involved in these detoxification processes. The aldose reductase AKR1B is a member of one of the largest families in this superfamily. Human AKR1B10 is a homolog of rat AKR1B8 and is expressed primarily in the small intestine and colon in humans.[21)]

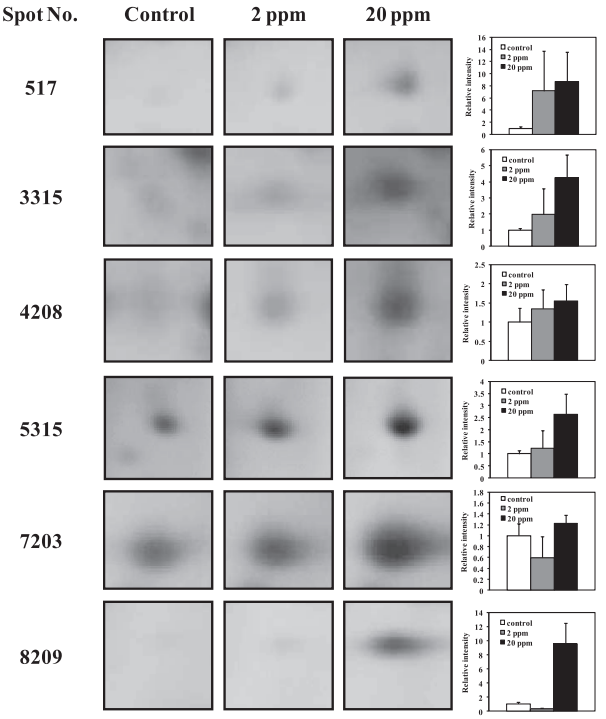

Fig. 2. A Representative 2-DE Image of the Entire Gel of BALF (Control Group) Proteins with Differentially Expressed Spots

The numbered proteins are identified as described in Table 1.

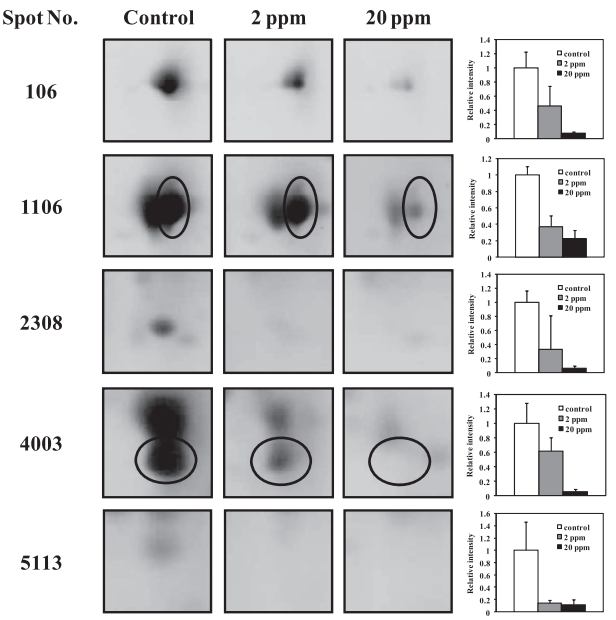

Fig. 3. The Images of Protein Spots Were Analyzed Using PDQuest (Version 7.0, Bio-Rad) Software Program

The 2-DE image demonstrated the BALF proteome pattern of unexposed control rats. The images of each up-regulated spot were compared at increased formaldehyde concentrations. Spot volumes were calculated by normalized versus total spot volumes. The quantity presented by each spot is expressed as a relative intensity compared with the unexposed control (right panel graph). Four rats were used for each group and one 2-DE gel made from one rat's BALF. Data represent the means ± S.D. (n = 4).

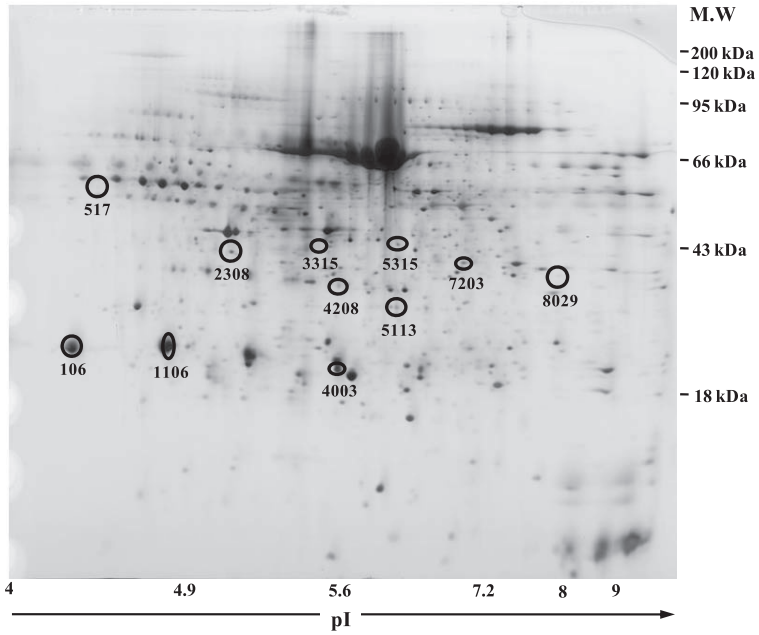

Fig. 4. The Images of Protein Spots Were Analyzed Using PDQuest (Version 7.0, Bio-Rad) Software Program

The 2-DE image demonstrated the BALF proteome pattern of unexposed control rats. The images of each down-regulated spot were compared at increased formaldehyde concentrations. Spot volumes were calculated by normalized versus total spot volumes. The quantity presented by each spot is expressed as a relative intensity compared with the unexposed control (right panel graph). Four rats were used for each group and one 2-DE gel made from one rat's BALF. Data represent the means ± S.D. (n = 4).

Table 1. Up- and Down-regulated Protein Spots in Rat BALF Following Exposure to Formaldehyde

Differentially expressed protein spots were analyzed by MALDI-TOF.

Spot No.	theoretic		up-regulated proteins	coverage	database
	mass (kDa)	isoelectric Point		(%)	accession No.
517	45.65	5.40	SPI-3 serine protease inhibitor (Serpin A3N)	36	GI:57235
3315	42.91	5.60	similar to SPI6 (Serpin B9)	43	GI:56090431
4208	30.43	7.20	preprohaptoglobin	35	GI:204657
5315	42.88	5.90	serine proteinase inhibitor, clade B, member 1A (Serpin B1A)	46	GI:72255515
7203	39.15	7.00	Annexin 1	42	GI:38197394
8209	36.25	7.10	aldo-keto reductase family 1, member B8 (AKR1B8)	54	GI:27465603

Spot No.	theoretic		down-regulated proteins	coverage	database
	mass (kDa)	isoelectric Point		(%)	accession No.
106	27.87	6.20	Palate, lung and nasal epithelium clone (PLUNC)	24	GI:25282405
1106	27.87	6.20	Palate, lung and nasal epithelium clone (PLUNC)	18	GI:25282405
2308	43.00	5.10	unidentified		
4003	27.87	6.20	Palate, lung and nasal epithelium clone (PLUNC)	18	GI:25282405
5113	70.70	6.10	serum albumin precursor	22	GI:124028612

AKR1B10 is highly over-expressed in non-small cell lung carcinoma,[22] suggesting that it may be a diagnostic marker. Thus, AKR1B10 was initially selected to be further characterized.

We investigated the change in the levels of AKR1B10 in the culture medium of A549 cells following exposure to formaldehyde. To establish an optimal concentration of formaldehyde for the release of AKR1B10, A549 cells were incubated with media containing formaldehyde (0, 25, 50, 100, 250, and 500 μM) and cell viability was determined by using the MTT colorimetric assay. Culture media containing released proteins were collected by TCA precipitation and separated by SDS-PAGE. The lev-

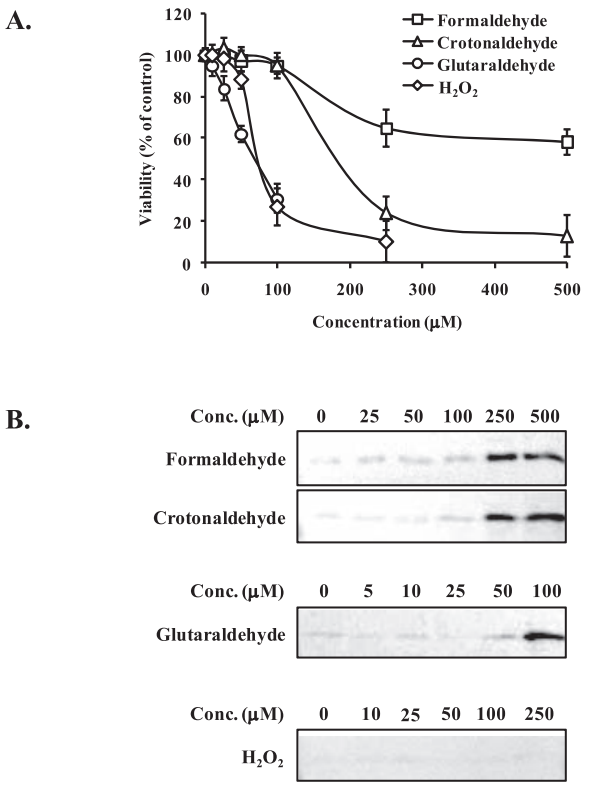

Fig. 5. Analysis of the cytotoxicity and AKR1B10 release after various aldehyde species and H_2O_2 exposure in A549 cells

(A) Acute cytotoxicity of various aldehyde species and H_2O_2 in A549 cells. The cells were incubated with increasing concentration of each chemical in serum-free medium for 24 hr, after which their viability was determined by the MTT assay. Results are expressed as the percent of viable cells compared to the control cells. (B) Cell culture media were precipitated with 10% TCA. Protein pellets were solubilized and separated by SDS-PAGE. Release of AKR1B10 was determined by Western blotting.

els of AKR1B10 in the cell lysate did not change (data not shown), while the levels of AKR1B10 in the culture media were significantly increased at 250 and 500 μM formaldehyde (Fig. 5). We also observed a concomitant increase in formaldehyde-induced cell death.

Crotonaldehyde and glutaraldehyde known as pollutants of the sick building syndrome were used, and cell death induced by both of them was increased in a dose-dependent manner (Fig. 5A). Similar to formaldehyde, the levels of AKR1B10 in cell lysate were not changed by exposure to crotonaldehyde or glutaraldehyde (data not shown), while the levels of this reductase in the culture media were significantly increased (Fig. 5B).

We examined whether cell death caused the release of AKR1B10 into the culture media. We induced cell death by subjecting the cells to H_2O_2 insult and examined the levels of AKR1B10 in the media. As shown in Fig. 5, H_2O_2 did not induce the release of AKR1B10, while, formic acid, a metabolite of formaldehyde, induced cell death, but did not cause the release of AKR1B10 into the media (data not shown).

DISCUSSION

Formaldehyde is a ubiquitous toxic compound classified as a carcinogen by the IARC and is one of the major factors responsible for sick building syndrome. Formaldehyde is a genotoxic agent and increases the number of DNA-protein cross-links in the upper respiratory tract of monkeys[23] and in the nasal mucosa of rats.[24] In repeated inhalation toxicity and chronic inhalation studies in rats, the exposure of formaldehyde up to 20 ppm resulted in increased incidences of nasal squamous cell carcinomas.[25]

BALF contains many proteins, which are either derived from the circulation or are locally released by inflammatory or epithelial cells.[26, 27] The cellular and biochemical factors indicative of alterations in inflammation and lung injury in response to various toxic agents can be detected in BALF; However, it may be difficult to detect and characterize the numerous proteins present in this fluid.

The present study was conducted to identify and quantify altered proteins in BALF following exposure of rats to formaldehyde using high-resolution 2-DE and MALDI-TOF analysis. Eleven protein spots were differentially expressed; six were up-regulated and were identified as the inflammatory proteins serpin A3N, serpin B9, and serpin B1A, the anti-inflammatory protein annexin I, the plasma protein preprohaptoglobin, and the metabolic enzyme AKR1B8.

The serpin superfamily comprises sixteen classes (A–P) of serine protease inhibitors. Each member of the serpin superfamily plays a central role in the regulation of inflammation, coagulation, and fibrinolytic cascades.[28] Serpin A3N is synthesized in the rat liver, and its expression is transiently increased during an acute inflammatory response.[29, 30] Serpin A1 and serpin A3K are secreted and are detected in serum, whereas the secretion of serpin A3N is controversial. The up-regulation of serpins may be associated with lung injury by influencing the inflammatory response.

Annexins, a group of calcium-dependent and

phospholipid-binding proteins consisting of various subtypes, mediate the anti-inflammatory effects of glucocorticoids in a variety of *in vivo* and *in vitro* models of inflammation.[31] Interestingly, significant amounts of annexin I exist in BALF from normal volunteers and patients with lung disease.[32] Moreover, macrophages and alveolar type II epithelial cells synthesize and secrete small amounts of annexin I in the lower respiratory tract.[33,34] In the present study, the levels of annexin I were elevated in BALF in the high exposure group.

Preprohaptoglobin is the primary translation product of haptoglobin. Haptoglobin is a plasma protein synthesized in the liver and sequesters free hemoglobin that may be released into the circulation from erythrocytes as a result of trauma or inflammation.[35] It is suggested that preprohaptoglobin may be up-regulated and is associated with lung injury by influencing the inflammatory response.

The AKR enzyme is a member of the nicotinamide adenine dinucleotide (NAD(P)H)-dependent oxidoreductase superfamily that functionalizes carbonyl groups. AKR1B10 is a member of this superfamily and reduces aromatic and aliphatic aldehyde substrates.[21] In normal tissues, this protein is primarily expressed in the small intestine and colon, with lower levels observed in the liver, thymus, prostate, and testis. Interestingly, this gene was over-expressed in approximately 84.4% of lung squamous cell carcinomas and 29.2% of lung adenocarcinomas in smokers.[21,22] Our results showed an elevated level of AKR1B8 protein in BALF in the high exposure group, suggesting that AKR1B8 could characterize a subset of carcinomas associated with formaldehyde inhalation. AKR1B8 expressed in *Rattus norvegicus* has less than 82% homology with AKR1B10 in *Homo sapiens*.[36] Thus, we examined whether treatment with formaldehyde induces the release of AKR1B10 into the culture medium of A549 cells. We found that a significant amount of AKR1B10 protein was detected in the media following treatment with formaldehyde, but not by H_2O_2 or formic acid. Our results suggest that AKR1B10 may be a biomarker of lung injury due to formaldehyde inhalation.

Five proteins were down-regulated following exposure to formaldehyde: the plasma protein serum albumin precursor, the inflammatory protein palate, lung, and nasal epithelium clone (PLUNC), and an unidentified protein.

Albumin is a major protein detected in BALF. The most abundant proteins in BALF are typical serum proteins. Several lung inflammatory conditions may accumulate and activate immune and inflammatory cells, which may damage the pulmonary tissue, in particular the alveolar-capillary barrier, leading to its dysfunction and to an increase in its permeability to solutes and plasma proteins.[37,38] Another down-regulated protein, PLUNC, is a small and secreted protein, which is expressed in the oropharynx and upper airways of humans, mice, and rats.[39,40] Secreted PLUNC has been detected in bronchial mucus and nasal secretions, and is up-regulated after irritation of the airway.[41,42] According to a recent study, a decrease in the short isoform of PLUNC was detected in smokers when compared with nonsmokers.[43] Although the exact function of this protein is unclear, it is thought to act in the inflammatory response to inhaled irritants such as tobacco smoke. In the present study, the amount of secreted PLUNC was decreased following inhalation of formaldehyde in a dose-dependent manner.

In summary, we examined the changes in BALF protein levels following formaldehyde inhalation. Proteomic analysis using high-resolution 2-DE resolved approximately 910 spots and showed that six proteins were up-regulated and five proteins were down-regulated following exposure to formaldehyde. Among these, AKR1B10 was further confirmed in A549 cells by Western blotting analysis and is suggested to be a candidate biomarker in BALF. The findings of this study contribute to a better understanding of the mechanisms involved in the pathogenesis of formaldehyde inhalation.

Acknowledgement This work was supported by the South Korean Ministry of Environment as "The Eco-Technopia 21 Project."

REFERENCES

1) Kim, W. J., Terada, N., Nomura, T., Takahashi, R., Lee, S. D., Park, J. H. and Konno, A. (2002) Effect of formaldehyde on the expression of adhesion molecules in nasal microvascular endothelial cells: the role of formaldehyde in the pathogenesis of sick building syndrome. *Clin. Exp. Allergy*, **32**, 287–295.
2) Kita, T., Fujimura, M., Myou, S., Ishiura, Y., Abo, M., Katayama, N., Nishitsuji, M., Yoshimi, Y., Nomura, S., Oribe, Y. and Nakao, S. (2003) Potentiation of allergic bronchoconstriction by repeated ex-

posure to formaldehyde in guinea-pigs in vivo. *Clin. Exp. Allergy*, **33**, 1747–1753.

3) Alexandersson, R. and Hedenstierna, G. (1988) Respiratory hazards associated with exposure to formaldehyde and solvents in acid-curing paints. *Arch. Environ. Health*, **43**, 222–227.
4) Godish, T. (1989) Formaldehyde exposures from tobacco smoke: a review. *Am. J. Public Health*, **79**, 1044–1045.
5) Im, H., Oh, E., Mun, J., Khim, J. Y., Lee, E., Kang, H. S., Kim, E., Kim, H., Won, N. H., Kim, Y. H., Jung, W. W. and Sul, D. (2006) Evaluation of toxicological monitoring markers using proteomic analysis in rats exposed to formaldehyde. *J. Proteome Res.*, **6**, 1354–1366.
6) The International Agency for Research on Cancer (IARC) (1995) Wood dust and formaldehyde. *IARC. Monogr. Eval. Carcinog. Risks. Hum.*, **62**, 217–375.
7) Medinsky, M. A. and Bond, J. A. (2001) Sites and mechanisms for uptake of gases and vapors in the respiratory tract. *Toxicology*, **160**, 165–172.
8) Sul, D., Kim, H., Oh, E., Phark, S., Cho, E., Choi, S., Kang, H. S., Kim, E. M., Hwang, K. W. and Jung, W. W. (2007) Gene expression profiling in lung tissues from rats exposed to formaldehyde. *Arch. Toxicol.*, **81**, 589–597.
9) Liteplo, R. G. and Meek, M. E. (2003) Inhaled formaldehyde: exposure estimation, hazard characterization, and exposure-response analysis. *J. Toxicol. Environ. Health B Crit. Rev.*, **6**, 85–114.
10) Metz, B., Kersten, G. F., Hoogerhout, P., Brugghe, H. F., Timmermans, H. A., de Jong, A., Meiring, H., ten Hove, J., Hennink, W. E., Crommelin, D. J. and Jiskoot, W. (2004) Identification of formaldehyde-induced modifications in proteins: reactions with model peptides. *J. Biol. Chem.*, **279**, 6235–6243.
11) Kilburn, K. H. (1994) Neurobehavioral impairment and seizures from formaldehyde. *Arch. Environ. Health*, **49**, 37–44.
12) Sakanashi, T. M., Rogers, J. M., Fu, S. S., Connelly, L. E. and Keen, C. L. (1996) Influence of maternal folate status on the developmental toxicity of methanol in the CD-1 mouse. *Teratology*, **54**, 198–206.
13) Yu, P. H., Wright, S., Fan, E. H., Lun, Z. R. and Gubisne-Harberle, D. (2003) Physiological and pathological implications of semicarbazide-sensitive amine oxidase. *Biochim. Biophys. Acta*, **1647**, 193–199.
14) Noël-Georis, I., Bernard, A., Falmagne, P. and Wattiez, R. (2001) Proteomics as the tool to search for lung disease marker in bronchoalveolar lavage. *Dis. Markers*, **17**, 271–284.
15) Plymoth, A., Löfdahl, C. G., Ekberg-Jansson, A., Dahlbäck, M., Lindberg, H., Fehniger, T. E. and Marko-Varga, G. (2003) Human bronchoalveolar lavage: biofluid analysis with special emphasis on sample preparation. *Proteomics*, **3**, 962–972.
16) Noël-Georis, I., Bernard, A., Falmagne, P. and Wattiez, R. (2002) Database of bronchoalveolar lavage fluid proteins. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **771**, 221–236.
17) Oakley, B. R., Kirsch, D. R. and Morris, N. R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.*, **105**, 361–363.
18) Shevchenko, A. and Shevchenko, A. (2001) Evaluation of the efficiency of in-gel digestion of proteins by peptide isotopic labeling and MALDI mass spectrometry. *Anal Biochem.*, **296**, 279–283.
19) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 680–685.
20) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
21) Cao, D., Fan, S. T. and Chung, S. S. (1998) Identification and characterization of a novel human aldose reductase-like gene. *J. Biol. Chem.*, **273**, 11429–11435.
22) Fukumoto, S., Yamauchi, N., Moriguchi, H., Hippo, Y., Watanabe, A., Shibahara, J., Taniguchi, H., Ishikawa, S., Ito, H., Yamamoto, S., Iwanari, H., Hironaka, M., Ishikawa, Y., Niki, T., Sohara, Y., Kodama, T., Nishimura, M., Fukayama, M., Dosaka-Akita, H. and Aburatani, H. (2005) Overexpression of the aldo-keto reductase family protein AKR1B10 is highly correlated with smoker's non-small cell lung carcinomas. *Clin. Cancer Res.*, **11**, 1776–1785.
23) Casanova, M., Morgan, K. T., Steinhagen, W. H., Everitt, J. I., Popp, J. A. and Heck, H. D. (1991) Covalent binding of inhaled formaldehyde to DNA in the respiratory tract of Rhesus monkeys: pharmacokinetics, rat-to-monkey interspecies scaling, and extrapolation to man. *Fundam. Appl. Toxicol.*, **17**, 409–428.
24) Casanova, M., Morgan, K. T., Gross, E. A., Moss, O. R. and Heck, H. A. (1994) DNA-protein cross-links and cell replications at specific sites in the nose of F344 rats exposed subchronically to formaldehyde. *Fundam. Appl. Toxicol.*, **23**, 525–536.
25) Kerns, W. D., Pavkov, K. L., Donofrio, D. J., Gralla, E. J. and Swenberg, J. A. (1983) Carcinogenicity of formaldehyde in rats and mice after long-term in-

halation exposure. *Cancer Res.*, **43**, 4382–4392.
26) Winterbauer, R. H., Lammert, J., Selland, M., Wu, R., Corley, D. and Springmeyer, S. C. (1993) Bronchoalveolar lavage cell populations in the diagnosis of sarcoidosis. *Chest*, **104**, 352–361.
27) Bell, D. Y., Haseman, J. A., Spock, A., McLennan, G. and Hook, G. E. (1981) Plasma proteins of the bronchoalveolar surface of the lungs of smokers and nonsmokers. *Am. Rev. Respir. Dis.*, **124**, 72–79.
28) Gooptu, B. and Lomas, D. A. (2008) Polymers and inflammation: disease mechanisms of the serpinopathies. *J. Exp. Med.*, **205**, 1529–1534.
29) Hill, R. E. and Hastie, N. D. (1987) Accelerated evolution in the reactive centre regions of serine protease inhibitors. *Nature*, **326**, 96–99.
30) Baumann, H., Hill, R. E., Sauder, D. N. and Jahreis, G. P. (1986) Regulation of major acute-phase plasma proteins by hepatocyte-stimulating factors of human squamous carcinoma cells. *J. Cell Biol.*, **102**, 370–383.
31) Yang, Y. H., Morand, E. F., Getting, S. J., Paul-Clark, M., Liu, D. L., Yona, S., Hannon, R., Buckingham, J. C., Perretti, M. and Flower, R. J. (2004) Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis Rheum.*, **50**, 976–984.
32) Ambrose, M. P. and Hunninghake, G. W. (1990) Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. *J. Appl. Physiol.*, **68**, 1668–1671.
33) Ambrose, M. P. and Hunninghake, G. W. (1990) Corticosteroids increase lipocortin I in alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **33**, 349–353.
34) Ambrose, M. P., Bahns, C. L. and Hunninghake, G. W. (1992) Lipocortin I production by human alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.*, **6**, 17–21.
35) Hanley, J. M., Haugen, T. H. and Heath, E. C. (1983) Biosynthesis and processing of rat haptoglobin. *J. Biol. Chem.*, **25**, 7858–7869.
36) Hyndman, D. J. and Flynn, T. G. (1998) Sequence and expression levels in human tissues of a new member of the aldo-keto reductase family. *Biochim. Biophys. Acta*, **1399**, 198–202.
37) Delacroix, D. L., Marchandise, F. X., Francis, C. and Sibille, Y. (1985) Alpha-2-macroglobulin, monomeric and polymeric immunoglobulin A, and immunoglobulin M in bronchoalveolar lavage. *Am. Rev. Respir. Dis.*, **132**, 829–835.
38) Valeyre, D., Soler, P., Basset, G., Loiseau, P., Pre, J., Turbie, P., Battesti, J. P. and Georges, R. (1991) Glucose, K^+, and albumin concentrations in the alveolar milieu of normal humans and pulmonary sarcoidosis patients. *Am. Rev. Respir. Dis.*, **143**, 1096–1101.
39) Weston, W. M., LeClair, E. E., Trzyna, W., McHugh, K. M., Nugent, P., Lafferty, C. M., Ma, L., Tuan, R. S. and Greene, R. M. (1999) Differential display identification of plunc, a novel gene expressed in embryonic palate, nasal epithelium, and adult lung. *J. Biol. Chem.*, **274**, 13698–13703.
40) Bingle, C. D. and Bingle, L. (2000) Characterisation of the human plunc gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. *Biochim. Biophys. Acta*, **1493**, 363–367.
41) Sung, Y. K., Moon, C., Yoo, J. Y., Moon, C., Pearse, D., Pevsner, J. and Ronnett, G. V. (2002) Plunc, a member of the secretory gland protein family, is upregulated in nasal respiratory epithelium after olfactory bulbectomy. *J. Biol. Chem.*, **277**, 12762–12769.
42) Lindahl, M., Stahlbom, B. and Tagesson, C. (2001) Identification of a new potential airway irritation marker, palate lung nasal epithelial clone protein, in human nasal lavage fluid with two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight. *Electrophoresis*, **22**, 1795–1800.
43) Steiling, K., Kadar, A. Y., Bergerat, A., Flanigon, J., Sridhar, S., Shah, V., Ahmad, Q. R., Brody, J. S., Lenburg, M. E., Steffen, M. and Spira, A. (2009) Comparison of proteomic and transcriptomic profiles in the bronchial airway epithelium of current and never smokers. *Public Library of Science One*, **4**, e5043.