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Oxidative Stress in C100 Cells Induced by Combined Treatment of Benzo(*a*)pyrene and/or 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin(TCDD)

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Abstract : When an organism is exposed to various toxicants chronically, reactive oxygen species(ROS) are accumulated and eventually result in several biological effects from gene expression to cell death. In the present study we investigated the oxidative damage of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin(TCDD) and/or benzo(*a*)pyrene (B(*a*)P) in C100 cells. C100 cells treated with TCDD(30 nM) and B(*a*)P(3 μM) underwent diverse oxidative stress as determined through thiobarbituric acid-reactive substances(TBARS) formation, DNA fragmentation, DNA single strand break(SSB) assay, immunohistochemical staining of 8-hydroxy-2′-deoxyguanosine(8-OHdG), and mRNA expressions of antioxidant enzymatic genes such as Cu/Zn-SOD gene, GPx(glutathione peroxidase 5) gene, and catalase gene. Lipid peroxidation in C100 cells was determined through measuing the formation of TBARS. For theat, the cells were pretreated with TCDD(30 nM) and/or B(a)P(3 μM) for 0.5, 1, 2 and 4 days. TBARS formation was increased in TCDD(30 nM) and B(a)P(3 μM) and mixture(30 nM TCDD+3 μM B(a)P) and positive control treatment groups comparing to the controls. Mixture treatment induced more DNA fragmentation than the single treatment group at day 6. Also, SSB in all treatment groups was clearly observed when compared with the negative control group. As with the expression of antioxidant enzyme, GPx 5mRNA, B(*a*)P alone and mixture(30 nM TCDD+3 μM B(a)P) treatment were higher comparing to those of the negative control and TCDD treatment groups. Our results suggest that exposure of C100 cells to mixture of TCDD and B(*a*)P leads to significant oxidative damage comparing to the exposures to the individual chemicals. Mechanisms of action are discussed. Additional studies are needed to elucidate the detailed mechanism of mixture-induced toxicity.

Key words : Benzo(*a*)pyrene, 2,3,7,8-TCDD, C100 cells, mixture toxicity, oxidative stress

Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin(TCDD)and benzo (*a*)pyrene[B(*a*)P)] are environmental toxicants of major public concern. TCDD is one of the most intensively studied chemicals due to its toxic potential and carcinogenicity.

Previous laboratory observations showed that TCDD induced cancer and several other toxic effects including dermal, immune, reproductive, developmental, and endocrine effects in experimental animals [28]. Among the adverse effects induced by TCDD in subcellular levels, reactive oxygen species(ROS), DNA break, 8-oxodG and lipid peroxidation have been most commonly noted [6, 7, 28].

B(*a*)P is also very important environmental toxicant and has associated with many biological effects including mutagenesis and carcinogenesis. This extremely potent carcinogen is found in coal tar, cigarette smoke, exhaust fumes from internal combustion engines, and smoke from other processes involving the burning of organic material [1]. This compound can cause extensive DNA fragmentation via the generation of ROS [5].

Combined treatment of TCDD and B(*a*)P was reported

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to induce calcium elevation with increased tyrosine phosphorylation in human lymphocytes [22]. Both contaminants appeared to mimic signaling through insulin-like growth factor-I receptor [24]. Since both chemicals are likely to occur in mixture in many situations, the combined toxicity of these compounds deserves special attention. However, the precise mechanism of such combined toxicity is yet to be elucidated. In the present study we investigated the combined toxicity of TCDD and/or B(*a*)P on C100 cells derived from SV 40-transformed baby hamster kidney.

Materials and Methods

Chemicals, reagents and media

TCDD(CAS 1746-01-6) was purchased from Supelco (Bellefonte, PA, USA). B(*a*)P(CAS 50-32-8) and Phorbol 12-myristate 13-acetate(PMA) were purchased from Sigma(St. Louis, MO, USA). The test compounds, TCDD (purity: >98%) and B(*a*)P(purity: >97%) were dissolved in ethanol(Hayman, England, purity: >99.9%) with a final maximum ethanol-concentration in the medium of 1%. PMA was used as positive control. Ethanol was utilized in negative control. Minimal essential medium (MEM), fetal bovine serum(FBS) and antibiotics (gentamicin sulfate) were obtained from Gibco BRL (Gaithersburg, MD, USA).

Cells and culture conditions

C100 cell lines was derived from the SV 40-transformed baby hamster kidney and obtained from Dr. Simoni(Stanford University, USA). Cells were maintained as monolayers in MEM supplemented with nonessential amino acids, 10%(v/v) delipidated FBS and 5 mg/100 m*l* gentamicin sulfate at 37°C and 5% CO_2 atmosphere. The lipids in FBS were removed as described by Cham and Knowles [2]. Delipidated FBS were filtered and stored at 4°C. When cells were 85% compacted in culture flask, they were harvested and used for various assays. The cells were treated with 30 nM TCDD and 3 μM B(*a*)P for 12 h, 1, 2, 4 and 7 days, respectively.

Determination of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) uptake (Promega, Madison, WI, USA) as described by Madesh *et al*. [14]. C100 cells seeded at the proper cell density (4×10^4 cell/well) to flat-bottomed 96-well plates with 100 μ*l* of medium/well, and allowed to attach and grow overnight at 5% CO_2 and 37°C atmosphere. The culture medium was replaced with fresh medium containing 30 nM TCDD and/or 3 μM B(*a*)P. After the exposure duration of 12 h, 1, 2, 4, and 7 days, MTT was added and incubated at 37°C for 4 h. The plate was read at 590 nm in a microplate reader(Molecular Devices, Sunnyvale, USA). Assays were performed in triplicate to ensure minimal variability in results.

Measurement of lipid peroxidation

Lipid peroxidation in C100 cells was determined through measuring the formation of TBARS as described by Mihara and Uchiyama [18]. For that, the cells were pretreated with TCDD(30 nM) and/or B(*a*)P(3 μM) for 0.5, 1, 2, and 4 days, respectively. Then the cells were washed twice with ice-cold PBS-buffer and thereafter re-suspended in 50 mM PBS(pH 6.8) containing 1 m*l* 2% sodium dodecylsulfate(SDS), 0.5 m*l* cell suspension, 1 m*l* 0.6% thiobarbituric acid(TBA) and 3 m*l* 1.0% phosphoric acid. The mixtures were then heated to boiling for 45 min, and TBA adducts were extracted with 3 m*l* 2-butanol. The concentrations of TBARS were determined on a spectrophotometer(Jasco, V-550, Tokyo, Japan) using a wavelength of 535 nm, and a molar absorptivity constant of $1.56\times10^5\ M^{-1}\ cm^{-1}$. The concentration of TBARS was represented as nmol of malondialdehyde/m*l*. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane.

Analysis of DNA fragmentation

C100 cells were harvested and washed twice with ice-cold PBS for 2 min. DNA was extracted using DNAzol Reagent(Gibco BRL, Cheshire, UK) according to the manufacturer's protocol. DNA sample in a loading buffer [(50 mM Tris, 10 mM EDTA, 1%(w/v) low melting point agarose, 0.25%(w/v) bromophenol)] was loaded onto solidified 1.8%(w/v) agarose gel containing 0.1 μg/m*l* ethidium bromide. A 100 bp DNA ladder standard marker(Promega) was also loaded to help verify the size of the products. Agarose gels were run at 50 V for 90 min in 1×TBE buffer. Finally, gels were visualized and photographed by computerized UV densitometer (Bio-Rad, Hercules, CA, USA).

Determination of DNA cleavage assay

To measure the DNA damage in C100 cells, an

analysis of SSB was undertaken by using pBR322 DNA(Takara, OTSU, Shiga, Japan) which contained circular form. DNA cleavage assays were performed in relaxation buffer(100 μ*l*; 50 mM Tris-HCl(pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM EDTA, 0.4 μg of supercoiled pBR322 DNA). Test chemicals were added at various times and they were incubated for 15 min at 37°C. Reactions were terminated by adding 10%(w/v) SDS to a final concentration of 0.75% and incubated for another 30 minutes in the presence of proteinase K(0.5 mg/m*l*)(Promega, Madison, USA). Then, DNA loading buffer was added and DNA was separated on 1.2% agarose gels containing 0.1 μg/m*l* ethidium bromide. Gels were scanned and DNA bands were quantified using Kodak Digital Science 1D gel analysis program(Eastman Kodak Company, New Haven, CT, USA).

Measurement of 8-OHdG adduct by immunoperoxidase staining

Induction of 8-OHdG in the nucleus of cells treated with test chemicals through cytoplasm was quantified with immunoperoxidase staining using monoclonal antibody for 8-OHdG(provided by Dr. Santella, at Columbia University, NY, USA). After treatment, culture slides were rinsed twice with 1×PBS and fixed with 75% ethanol at 20°C. Fixed cultures were treated with RNAse(100 μg/m*l*) in Tris buffer(pH 7.5; 10 mM Trizma Base, 1 mM EDTA, and 0.4 M NaCl) at 37°C for 1 h. After washing with PBS-buffer, cells were treated with proteinase K(10 μg/m*l*) at room temperature for 7 min. After re-washing with PBS-buffer, DNA was denatured with 4 N HCl for 7 min at room temperature. The cells were treated with 10% normal horse serum in 10 mM Tris(pH 7.5) at 37°C for 1 h to block nonspecific binding sites and then incubated overnight with primary antibody IF7(1:30 dilution of hybridoma supernatant) at 4°C [26]. Negative controls included slide cells staining in the following ways: one with non-specific antibody recognizing DNA damage produced by 8-OHdG adducts, two without primary antibody 1F7. After washing with PBS, cells were treated with goat antimouse IgG conjugated to biotin at 37°C for 30 min. Endogenous peroxidase was blocked by treating the cells with 3% H_2O_2 in methanol for 30 min at room temperature. After washing with PBS, ABC reagent, avidin conjugated to horseradish peroxidase was added, and the slides were incubated for 30 min at 37°C, followed by PBS and 1% Triton X-100 in PBS washes. To localize peroxidase, cells were treated with diaminobenzidine for 10 min at room temperature. Finally, slides were washed with distilled water, dehydrated with a series of 95 and 100% ethanol and xylene washes, mounted with cover glass using Permount(Fisher, New Jersey, USA), and quantified with a Cell Analysis System CAS 200 microscope(Becton Dickinson, San Jose, CA, USA). The relative intensity of nuclear staining of 30-50 randomly selected cells was measured using the Cell Measurement Program software package(Becton Dickinson, San Jose, USA). The presented data are the object average absorbance multiplied by 1000.

Measurement of antioxidant gene expression by RT-PCR

The mRNA was extracted using TRI reagent(Sigma) according to the manufacturer's protocol. The mRNA levels were shown using Access RT-PCR System(Takara, Tokyo, Japan) in accordance to the manufacturer's manual. The number of amplification cycles was previously determined to keep amplification in the linear range to avoid the plateau effect associated with increased number of PCR cycles. The RT-PCR procedure was done as previously described [4, 16, 17]. One μM total RNA and 0.15 μM primers were used for RT-PCR amplification using an RT-PCR kit(Takara, Tokyo, Japan). The PCR amplification was performed for Cu/Zn-SOD, GPx 5, and catalase, with primers; Cu/Zn-SOD gene (487 bp): sense 5'-CTA ACT CAA GCA TGG CGA TGA AA-3' and antisense 5'-ACA CAG GGA ATG TTT ACT GCG C-3', GPx 5 gene (504 bp): sense 5'-GCT GCT CAT TGA GAA TGT CGC-3' and antisense 5'-AAT CAG GTG TTT CTC CGT GCA A-3', catalase gene (248 bp): sense 5'-AGG CTC TTC TGG ACA AGT ACA ACG-3' and antisense 5'-CAC TCT AGA AGC CCG GAT TAT CG-3'. The β-actin primers (541 bp), used for internal control, had the sequences 5'-CCT GAC CCT GAA GTA CCC CA-3' (sense) and 5'-CGT CAT GCA GCT CAT AGC TC-3' (antisense). The primers were synthesized by Bioneer(Daejon, Korea). The 30-cycle RT-PCR was performed at 95°C for 30 sec, 59°C for 45 sec, and 70°C for 1 min. Aliquots of 10 μ*l* gene primer RT-PCR reactions, from 50 μ*l* total PCR reaction volume, were separated through electrophoresis on a 2% agarose gel and then stained with a 0.1%(v/v) ethidium bromide stain. The ϕX174/Hae III marker (Promega) was also loaded to help verify the size of

the amplified products. The intensity of each band was measured quantitatively using a computerized densitometer (Bio-Rad, Herclues, USA).

Results

Cytotoxicity and lipid peroxidation

For the first 4 days, the cell viability was maintained at approximately 90%, but was significantly decreased at Day 7(data not shown). Increased lipid peroxidation (TBARS formation) was observed in all treatments comparing to the negative control group(Table 1). The time-dependent formation of TBARS was observed in mixture and B(*a*)P groups, which was increased approximately 1.2 times at Days 2 and 4. Formation of TBARS in TCDD treatment showed a maximum increase of approximately 1.5-fold as compared with the corresponding controls at Day 4(Table 1).

Table 1. Effects of Measurement of lipid peroxidation level by forming thiobarbituric acid reactive substance assay in C100 cells treated with TCDD and/or B(*a*)P

Groups	Time (day)	TBARS forming (nM)
Negative	0.5	0.10774±0.153
	1	0.43967±0.053
	2	0.57605±0.154
	4	1.05398±0.097
B(*a*)P	0.5	0.13633±0.162
	1	0.49591±0.091*
	2	0.70145±0.073*
	4	1.27060±0.095*
TCDD	0.5	0.16325±0.029
	1	0.49997±0.091*
	2	0.62255±0.095
	4	1.32958±0.090**
Mixture	0.5	0.15937±0.132
	1	0.49988±0.091*
	2	0.60100±0.070
	4	1.32250±0.105**
PMA	0.5	0.17568±0.146
	1	0.50186±0.002*
	2	0.76100±0.063*
	4	1.28865±0.169

C100 cells were treated with 30 nM TCDD and/or 3 μM B(A)P for 12 hr, 1 day, 2 days, 4 days. Each value represents the mean±S.D. (n=5)

(Negative: vehicle control with treatment of ethanol, B(a)P: treatment of 3 μM benzo(a)pyrene, TCDD: treatment of 30 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Mixture: co-treatment of 30 nM TCDD and 3 μM B(a)P, PMA: positive control)

*, P<0.05; compared with each groups with control group.

**, P<0.01; compared with each groups with control group.

DNA fragmentation and DNA-single strand break

As shown in Fig. 1, cells incubated for 6 and 7 days exhibited an increased amount of low-molecular-weight DNA, which was electrophoresed in a dense ladder pattern. In contrast, the rest of treatments incubated for 12 h, 1, 2 and 4 days did not show such pattern(data not shown). The mixture treatment group induced more apoptosis than the single treatment group at Day 6 {Fig. 2(A)}. In Fig. 2 DNA damage was not detected after 12-hr exposure to chemicals, but SSB was clearly demonstrated after one-day exposure to test compounds. As time went by, the forms II and I were gradually attenuated. At Day 7{Fig. 2(B)}, they became dim, and the relative intensity of the band was decreased.

8-OHdG DNA adducts

As a control for day-to-day variation in immunoperoxidase staining, C100 cells treated with/without each chemical were stained with each batch of all slides. Representative immunoperoxidase staining of 8-OhdG on C100 cells is illustrated in Fig. 3. Specific

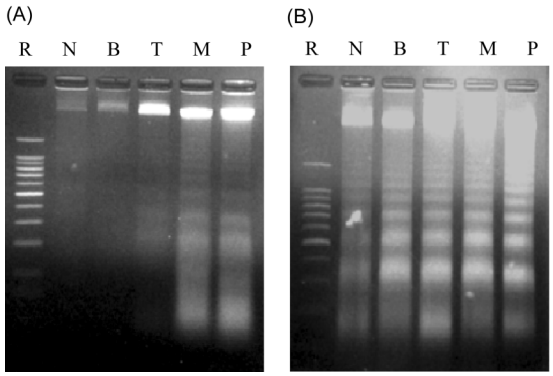

Fig. 1. DNA fragmentation induced by TCDD and/or B(*a*)P in C100 cells after treating for 6 {panel A} and 7 days{panel B}. The data are presented as means±S.D.(n=3). R, 100 bp molecular ruler; N, negative control; B, 3 μM B(*a*)P; T, 30 nM TCDD; M, TCDD+B(*a*)P; P, positive control.

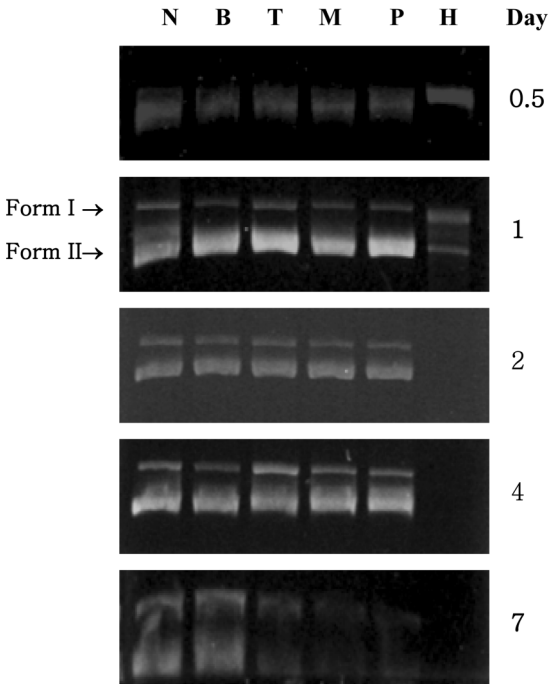

Fig. 2. Agarose gel electrophoretic pattern depicting Forms I and II of DNA. The data are presented as means±S.D. (n=3). N, 100 bp negative control; B, 3 μM B(*a*)P; T, 30 nM TCDD; M, TCDD+B(*a*)P; P, positive control; H, H_2O_2.

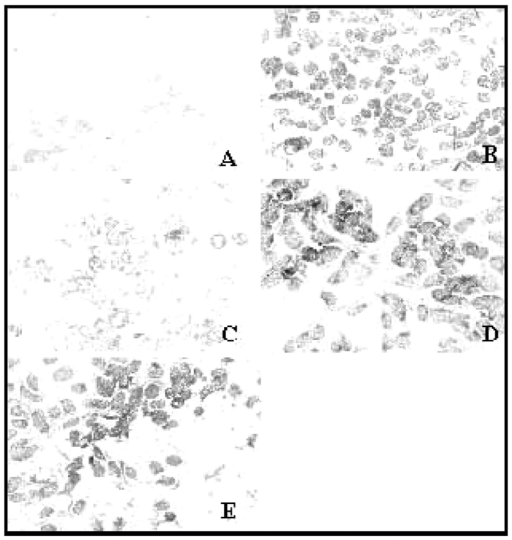

Fig. 3. Representative immunoperoxidase staining of 8-OHdG on C100 cells. C100 cells were treated with 30 nM TCDD and/or 3 μM B(*a*)P for 12 h, 1 day, 2 days, 4 days and 7 days with antiserum 1F7 recognizing 8-OHdG(X 200). The data are presented as means±S.D.(n=3). A, negative control; B, positive control; C, B(*a*)P; D, TCDD; E, TCDD+B(*a*)P.

nuclear staining was observed in TCDD and mixture groups at Day 4{Fig. 3(C) and (D)}, but not in the ethanol-treated group{Fig. 3(A)}. There was no evident variation in staining intensity on different days(data not shown). The immunoreactivity of 8-OHdG was localized mainly on nuclear and perinuclear sites, specifically.

Expression of antioxidant enzyme

Gels corresponding to transcripts encoding antioxidant enzymes are shown in Figs. 4, 5 and 6. The sizes of amplicons were 487 bp, 504 bp and 248 bp for Cu/Zn-SOD, GPx 5, and catalase transcripts, respectively. β-Actin internal PCR control was positive in all RT-PCR experiments. Expression profiles of transcripts encoding for antioxidant enzymes were different between TCDD and B(*a*)P groups. In all treatment groups except the negative control group, transcripts encoding for Cu/Zn-SOD were generally detected until Day 4 and expression level was the lowest at Day 7(Fig. 4). GPx 5 mRNA expression of B(*a*)P and mixture groups is higher compared to negative and TCDD groups(Fig. 5). Consistent declined transcription of the GPx 5 and

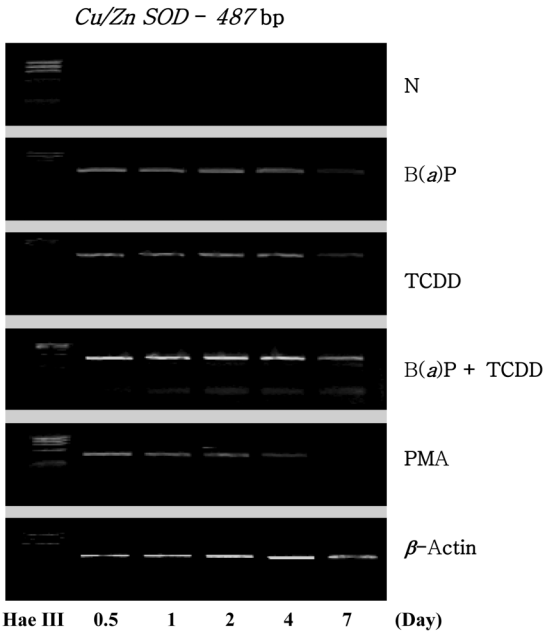

Fig. 4. RT-PCR analysis of Cu/Zn SOD mRNA in C100 cells treated with TCDD and/or B(*a*)P. Representative RT-PCR reactions from n=3 cells for the groups are shown. N, negative control; PMA, positive control; Hae III, *Hae*III/ Φ174 marker.

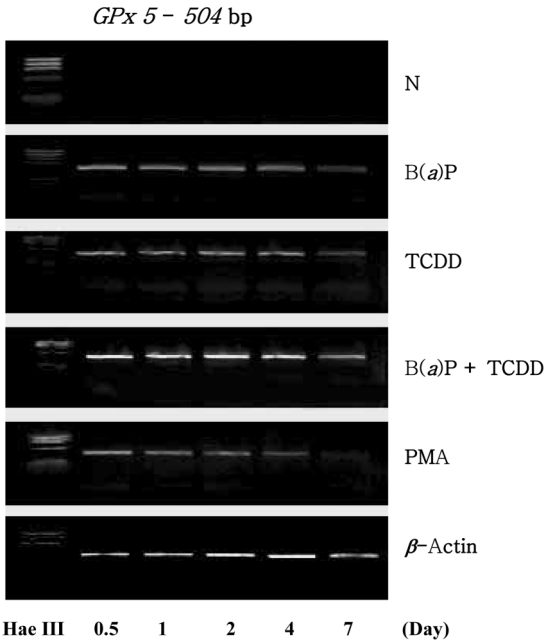

Fig. 5. RT-PCR analysis of GPx 5 mRNA in C100 cells treated with TCDD and/or B(*a*)P. Representative RT-PCR reactions from n=3 cells for the groups are shown. N, negative control; PMA, positive control; Hae III, *Hae*III/ Φ174 marker.

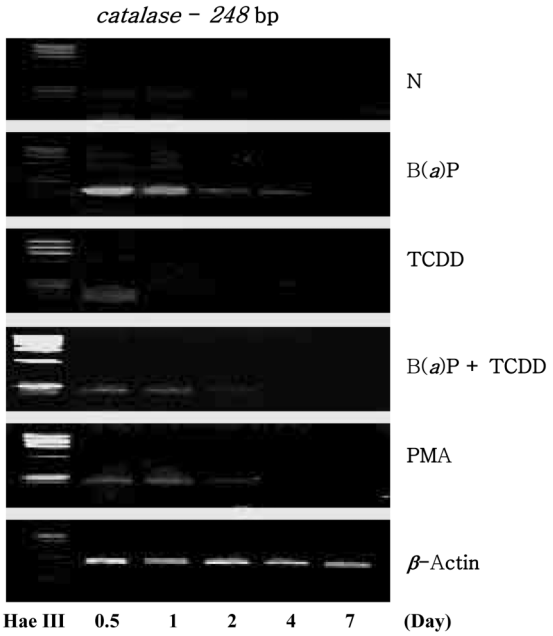

Fig. 6. RT-PCR analysis of catalase mRNA in C100 cells treated with TCDD and/or B(*a*)P. Representative RT-PCR reactions from n=3 cells for the groups are shown. N, negative control; PMA, positive control; Hae III, *Hae*III/Φ174 marker.

catalase transcripts was demonstrated(Fig. 5 and 6). In all groups, Cu/Zn-SOD was expressed at relatively high levels compared to the expressions of GPx 5 and catalase in a time-dependent manner. Expression of both Cu/ Zn-SOD and catalase in the TCDD group was stronger than that of the B(*a*)P group(Fig. 4 and 6).

Discussion

It is suggested that ROS may be involved in the apoptotic phenomenon and in the pathogenesis of cancer related with exposure to dioxin and other toxicants [21, 25]. It is well documented that ROS can cause cell death via apoptosis in cells [8, 10, 11, 12, 19]. Involvement of ROS in the pathogenesis of cancer by TCDD has been suggested [7, 28]. We hypothesized that ROS might be implicated in TCDD and/or B(*a*)P-induced toxicities, and TCDD and B(*a*)P might have synergistic interaction. Accumulating evidence indicated that TCDD and/or B(*a*)P induces generation of ROS in many rodent and human cell systems [4, 7, 13].

MTT assay provides a sensitive measurement of normal metabolic status of cells, particularly that of mitochondria, and is hence indicator of early cellular redox changes [9]. There was a significant decrease in cell viability indicating TCDD and their mixture treatment induced cell apoptosis at Day 6. At Day 7, however, all treatment induced cell death. Jose *et al*. [15] noted that superoxide anions and hydroxyl radicals were involved in dioxin-induced cytotoxicity. On the basis of results mentioned above, cell death induced by TCDD(30 nM) and/or B(*a*)P(3 μM) may be implicated in the generation of ROS.

The purpose of measuring thiobarbituric acid reactive substances is to screen and monitor lipid peroxidation, a major indicator of oxidative stress. This assay has been used for the measurement of anti-oxidant activity of several compounds [6]. Previous studies have shown the ability of TCDD to induce oxidative stress and lipid peroxidation [7]. Our study also demonstrated that individual and mixture exposure to TCDD(30 nM) and B(*a*)P(3 μM) could result in a significant increase of lipid peroxidation in a time-dependent manner. However, it should be noted that such increase was not seen at Day 7(Fig. 1). This result suggests that there may be certain threshold for cell survival in our system. Therefore, the cells at Day 7 or later may die of excess lipid peroxidation.

The free radical-induced DNA strand break occurs only if the sugar moiety is ultimately damaged [12]. A majority of free radicals react with protein and lipid, while some others may react directly with DNA by adding to the double and single bonds. This mechanism can abstract hydrogen atoms leading to the formation of deoxyribose radicals and eventually lead to strand breaks [20]. Our study showed that TCDD and/or B(*a*)P induced strand breaks in a time-dependent manner (Fig. 1). The DNA damage was greater in mixture treatment than in control and single treatments, which was verified by gel electrophoresis. Fig. 2 also demonstrates that DNA was broken into low-molecule nuclear fragmentations. These data suggest that ROS induced by TCDD and/ or B(*a*)P gives nick to the supercoil form DNA and eventually makes DNA very low molecular DNA fragmentations. Our study indicates that the cell under stress surpass the limits of cell defense ability as time goes by and undergoes apoptosis.

Although there are many documents demonstrating mutations due to reaction with DNA [10, 23, 26, 27], little is known about oxidative DNA damage directly [20]. In the present study, we investigated the expression of 8-hydroxydeoxyguanosine(8-OHdG), a biomarker of oxidative DNA damage, in TCDD(30 nM) and/or B(*a*)P (3 μM) treatment and compare its expression with negative control. 8-OHdG expressions were immuno-peroxidase staining-examined using a monoclonal antibody against 8-OHdG in C100 cells. In negative control, no 8-OHdG expressions were observed{Fig. 3(A)}. On the other hand, nuclear expression of 8-OHdG was detected in TCDD(30 nM) and/or B(*a*)P(3 μM) treatment groups {Fig. 3(C),(D), and (E)}. The positive control was predominantly observed in the marginal areas of C100 cells{Fig. 3(B)}. Our study showed that oxidative DNA damage was generated in TCDD and/or B(*a*)P treatment, suggesting 8-OHdG as a reliable marker for oxidative DNA damage.

Under normal conditions, cells have defense mechanisms, including nonenzymatic antioxidants such as glutathione, and enzymatic ones such as SOD, GPx and catalase, against ROS [16]. Among them, GPx and SOD are the major enzymes protecting mammalian cells against oxygen toxicity and lipid peroxidation [17]. SOD is considered as the first line of defense against oxidative toxic damage and catalase in peroxisomes. GPx in mitochondria plays an important role in the conversion of H_2O_2 to H_2O and O_2 [15]. Our study showed descending induction of GPx 5 and catalase at the transcriptional level in response to TCDD and/or B(*a*)P in a time-dependent manner (Fig. 5 and 6). However, the transcription of Cu/Zn-SOD genes was higher than other two genes in all treatment period(Fig. 4). Such unbalanced induction of Cu/Zn-SOD, GPx 5 and catalase might be the underlying basis of mixture-induced cytotoxicity in our system. Our results strongly suggest that harmonized actions of several antioxidant enzymes are critical for maintaining healthy cellular environment.

In conclusion, we found that oxidative stress might be a critical factor for combined toxicity of TCDD and B(*a*)P. Our results also suggest that the exposure to mixture of TCDD and B(*a*)P was caused more potent toxicity than single exposures. Additional extensive studies are needed to elucidate the detailed mechanism of the mixture-induced toxicity.

Acknowledgments

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