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Benzo[*a*]pyrene-induced DNA damage and p53 modulation in human hepatoma HepG2 cells for the identification of potential biomarkers for PAH monitoring and risk assessment

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Abstract

To identify potential biomarkers for the monitoring and risk assessment of benzo[*a*]pyrene (BaP), the oxidative stress-related DNA damage and p53 modification were investigated in human hepatoma HepG2 cells. Benzo[*a*]pyrene exposure induced a decrease in the cell viability, but increased the antioxidant enzyme activity as well as the DNA and lipid damage. The p53 protein activation appeared to have been a downstream response to the benzo[*a*]pyrene-induced DNA damage, suggesting p53 plays important roles in the defense against benzo[*a*]pyrene-induced genotoxicity. The response of phosphorylated p53 may be more sensitive towards benzo[*a*]pyrene exposure than normal p53. Following DNA damage, the activation of p53 acts as a transcriptional regulator of several target genes, including, p21 protein; a gene that encodes the Cdk inhibitor and is induced by exposure to benzo[*a*]pyrene. The p53 mRNA level was increased after the treatment of cells with benzo[*a*]pyrene, as well as following the induction of p53 protein, suggesting the benzo[*a*]pyrene-stimulated p53 accumulation may also be transcriptionally induced. The overall results suggest that benzo[*a*]pyrene leads to serious DNA damage, which leads to the transcription of the p53 gene; that the subsequent p53 protein accumulation up-regulates the cellular p21 protein. Oxidative DNA damage and p53 accumulation seem to be related to benzo[*a*]pyrene toxicity; however, their potential as biomarkers in environmental monitoring and risk assessment needs to be validated in the context of their specificity and sensitivity.
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Keywords: Benzo[*a*]pyrene; DNA damage; p53; p21; Oxidative stress

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental agents that are commonly believed to significantly contribute to human cancers. Although chemically inert, they undergo metabolic activation to diol-epoxides within mammalian cells, which bind covalently to cellular macromolecules, including DNA, thereby causing errors in DNA replication and mutation, initiating the carcinogenic process (Pillips, 1999). Among PAHs, benzo[*a*]pyrene (BaP) is the most commonly studied and measured. Benzo[*a*]pyrene is not manufactured and has no industrial use, but is ubiquitously distributed throughout the environment as a consequence of its formation during the combustion of

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organic matter. The carcinogenic and mutagenic effects of BaP have been extensively investigated in mammalian and other animal cell systems (Harvey, 1991; Pei et al., 1999).

During the metabolic process, BaP produces reactive oxygen species (ROS) via cytochrome P-4501A1 (CYP1A1) (Safe, 1995; Burczyniski and Penning, 2000). These ROS and metabolites can cause oxidative DNA damage and form adducts with DNA, starting the mutagenic chain of events responsible for tumor initiation. Therefore, it is important to understand the mechanism of PAH-induced oxidative stress causing the stress signals that result in the activation of transcription factors such as, tumor suppressor p53 (Ueno et al., 1999).

p53 is the most frequent target of 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 and apoptosis under conditions of environmental stresses (Levine, 1997; Oren, 1999; Ljungman, 2000). Following DNA damage, p53 is phosphorylated and acetylated at a number of sites. Its phosphorylation represents an early cellular response to a variety of genotoxic stresses, promoting both the accumulation and functional activation of p53 (Shieh et al., 1997). Besides phosphorylation and acetylation, the binding of p53 to DNA is also modulated by a redox regulation mechanism (Gaiddon et al., 1999). The increased cellular p53 protein levels exposed to various genotoxic agents are due mainly to an increase in the stability of p53 protein rather than an increase in the levels of p53 mRNA. However, it has been suggested that an increase in p53 protein stability is not solely responsible for the recruitment of p53 in response to genotoxic stress; it is more likely that the p53 genotoxic stress response is a complex cellular process regulated at transcriptional mRNA stability levels.

To identify potential biomarkers for BaP monitoring and risk assessment, this study investigated the toxic mechanisms of BaP on human hepatoma HepG2 cells, focusing on its involvement in oxidative stress-related DNA damage and p53 as well as its related protein modifications.

2. Materials and methods

2.1. Cell culture and treatment

The human hepatoma HepG2 cells were maintained in DMEM (GIBCO BRL Life Technologies, Rockville, MD, USA), supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics, at 37 °C in a CO_2 atmosphere. The benzo[*a*]pyrene (Sigma, St. Louis, MO, USA,), dissolved in dimethyl sulfoxide (DMSO), was treated to the cells. The treated and control cells incubated for 24 h, and then harvested for the analysis.

2.2. Cell viability assay

The cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltera zolium bromide (MTT) reagent, as described by Mosmann (1983).

2.3. Flow cytometry

Flow cytometry was performed on the BaP treated and control cells, and the effects on apoptosis observed. Propidium iodide (PI) stained cells were analyzed using a flow cytometer (BD Science, San Jose, CA, USA), as described by Nicoletti et al. (1991). The effect on apoptosis was determined by the increase in the proportion of sub G1 hypo-diploid cells.

2.4. DNA gel electrophoresis

Apoptosis in BaP treated cells was also evaluated by electrophoretic demonstration of DNA fragmentation. After treatment, genomic DNA was extracted from the cells using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). DNA was subjected to electrophoresis on a 1.5% agarose gel containing 0.1 g/ml ethidium bromide, and visualized under ultraviolet light.

2.5. Catalase activity

The rate of H_2O_2 degradation (measured at 240 nm) was used to quantify the catalase activity on BaP treated and control cells, as described by Aebi (1984).

2.6. Lipid peroxidation

The lipid peroxidation level was determined by measuring the concentration of malonaldehyde (MDA), using the thiobarbituric acid (TBA) method described by Uchiyama and Mihara (1978).

2.7. Comet assay

The Comet assay was performed on BaP treated and control cells using the image analysis system (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK), as described by Tice et al. (2000).

2.8. Western blotting

For Western blotting analysis, aliquots of the cell lysates, prepared in a 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, were subjected to electrophoresis on a 10% sodium dodecyl sulfate

(SDS)-polyacrylamide gel, and the gel transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with a primary antibody for 1 h, subjected to further incubation with a secondary antibody and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK). The antibodies were purchased from Santa Cruz (for p53, CdK4 and Rb; Santa Cruz, CA, USA), Cell Signaling (for p-p53 and p21; Beverly, MA, USA) and Upstate (for a-p53; Lake Placid, NY, USA).

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase chain reactions were performed on BaP treated and control cells, using a two-steps method employing RT Premix and the RCR Premix Kit (Bioneer Co., Seoul, Korea). The PCR products were separated through electrophoresis on a 1.5% agarose gel (Promega, Madison, WI, USA) and visualized with ethidium bromide (Bioneer). All the tests were replicated at least three times. Primers for the detection of p53 (5′-TCT GGG ACA GCC AAG TCT GT-3′ and 5′-CAT CAC ACT GGA AGA CTC CA-3′) and p21 genes (5′-ACC CTC TCA TGC TCC AGC T-3′ and 5′-CCT TGT TCC GCT GCT AAT CA-3′) were designed based on sequences retrieved from GenBank^{TM}.

2.10. Band density analysis

Following the Western blotting and RT-PCR analyses, the relative densities of the protein and DNA bands were determined using an image analyzer, the Gel Documentation system (Vilber Lourmat TFX-20.M, Marne la Vallee, France), coupled to a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

2.11. Data analysis

Statistical differences between the control and treated cells were examined with the aid of a parametric *t*-test using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA). An alpha level of 0.05 was used to determine significance in all statistical analyses.

3. Results

The effect of BaP on cell viability was studied in HepG2 cells (Fig. 1). Statistically significant cell death occurred after BaP exposure, with 10 μM BaP treatment inducing a decrease of cell viability to almost 25% that of the control group.

To verify if the BaP-induced cell death was due to apoptosis, flow cytometry analysis was conducted using PI staining to quantify the number of cells with a sub-diploid DNA content. As shown in Fig. 2, 24 h after BaP treatment, the numbers of cells localized in the sub-diploid DNA peak was not significant. Because DNA ladder formation is an important phenomenon in apoptosis, DNA fragmentation was also examined. However, the DNA ladder, a typical apoptosis marker, was not observed with any of the treatments.

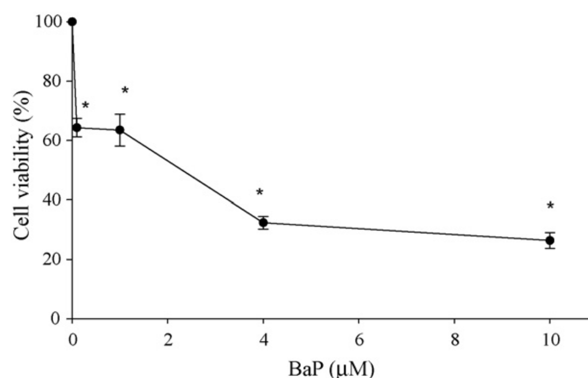

Fig. 1. Effects of BaP on cell viability. HepG2 cells were treated with different concentrations of BaP for MTT assay. The results are shown as the mean ± standard error of mean of the triplicate culture. The asterisk denotes the significant difference from the DMSO control group ($p < 0.05$).

Because PAHs are known to produce ROS, as well as metabolize into genotoxic intermediates via the CYP1A1 pathway, oxidative stress-related markers were examined (Fig. 3). The involvement of oxidative stress in BaP-induced toxicity was investigated by studying both the catalase activity and lipid peroxidation. An increase in catalase activity and MDA formation was observed in BaP-treated cells, but no concentration-dependent response was observed.

ROS and PAH metabolites can cause oxidative DNA damage and form adducts with DNA. Thus, DNA damage, especially DNA strand breaks, was measured using the Comet assay (Fig. 4). From the images of treated cells and the calculated tail, moments as well as the olive tail moments, the amount of DNA strand breaks increased at all BaP concentrations used.

Since p53 can play important roles in cell defense following exposure to a DNA damaging agent, and BaP caused significant DNA damage, as shown in Fig. 4, the cellular level of p53 was examined. The level of p53 protein was augmented in the BaP treated cells (Fig. 5). Because phosphorylation of the serine 15 residue of p53 is known to be a very early step in the activation of p53, the amount of p53 phosphorylation at serine 15 following BaP treatment was assessed using an antibody able to specifically recognize the phosphorylated serine 15 residue of p53. The phosphorylation

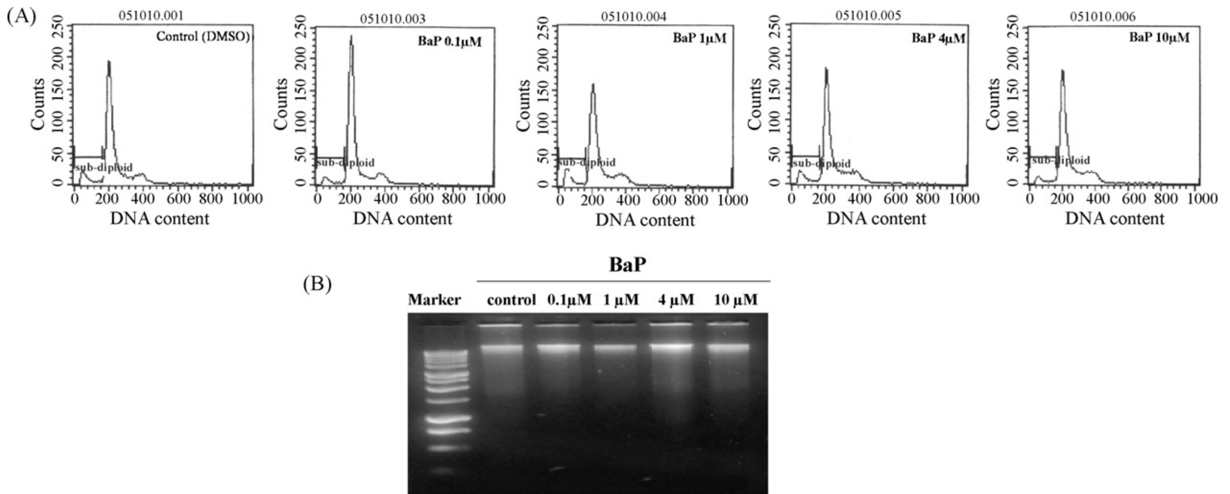

Fig. 2. Apoptosis analysis in BaP-treated HepG2 cells. The cells were treated with different concentrations of BaP for 24 h for (A) flow cytometry analysis and (B) a DNA fragmentation test.

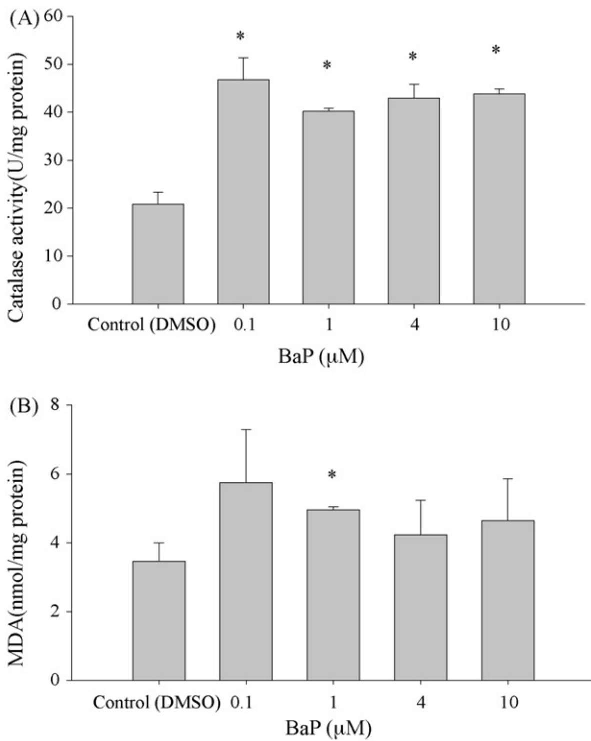

Fig. 3. Effects of BaP on catalase activity and MDA formation. Catalase activity and MDA formation were measured in HepG2 cells treated with different concentrations of BaP (0–10 μM, 24 h). The results are shown as the mean ± standard error of mean of the triplicate samples. The asterisk denotes the significant difference from the DMSO control group ($p < 0.05$).

of p53 at serine 15 increased, whereas the acetylation decreased following BaP treatment, which was also in a concentration-dependent manner (Fig. 5). The β-actin protein level remained unchanged following BaP exposure (data not shown).

Since the level of p53 protein was increased by BaP exposure, p53 target genes, such as, p21, were examined (Fig. 6). Treatment of the human hepatoma HepG2 cells with BaP led to a strong increase in the level of p21 protein.

The time-dependent effects of BaP on p53 and p21 are shown in Fig. 7. The level of p53 protein increased as early as 3 h after BaP treatment, and this augmentation

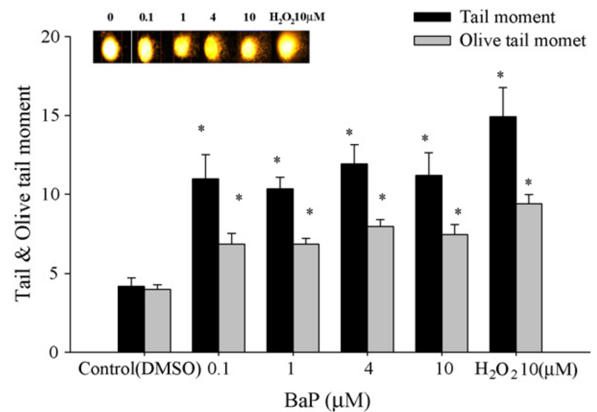

Fig. 4. Effects of BaP on DNA damage. The tail moment and the olive tail moment were measured in HepG2 cells treated with different concentrations of BaP (0–10 μM, 24 h) using Comet assay. The results are shown as the mean ± standard error of mean of triplicate slides. The asterisk denotes the significant difference from the DMSO control group ($p < 0.05$). Photographs are images of treated and control cells.

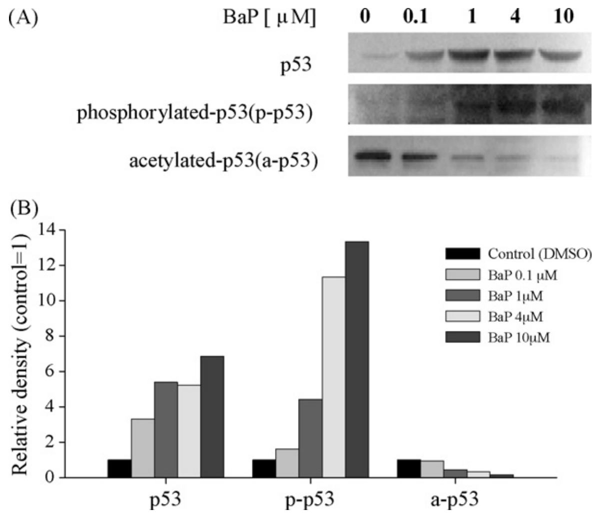

Fig. 5. Expression of p53, phosphorylated p53 (p-p53), and acetylated p53 (a-p53) proteins in HepG2 cells treated with different concentrations of BaP (0–10 μM, 24 h). Western blot analysis (A) and densitometry (B).

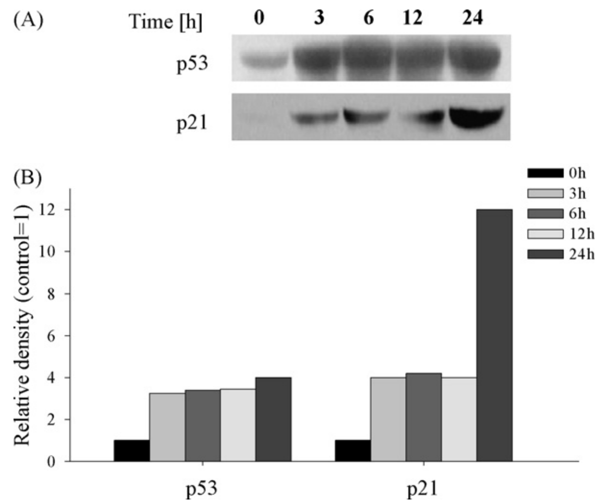

Fig. 7. Expression of p53 and p21 proteins at different time points (0–24 h) in HepG2 cells treated with BaP (4 μM). Western blot analysis (A) and densitometry (B).

remained until 24 h. The level of p21 protein also increased 3 h after treatment, but the highest level was reached 24 h after treatment.

Since the level of p21 protein increased significantly after BaP treatment, as shown in Figs. 6 and 7, the proteins considered related to p21 in cell cycle arrest when subjected to cellular stress, namely, CdK4 and Rb, were also examined (Fig. 8). The expressions of Cdk4 and Rb appeared to be slightly increased by exposure to BaP, especially in the case of Rb; however, the densitometric scan data analyzing band intensity compared to the baseline, revealed no differences between the control and those treated, as seen in Fig. 8(B).

To study if transcriptional activation is also involved in the recruitment of p53 in response to exposure to BaP, the levels of p53 mRNA were examined (Fig. 9). Since the p21 protein was strongly induced by exposure to BaP, concomitantly with p53, the p21 gene expression level was also studied. The levels of p53 and p21 mRNA were increased with exposure to 1, 4 and 10 μM BaP. The degree of increase in p53 was more significant than in p21 (two-fold and 1.4-fold average, respectively).

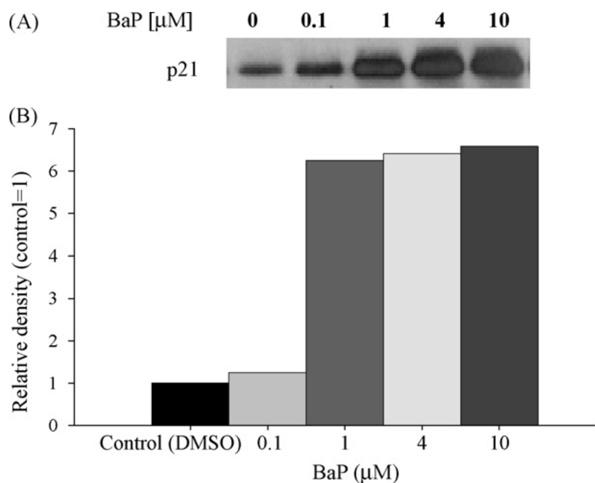

Fig. 6. Expression of p21 protein at different concentrations of BaP (0–10 μM, 24 h) treated HepG2 cells. Western blot analysis (A) and densitometry (B).

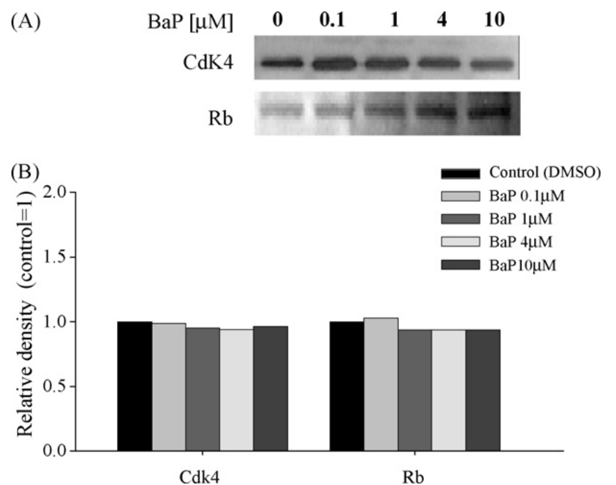

Fig. 8. Expression of Cdk and Rb proteins in HepG2 cells treated with different concentrations of BaP (0–10 μM, 24 h). Western blot analysis (A) and densitometry (B).

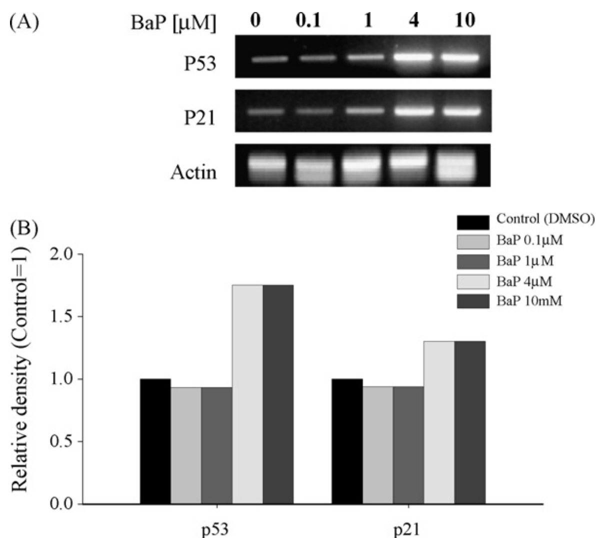

Fig. 9. Expression of p53 and p21 mRNA in HepG2 cells treated with different concentrations of BaP (0–10 μM, 24 h). Reverse transcription-polymerase chain reaction analysis (A) and densitometry (B).

4. Discussion

Oxidative stress-related DNA damage and p53 modification were investigated as potential toxic mechanisms of BaP, for the identification of potential biomarkers for BaP monitoring and risk assessment. Oxidative stress, which elicits a wide variety of cellular events, such as apoptosis, cell cycle arrest and the induction of antioxidant enzymes, is thought to be involved in the biotransformation of PAH via CYP1A1 (Safe, 1995; Burczyniski and Penning, 2000). BaP-induced oxidative stress may cause increased antioxidant enzyme activity (Fig. 3), whereas apoptosis is not always directly related to BaP-induced toxicity, as this study failed to prove the decreased cell viability (Fig. 1) was due to apoptosis (Fig. 2). In this study, formation of ROS by BaP exposure was not directly investigated, however, activation of oxidative stress defense mechanism and increase in its damage product (i.e. activation of catalase and increase of MDA, Fig. 3) may suggest BaP-induced ROS formation in a non-direct manner. ROS and PAH metabolites can also cause oxidative DNA damage, and BaP metabolites are well known to be capable of forming adducts with DNA, which may result in DNA strand breaks, as shown by this study (Fig. 4). Both the tail and olive tail moments, as measured using the Comet assay, increased significantly after exposure to all BaP concentrations, proving the strong genotoxic potential of this compound.

The induction of DNA damage by a variety of agents, such as BaP, is known to activate p53, a tumor suppressor, and oxidative stress also causes stress signals that result in the activation of p53 (Ueno et al., 1999). The recruitment of p53 in response to various genotoxic stresses is an important cellular response in maintaining the integrity of the genome. The activation of p53 seems to be a downstream response to BaP-induced DNA damage (Fig. 5); suggesting p53 plays important defensive roles against BaP-induced genotoxicity. The phosphorylation of the serine 15 residue of p53 is known as a very early step in the activation of p53. As the degree of p53 protein induction was increased 5–10-fold, and the phosphorylated p53 protein 10–15-fold (at high concentrations), suggests the response of phosphorylated p53 towards genotoxic agents could be more sensitive than that of normal p53. Previously, 3-methylcholanthrene, another PAH compound, was found to induce apoptosis, which was preceded by serine 15 phosphorylation and accumulation of p53 (Kwon et al., 2002). Nevertheless, the significance of the decrease in acetylated p53 following exposure to BaP (Fig. 5), as well as the way this process is regulated remain to be fully understood (Fig. 8).

The activation of p53 following DNA damage, in turn, acts as a transcriptional regulator of several target genes, with the gene encoding the Cdk inhibitor, p21, being one of the main targets. BaP-induced p21 protein has been observed in both concentration- (Fig. 6) and time-dependent studies (Fig. 7). The results of the time-course study suggest BaP-induced p53 protein accumulation could lead to an increase in the level of p21 protein (Fig. 7). Moreover, the accumulation of p21 protein following BaP treatment may be transcriptionally regulated, as both the p21 protein and p21 mRNA levels were increased following exposure to BaP (Fig. 9). The increase in the p21 protein might be related to cell cycle arrest, but the levels of the Cdk4 and Rb proteins were unchanged following exposure to BaP (Fig. 8). The significance of increased p21, with no modification of its downstream signaling proteins, Cdk4 and Rb, remains uncertain. Given the incomplete understanding of the meaning of p53 and p21 protein inductions following exposure to BaP, there is a need for further research to elucidate if BaP-induced p53 accumulation leads to cell cycle arrest through the p21 signaling pathway.

PAH-mediated p53 protein accumulation is known to be a post-transcriptional phenomenon, but more recent evidence suggests PAH-stimulated p53 accumulation may also be transcriptionally induced (Pei et al., 1999). The effects of PAHs on p53 expression have recently been investigated (Khan et al., 1997; Venkatachalam et al., 1997). In this study, the level of p53 mRNA increased about two-fold after the treatment of cells with BaP,

whereas the degree of p53 protein induction was more than 2-fold (5–10-fold); indicating p53 is regulated at both the protein and mRNA levels.

Overall, the results of this study suggest BaP leads to serious DNA damage, resulting in accumulation of p53 in the levels of both mRNA and protein. Furthermore, p53 activation in turn up-regulates the level of cellular p21 protein. Oxidative DNA damage and p53 accumulation seem to be related to the toxicity of BaP, however, their potential as biomarkers for the monitoring and risk assessment of BaP needs to be validated in the context of their specificity and sensitivity.

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