INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH), of which Benzo[a]pyrene (BaP) is the most commonly studied and measured, are ubiquitous environmental agents and they commonly believed to significantly contribute to human cancers. BaP is not manufactured and has no industrial use. It is ubiquitously distributed throughout the environment as a consequence of its formation during the combustion of organic matter. Carcinogenic and mutagenic effects of BaP have been well investigated in animals and other mammalian cell systems (Harvey, 1991; Pei et al., 1998). PAHs are themselves chemically inert and hydrophobic. However, they undergo metabolic activation in mammalian cells to diol–epoxides that bind to human cancers. BaP is not manufactured and has no industrial use. It is ubiquitously distributed throughout the environment as a consequence of its formation during the combustion of organic matter. Carcinogenic and mutagenic effects of BaP have been well investigated in animals and other mammalian cell systems (Harvey, 1991; Pei et al., 1998). PAHs are themselves chemically inert and hydrophobic. However, they undergo metabolic activation in mammalian cells to diol–epoxides that bind
covalently to cellular macromolecules, including DNA, thereby causing errors in DNA replication and mutation that initiate the carcinogenic process (Phillips, 1999).

The tumor suppressor p53 is the most frequent target genetic alterations in human cancers, with mutations occurring in almost 50% of all human tumors (Cariello et al., 1994). It has been suggested that p53 may play an important role in DNA repair, cell cycle arrest, apoptosis upon environmental stresses (Oren, 1999). The activated p53 induces several mechanism including protein stabilization and modification of the protein by phosphorylation and acetylation. The p53 can be acetylated in vivo in response to a variety of cellular stress signals (Sakaguchi et al., 1998; Pearson et al., 2000; Prives et al., 2001) and p53 is a transcription factor that recognizes specific binding sites within numerous target gene including mdm2, cyclin G, bax, and p21/WAF1/CIP1, which mediate p53-dependent cell cycle arrest and/or apoptosis (Sakaguchi et al., 1998; el-Deiry 1998; Bargonetti et al., 2002; Lagger et al., 2003). The cyclin dependent kinase inhibitor p21/WAF1/CIP1 is an important regulator of cell cycle progression, senescence, and differentiation (Appella et al., 2001). In this study, Bezo[a]pyrene toxicity was investigated on human hepatoma HepG2 cells, focusing on its effect on tumor suppressor protein, p53, to identify biomarkers for PAH risk assessment.

MATERIALS AND METHODS

Cell culture and cell treatment

The human hepatoma HepG2 cells were maintained in DMEM (GIBCO BRL. Life Technologies) supplemented with 10%(v/v) fetal bovine serum and 1% antibiotics at 37°C in CO2 atmosphere. BaP was dissolved in dimethyl sulfoxide (DMSO) as a 1000x stock. For BaP treatment, cells were 70~80% confluent, and the medium was exchanged with fresh medium (serum free) containing various concentrations of BaP in DMSO. Treated and control cells were incubated for 24 hours and harvested for the analysis.

MTT Assay

Cell viability was measured using MTT assay. Cells at the exponential phase were collected and transferred into each well (about 10^4 ~ 10^5 cells in 180 µL/well). The cells were incubated for various concentration and then 50 µL of 2 mg MTT(3-4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide) solution was added to each well (0.1 mg/well). After incubating for 4h, the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 µL of DMSO and the A 540 was read on a scanning multi-well spectrophotometer (Molecular Device Co., Sunnyvale, CA). All experiments were performed in triplicate.

Western blotting

Cells (2 x 10^6 cells/mL) were cultured with BaP and harvested at the indicated times. Cells were washed with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 50 mM NaF, 50 µg/mL leupeptin and 50 µg/mL aprotinin and incubated on ice for 30 min. After centrifugation (15,000 g for 10 min at 4°C), the supernatant was collected and protein contents in lysates were determined by Bradford analysis (Bio-Rad). Equal amounts of protein per lane were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After SDS–PAGE, the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody for 1 hour and then extensively washed with TTBS buffer. And the membranes were incubated for 1 hour with secondary antibody. After extensive washing with TTBS buffer, the immune complexes were detected by enhanced chemiluminescence detection system (Amersham) and exposed to X-ray film.
Chemicals

Benzo[a]pyrene was purchased from Sigma (B1760) and antibodies were purchased from SantaCruz, Cell Signaling and Upstate (Table 1).

Data analysis

After band intensity analysis (Kodak 1D 3.6), all analyses were performed using SigmaPlot 8.0 and SigmaStat 5.0.

RESULTS AND DISCUSSIONS

The effect of BaP on cell viability was studied in HepG2 cells. 10 \( \mu \)M of BaP treatment decreased in the cell viability to almost 25% of that of control by 24 h (Fig. 1). The cell death induced by BaP might be due to apoptosis, however, to prove this hypothesis, further analysis are needed.

In order to find out whether p53 protein is involved in the cell death induced by BaP, cellular p53 levels was measured using western blot analysis. The p53 protein level was augmented in BaP treatments (Fig. 2). Because the phosphorylation of the serine15 residue of p53 is known as a very early step in the activation of p53, we assessed the amount of p53 phosphorylated at serine 15 after BaP treatment, using an antibody that specifically recognizes the phosphorylated serine 15 residue of p53. The phosphorylation of p53 at serine 15 was induced by BaP treatment, in a concentration dependent manner (Fig. 2). Chemical response of phosphorylated p53 could be more sensitive than that of p53. However, the level of acetylated p53 at lysine 320, which is also an activated form of p53, decreased by BaP exposure (Fig. 2A.). The meaning of p53 acetylation and the way by which this process is regulated have not yet fully understood (8), therefore, further researches are needed to explain the result observed in this study. The \( \beta \)-actin protein level remained unchanged following BaP exposure (data not shown).

Induction of DNA damage by a variety of agents, such as BaP, is known to activate the tumor suppressor p53, which in turn acts as transcriptional regulator of several target genes. One of the main targets is the gene encoding the Cdk inhibitor, p21. Treatment of the human hepatoma HepG2 cells with BaP led to strongly increased p21 protein levels (Fig. 2). Increase of this protein might be related with cell cycle arrest, however the levels of Cdk4 and Rb proteins were not modified by BaP exposure (Fig. 2). The meaning of increase of p21, with no modification of its downstream signaling proteins, Cdk4 and Rb, remains uncertain. Further studies are needed to elucidate whether benzo[a]pyrene-induced p53 accumulation lead to cell cycle arrest through p21 signaling pathway.

Time dependent effects of BaP on p53, acetylated p53 and p21 were shown in Fig. 4. The p53 protein level increased as early as 3 h after BaP treatment and this augmentation remained until 24 h. Whereas, the peak of the p21 protein level occurred at 24 h after treatment. The results of time-course study
suggest that BaP–induced p53 protein accumulation could lead increase in the p21 protein level. Acetylated p53 level decreased by BaP treatment in time–course study (Fig. 3), as in exposure level study, shown in a 1. Given the as yet incomplete understanding of the meanings of p53 acetylation, further researches are needed to explain BaP–induced decrease of acetylated p53 observed in this study.

Table 2 shows Spearman correlation analysis between the different parameters tested in this study. Statistical study reveals that BaP exposure levels are negatively correlated with cell viability, and positively with phosphorylated p53 and p21 protein levels. Among 6 proteins tested, phosphorylated p53 and p21 are negatively correlated with cell viability, whereas positive correlation was observed between acetylated p21 protein and cell viability. These overall results suggest that exposure to BaP lead to cell death and the accumulation of the phosphorylated p53 and p21 proteins levels may be deeply involved in this process.

It is well known that metabolite of BaP are capable of binding to cellular macromolecules including DNA. Therefore, p53 activation and phosphorylation of serine 15 seems to be a downstream response to BaP–induced DNA damage and these results suggest that p53 plays important roles in defense against BaP induced genotoxicity. Taken into account overall results, p53 and p21 seems pertinent biomarkers for BaP exposure and after calibration and validation, these proteins could be used for PAH risk assessment. However, further work is necessary to eluci-
date the molecular mechanism leading to modification of the p53 and p21 proteins levels after BaP treatment.

**CONCLUSIONS**

BaP toxicity was investigated on HepG2 cells, focusing on its involvement in tumor suppressor protein, p53. HepG2 cell viability decreased upon BaP exposure. Concentration–dependant increase of protein induction was observed on p53, as well as on phosphorylated p53, whereas acetylated p53 level decreased by BaP treatment. P21 level increased by chemical treatment, in a concentration–dependant manner, whereas its downstream signaling proteins, such as, Cdk and Rb, did not changed by BaP exposure. These results suggest that p53 and p21 accumulation is an important phenomenon for BaP toxicity, and thus, concomitantly with chemical specific biomarker, these proteins could be identified as sensitive biomarkers for BaP–like PAH risk assessment.

Fig. 3. Concentration–dependent effect of BaP on p21, Cdk4 and Rb. Expression of p21, Cdk and Rb in different concentrations of BaP (0–10 µM, 24 h) treated HepG2 cells by Western blot analysis (A); densitometric analysis (B).

Fig. 4. Time–dependent effects of BaP on p53, acetylated p53 and p21. Expression of p53, acetylated p53 and p21 at different time points (0–24 h) in BaP (4 µM) treated HepG2 cells by Western blot analysis (A); densitometric analysis (B).
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REFERENCES


