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B6C3F1 mice exposed to ozone with 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone and/or dibutyl phthalate showed toxicities through alterations of NF-κB, AP-1, Nrf2, and osteopontin

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Toxic effects of ozone, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and/or dibutyl phthalate (DBP) were examined through NF-κB, AP-1, Nrf2, and osteopontin (OPN) in lungs and livers of B6C3F1 mice. Electrophoretic mobility shift assay (EMSA) indicated that mice treated with combination of toxicants induced high NF-κB activities. Expression levels of p105, p65, and p50 proteins increased in all treated mice, whereas IκB activity was inhibited in NNK-, DBP-, and combination-treated ones. All treated mice except ozone-treated one showed high AP-1 binding activities. Expression levels of *c*-fos, *c*-jun, junB, jun D, Nrf2, and OPN proteins increased in all treated mice. Additive interactions were frequently noted from two-toxicant combination mice compared to ozone-treated one. These results indicate treatment of mixture of toxicants increased toxicity through NF-κB, AP-1, Nrf2, and OPN. Our data could be applied to the elucidation of mechanism as well as the risk assessment of mixture-induced toxicity.

Key words: dibutyl phthalate (DBP), inhalation, mixture toxocity, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), Ozone

Introduction

Ozone (O_3) is a major environmental pollutant to which humans are routinely exposed, with over 60 cities of the United States exceeding the National Ambient Air Quality Standard, 0.12 ppm for a daily 1-h average [27,36]. At present, Korean Ambient Air Quality Standards (KAAQS) for ozone is set at 1-h/0.12-ppm and 8-h/0.06-ppm. Laboratory animal and human clinical studies have demonstrated that ozone caused reversible decrement in pulmonary function, increased permeability of the epithelium, influx of inflammatory cells, impaired pulmonary defense capacity, and tissue damage [29]. Several nitrosamines derived from tobacco alkaloids using laboratory animals have revealed to be carcinogenic [18,19]. Among them, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is not only a potent lung carcinogen in rodents, but also a likely causative factor in human lung carcinogenesis [18,19].

A wide range of use has been found for various phthalic acid esters (PAEs), with the largest portion being used as plasticizing agents for poly (vinyl chloride) products [3]. Recently, dibutyl phthalates (DBPs) were reported to be estrogenic in estrogen-responsive human breast cancer cells [14,15,16,17,20,22,24,35].

One of the most ubiquitous eukaryotic transcription factors that regulate the expression of genes involved in controlling cellular proliferation/growth, inflammatory responses, and cell adhesion is nuclear factor kappa-B (NF-κB) [6]. The functionally active NF-κB exists mainly as a heterodimer consisting of subunits of Rel family (*e.g.* Rel A, p65, p50, p52, c-Rel, and Rel B), which is normally sequestered in the cytoplasm as an inactive complex through binding with an inhibitory protein, IκB. Enzymatic phosphorylation of IκB with subsequent ubiquitination upon exposure to various extracellular stimuli causes proteosomes to rapidly degrade the inhibitory subunit. This process leads to the translocation of free NF-κB dimer to the nucleus, where it binds to a specific consensus motif in the promoter or enhancer regions of target genes, thereby regulating their expressions. Another transcription factor that has a central role in controlling the eukaryotic gene expression is activator protein 1 (AP-1). AP-1 is composed of c-jun and c-Fos proteins, which interact via a 'leucine-zipper' domain [1]. As with NF-κB, DNA binding of c-jun/c-Fos heterodimer is regulated in the intracellular redox state. At present, transactivation of AP-1 as well as NF-κB is

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considered to be required for TPA-stimulated cellular proliferation and transformation, and tumor promotion [23]. The transcription factor Nrf2 is a member of the "cap n collar" family, comprising p45-Nfe2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2 [9]. Among the members, p45-Nfe2, Nrf1, and Nrf2 were first cloned during the search for proteins that bind to the NFE2-AP1 motif, the core element on hypersensitive site 2 of the locus control region of the human-globin gene cluster. The NFE2-AP1 motif was later determined to share high sequence homology with the antioxidant response element. Furthermore, evidences revealed Nrf1 and Nrf2 play important roles in the cellular detoxification process. Nrf1 and Nrf2 transactivatiy reporter genes are linked to antioxidant response elements, and their expression sites coincide with those of several phase II detoxifying genes [11]. Nrf2 mediates the induction of detoxifying genes, NAD(P)H quinone oxidoreductase I and glutathione S-transferases (GSTs), by the phenolic antioxidant butylated hydoxyanisole. Nrf2 is essential for the protection against butylated hydroxytoluene-induced pulmonary injury, and regulates the expression of other detoxifying genes such as heme oxygenase I, catalase, superoxide dismutase I, UDP-glucuronosyl-transferase (UGT), and α-6-glutamylcysteine synthetase (GCS).

Osteopontin (OPN), also known as Eta-1 (early T-lymphocyte activation 1), is an arginine-glycine-aspartate (RGB)-containing phosphoprotein. Because the RGB sequence is an integrin-binding motif common to many extracellular matrix (ECM) proteins, OPN has been classified as an ECM protein. Although first identified in 1979, OPN has shown to be an important component of the early cellular immune responses recently [2].

Although the above-mentioned factors are known to be important for maintaining normal cell functions, few *in vivo* studies have been performed to examine their roles in toxicities induced by combination of toxicants. Therefore, to elucidate the basic mechanism of toxicity in the lungs and livers of B6C3F1 mice after 52-wk treatments of NNK and/or DBP on ozone inhalation, changes in the activation of NF-κB family, AP-1 family, Nrf2, and OPN were investigated using EMSA and Western blot.

Materials and Methods

Chemicals

NNK (CAS NO. 64091-91-4) was obtained from Chemsyn Science laboratories (Lenexa, USA), with over 99% purity as revealed through HPLC analysis (data not shown). Trioctanoin, obtained from Wako (Japan), was redistilled before use. DBP (CAS NO. 84-74-2) was obtained from Sigma (St. Louis, MO, USA). Diet containing DBP was freshly prepared every week. A predetermined amount of DBP was added to a small aliquot of ground basal diet, and hand-blended. This premix was then added to a preweighed ground basal diet and blended in a mill for 30 min.

Animals

B6C3F1 mice, 4-5-wk-old, were purchased from Seoul National University (SNU) Laboratory Animal Facility (Seoul, Korea) and were acclimated for about 7 days prior to the initiation of chemical exposure. Food and water were provided *ad libitum* except during the ozone exposure period. Rooms were maintained at 23 ± 2°C, with a relative humidity of 50 ± 20% and a 12-h light/dark cycle. All methods used in this study have been approved by the Animal Care and Use Committee at SNU and conform to the NIH guidelines (NIH publication No.86-23, revised 1985). The experimental groups were as follows: (a) unexposed group (control); (b) group exposed to 0.5 ppm ozone (ozone group); (c) group exposed to 1.0 mg NNK/kg body weight (NNK group); (d) group exposed to 5,000 ppm DBP (DBP group); (e) group exposed to 0.5 ppm ozone + 1.0 mg/kg NNK (ozone + NNK group); (f) group exposed to 0.5 ppm ozone + 5,000 ppm DBP (ozone + DBP group); (g) group exposed to 0.5 ppm ozone + 1.0 mg/kg NNK + 5,000 ppm DBP (three-combination group).

Exposures

Mice were exposed to ozone (0.50 ± 0.02 ppm) 6 h per day (between 9 : 00 AM and 3 : 00 PM), 5 days per wk for 32 or 52 wk in 1.5-m^3 whole-body inhalation exposure chambers (Dusturbo, Korea). Ozone (CAS NO. 10028-15-6), generated from pure oxygen using a silent electric arc discharge ozonator (Model KDA-8, Sam-Il Environment Technology, Korea), was mixed with the main stream of filtered air before entering the exposure chambers. Ozone concentrations in the chambers were monitored through a gas detection system equipped with an O_3 gas sensor (Analytical Technology, USA). O_3 gas sensor probes were placed in the breathing zone of the mice on the middle cage rack. Measurements were taken from 12 locations in each chamber to ensure the uniformity of ozone distribution, which was enhanced through a recirculation device. Airflow in the chambers was maintained at 15 changes per hour. During exposure, wire cages were used to allow visual observation of all individually housed animals. Before and after ozone exposures, the mice were housed five per cage in polycarbonate cages equipped with bottom wire nets. During the test periods, mice were subcutaneously injected with 1.0 mg NNK per kg body weight three times per week. They also received diets containing DBP at a concentration of 5,000 ppm for 52 wk. The concentration of each test material was determined based on the National Toxicology Program (NTP) carcinogenesis study [30,31].

Electophorelic mobility shift assay (EMSA)

Animals were sacrified by cervical dislocation. Lungs and

livers were excised prior to storage at −70°C. They were then placed in 2 ml of hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and homogenized in an ice bath using a Polytron tissuemizer. To the homogenates, 125 ml of 10% Nonidet P-40 solution was added, and the mixture was centrifuged for 30 s at 14,800 × g. The pelleted nuclei were washed once with 400 ml of buffer A and 25 ml of 10% NP-40, centrifuged, resuspended in 50 ml of solution consisting of 50 mM HEPES (pH 7.8), 50 mM KCl, 0.3 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol, mixed for 20 min, and centrifuged for 5 min at 4°C. The supernatant containing nuclear proteins was collected and stored at −70°C after determining the protein concentration. EMSA was performed for NF-κB binding according to the manufacturer's protocol using a gel shift assay system (Promega, USA). Briefly, a 22-bp NF-κB double-strand oligonucleotide (Promega, USA) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick spin column (Pharmacia Biotech, Sweden). The binding reaction was carried out in 25 ml of the mixture containing 5 ml of incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 mg of nuclear extracts, and the labeled probe. After 20 min incubation at room temperature, 2 ml of 0.1% bromophenol blue was added to the mixture, and samples were electrophoresed through a 4% non-denaturing polyacrylamide gel at 350 V. Finally, the gel was dried and exposed to an X-ray film. EMSA for measuring AP-1-DNA binding activity was conducted in the same manner as that applied to the NF-κB-DNA binding assay except for the use of 21 base pairs of AP-1 double-stranded oligonucleotide (Promega, USA).

Western blot for NF-κB family (p105, p65, p50 and IκB-α), AP-1 family (*c*-fos, *c*-jun, jun B, jun D), Nrf-2, and OPN protein levels

All tissues were homogenized with lysis buffer [50 mM Tris at pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% sodium dodecyl sulfate (SDS), 100 μg/mL phenylmethyl-sulfonylfluoride (PMSF), 1 μL/mL of aprotinin, 1% igapel 630 (Sigma, USA), and 0.5% deoxycholate] and centrifuged at 14,000 × g for 30 min. Protein concentration was determined using a Bradford analysis kit (Bio-Rad, USA). Equal amount of proteins were separated on an SDS-12% polyacrylamide gel and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia, USA). The blots were blocked for 2 h at room temperature with a blocking buffer (10% nonfat milk in TTBS buffer containing 0.1% Tween 20). The membranes were incubated for 1 h at room temperature with specific antibodies. Mouse, goat or rabbit antibodies against p105, p65, p50, IκB-α, c-fos, c-jun, jun B, jun D, Nrf-2, actin (Santa Cruz Biotechnology, USA), and OPN were used at dilutions specified by the manufacturer. After washing with TTBS, the membranes were reincubated with anti-mouse, goat or rabbit horseradish peroxidase (HRP)-labeled secondary antibody and visualized using an ECL detection kit (Amersham Pharmacia, USA).

Results

DNA-binding activity of NF-κB

Effects of exposure to various combinations of ozone, NNK, and DBP on DNA-binding activity of NF-κB in the lungs and livers of B6C3F1 mice are shown in Fig. 1. DBP, ozone + NNK, ozone + DBP, and three-combination groups showed higher NF-κB-DNA-binding activity than the other groups. NNK induced higher NF-κB-DNA-binding activity than ozone. A similar pattern of binding activity was observed in the livers of 52-wk exposed mice. Among various groups, DBP, ozone + DBP, and three-combination groups showed high activities (Fig. 1).

Alterations of p105, p65, p50 and IκB-α protein levels

To determine the changes in the expressions of NF-κB family, which may be responsible for toxicity, we analyzed p105, p65, p50, and IκB-α protein expressions in lung and liver tissues by Western blotting. The expression levels of p105, p65, and p50 proteins were highest in all three-combination groups after 52-wk exposures. All combination groups caused the degradation of IκB-α in lungs and livers (Fig. 2).

DNA-binding activity of AP-1

Specific AP-1-binding activity was detected in the lungs

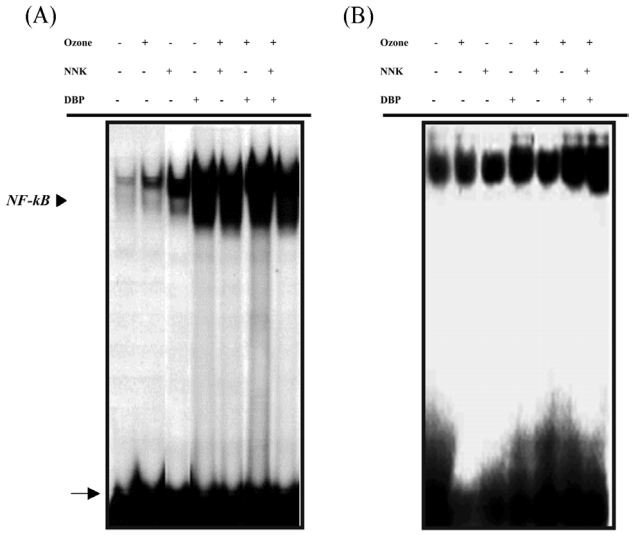

Fig. 1. Representative DNA-binding activity of transcription factor NF-κB. Nuclear extracts of mouse lung (A) and liver (B) exposed to toxicants for 52 wk were subjected to EMSA as described in Materials and Methods.

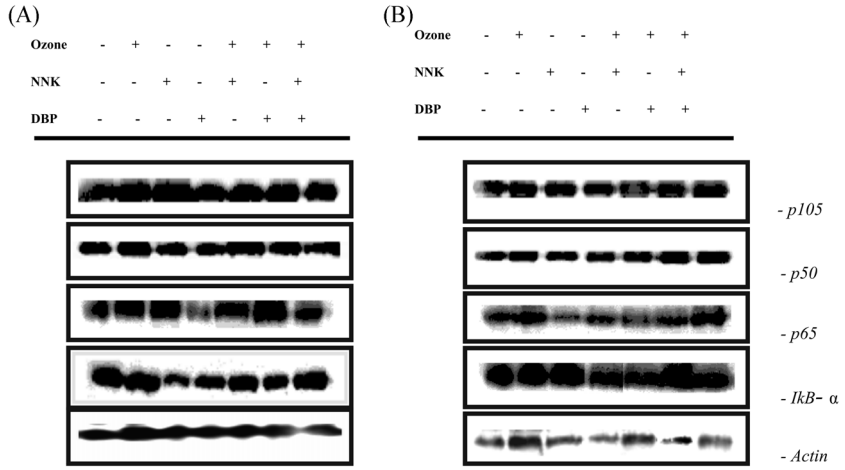

Fig. 2. Expressions of NF-κB family, p105, p50, p65, and IκB in toxicant-exposed lungs (A) and livers (B) for 52 wk. Proteins were isolated and prepared for Western blotting analysis using appropriate primary antibodies and secondary HRP conjugates as described in Materials and Methods.

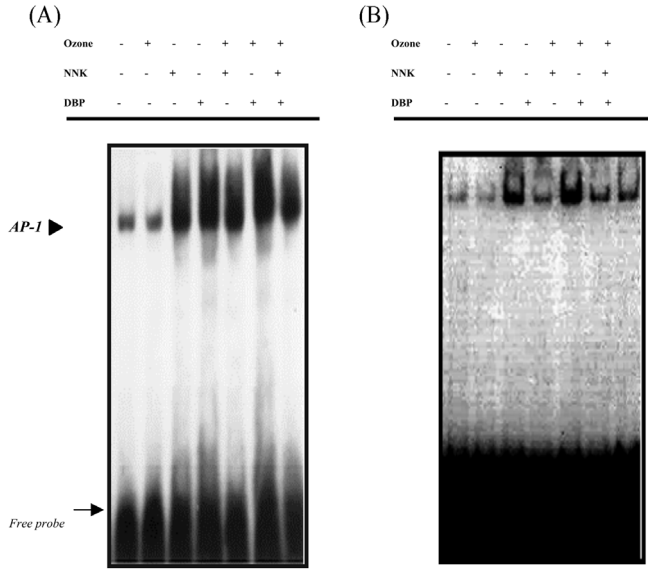

Fig. 3. DNA-binding activity of transcription factor AP-1. Nuclear extracts of lung (A) and liver (B) exposed to toxicants for 32 wk were subjected to EMSA as described in Materials and Methods.

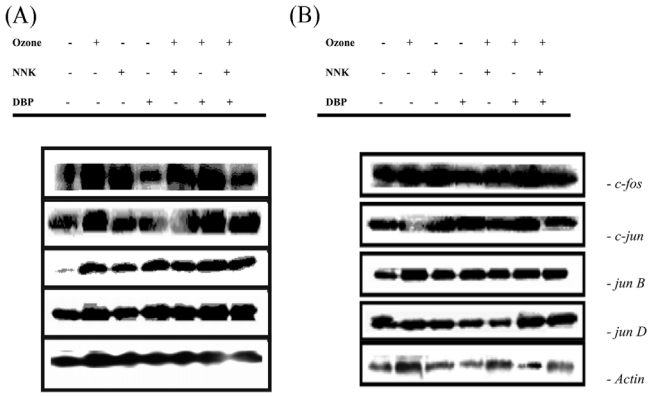

Fig. 4. Expressions of AP-1 family, c-fos, c-jun, jun B, and jun D, in toxicant-exposed lungs (A) and livers (B) for 52 wk. Proteins were isolated and prepared for Western blotting analysis using appropriate primary antibodies and secondary HRP conjugates as described in Materials and Methods.

and livers of 52-wk-exposed NNK, DBP, ozone + NNK, ozone + DBP, and three-combination groups. In particular, pulmonary AP-1 activation showed higher increase in the combination than in the ozone group. Furthermore, hepatic AP-1-DNA-binding activity was highest in the NNK and ozone + NNK groups, whereas exposure to ozone only did not induce high activity (Fig. 3).

Changes in *c*-fos, *c*-jun, jun B, and jun D protein levels

To examine whether changes in the expressions of AP-1 family could be involved with toxicity, *c*-fos, *c*-jun, jun B, and jun D expressions in lung and liver tissues were analyzed by Western blotting. The expression levels of the analyzed proteins increased in all treated lungs and livers. Interestingly, however, treatment with DBP only decreased the *c*-fos expression in both lungs and livers (Fig. 4).

Changes in Nrf-2 and OPN protein levels

In general, the expression levels of Nrf-2 and OPN proteins increased in all treated lungs and livers. However, pulmonary and hepatic Nrf-2 expressions decreased in three-combination group. On the other hand, combined treatment dramatically increased the pulmonary OPN expression. Such pattern was also observed in the livers (Fig. 5).

Discussion

NF-κB is normally maintained in an inactive state in the

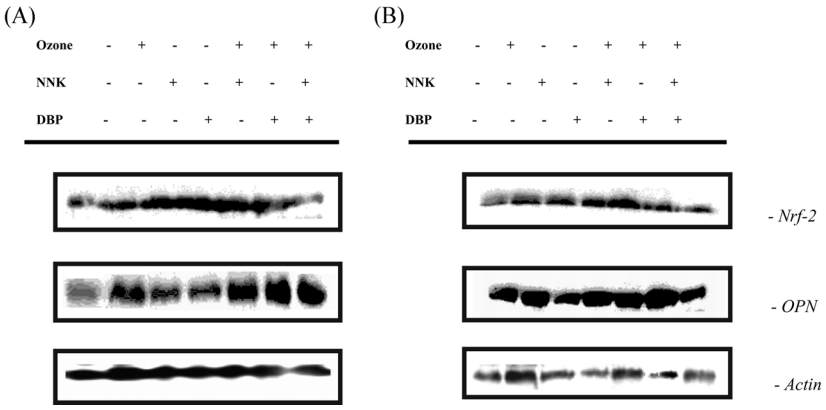

Fig. 5. Expressions of Nrf-2 and OPN of lungs (A) and livers (B) treated for 52 wk. Proteins were isolated and prepared for Western blotting analysis using appropriate primary antibodies and secondary HRP conjugates as described in Materials and Methods.

cytoplasm by the IκB inhibitory proteins that prevent NF-κB from entering into the nucleus to initiate gene transcription [4,5]. Upon stimulation, however, a cascade of reactions is induced, leading to the phosphorylation and ubiquitination of IκB, and eventually to the degradation of IκB by the proteosome. NF-κB is then released and translocated into the nucleus to activate the transcription of various genes. Therefore, to determine the activation mode of NF-κB, DNA-binding activity of NF-κB in the nuclear extracts of lung and liver tissues was analyzed through the gel shift assay using that ^{32}P-labelled NF-κB-specific oligonucleotide. NF-κB is a set of heterogeneous transcription factors [4,5]. Members of the NF-κB/Rel family, including p105, p65, and p50 proteins, form homodimers or heterodimers with other family members, which permit the generation of numerous distinct transcription factors having variable DNA-binding affinities and transactivation activities toward various NF-κB sites [33,37]. Results revealed the expression levels of p105, p65, and p50 proteins increased in all treated lungs and livers, whereas degradation of IκB-α occurred, particularly in combination groups. A number of studies showed that NF-κB was required for the induction and/or maintenance of tumor phenotype [13]. Furthermore, expression of nondegradable mutants of IκB-α and antisense RNA inhibition of NF-κB are known to induce tumor regression [28]. Our results are in well agreement with the above lines of evidences; it could, therefore, be concluded such low degradation of IκB as well as increased expression of NF-κB family were responsible for combination-induced toxicity. In fact, our previous results showed that NNK, ozone + NNK, and three-combination treatments induced pulmonary neoplasm, whereas DBP and three-combination treatments caused oviduct carcinoma in female mice exposed to the toxicants for 52 wk. Incidences of lesions in the tested organs of two- or three-combination groups were higher than that of ozone group (unpublished data). Moreover, we previously showed, through chromosome aberration and supravital micronucleus assays, that additive and/or synergistic responses occurred in both mice sexes exposed to the combination of ozone, NNK, and DBP for 16, 32, and 52 wk compared to single exposure to ozone, NNK or DBP [25,26]. Thus, high mutations and decreased degradation of IκB could be important factors in the combination-induced toxicity [7].

AP-1, a heterodimetric nuclear transcription factor, contains proteins of two major families, jun and cfos, consisting of *c*-jun, jun B, jun D, *c*-fos, fos B, (Fos-related antigen-1) Fra-1, and Fra-2 family members. Many stimuli including tumor promoters regulate binding of AP-1 to the consensus AP-1-binding sequence and stimulate target gene transcription. Moreover, some of these AP-1-regulated gene transcripts may mediate neoplastic formation [23]. In the present study, specific AP-1-binding activity as well as expression of AP-1 family proteins in ozone, DBP, ozone + DBP, ozone + NNK, and three-combination groups were evaluated. Recently, Young *et al.* [38] suggested the possibility of AP-1 as a promising target for cancer chemoprevention, because the dominant negative c-jun can protect against HPV-16 E7-enhanced tumorigenesis in mice. Furthermore, activation of AP-1 family, such as jun-D and jun-B, is known to inhibit apoptosis [21]. Our findings also indicate that activation of AP-1 family can be one of the leading causes of combination-induced toxicity. That is, at initial stage, activation of AP-1 family may induce certain degree of protection against combination-induced toxicity; however, continuous chemical stress over threshold finally induces critical lesions such as genotoxicities, pulmonary cancer as well as oviduct cancer.

Nrf-2 has been shown to transactivate reporter genes linked to the antioxidant response element and plays a role in the induction of phase II detoxifying genes [11]. OPN is a multifunctional protein involved in bone mineralization, cell adhesion, migration, and transformation [12]. It is expressed

in various cells including tumor cells and macrophages. OPN expression has been linked to tumorigenesis and metastasis in several experimental animal models and human cancers [8,32]. Chambers *et al*. [10] reported that OPN was expressed in lung cancer tissues, and revealed a significant association between OPN-immunopositivity of the tumor and patient survival. In our study, the expression levels of Nrf-2 and OPN proteins increased in all treated lungs and livers, strongly suggesting that such transcription factors could be important for chemically induced toxicity. This hypothesis is well supported by recent findings that OPN-integrin interaction is an important step in tumorigenesis and metastasis of cancer cells [13].

In conclusion, altered activation of NF-κB and AP-1, and altered expression of their families in the lungs and livers may be the underlying mechanism of action of toxicants-induced toxicities in this study. Further studies are undergoing to elucidate the relative molecular roles of above important biological factors.

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