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Role of Cell Signalling Involved in Induction of Apoptosis by Benzo[a]pyrene and Cyclopenta[c,d]pyrene in Hepa1c1c7 Cells

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Abstract The reactive metabolites of benzo[a]pyrene (B[a]P) and cyclopenta[c,d]pyrene (CPP) induced an accumulation/phosphorylation of p53 in Hepa1c1c7 cells, whereas inhibition of p53 reduced the apoptosis. Judged by the inhibiting effect of wortmannin, phosphatidyl-inositol-3 (PI-3) kinases such as DNA-dependent protein kinase (DNA-PK), ATM (ataxia-telangiectasia mutated), and/or ATR (ATM related kinase), appeared to be involved in the DNA damage recognition and the B[a]P-/CPP-induced accumulation of p53. B[a]P and CPP also induced phosphorylation of jun-N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK). While inhibition of JNK had no effects on the B[a]P-/CPP-induced apoptosis, inhibition of p38 MAPK activity reduced this effect. Interestingly, survival signals such as phosphorylation of Akt and Bad seemed to be induced by the B[a]P-/CPP-compounds. Furthermore, also extracellular signal-regulated kinase (ERK)1/2 was activated and seemed to function as a survival signal in B[a]P-/CPP-induced apoptosis. J. Cell. Biochem. 93: 1143–1154, 2004.

Key words: apoptosis; PAH; PI-3 kinases; MAP kinases

Polycyclic aromatic hydrocarbons (PAH) constitute a large class of structurally related chemicals with two or more benzene rings. These compounds are formed during incomplete combustion of organic matter and are widespread in the atmosphere, food, and drinking water [IARC, 1983; Knize et al., 1999]. Cyclopenta polycyclic aromatic hydrocarbons (CP-PAH) are a related class of environmental contaminants, which contain an ethylene fragment fused to an unsubstituted PAH, creating an unsaturated 5-membered ring. CP-PAH have been found in a variety of environmental sources including gasoline and diesel exhaust, carbon black, coal combustion emissions, and cigarette smoke [Spitzer and Dannecker, 1983; Tong and Karasek, 1984; Grimmer et al., 1985; IARC, 1986; Schmidt et al., 1986]. Interestingly, the CP-PAH, cyclopenta[c,d]pyrene (CPP; Fig. 1) has been detected in diesel and automobile exhaust at levels 8–10 times higher than benzo[a]pyrene (B[a]P; Fig. 1) [IARC, 1989].

It has long been known that several of the PAH such as B[a]P are carcinogenic. To become carcinogenic B[a]P must first be metabolised by cytochrome P450 monooxygenases (CYP-enzymes; most often CYP1A1) to reactive diol-epoxide metabolites. The reactive metabolites may bind covalently to DNA and cause mutations and cancer [Nebert et al., 2000]. Like

Abbreviations used: αNF, α-naphthoflavone; ATM, ataxia-telangiectasia mutated; ATR, ATM related kinase; B[a]P, benzo[a]pyrene; CPP, cyclopenta[c,d]pyrene; CP-PAH, cyclopenta polycyclic aromatic hydrocarbons; DMSO, dimethyl sulfoxide; DNA-PK, DNA-dependent protein kinase; ERK, extracellular signal-regulated kinase; FBS, foetal bovine serum; HRP, horseradish peroxidase; MAPK, mitogen activated protein kinase; MDM2, murine double minute 2; NFκB, nuclear factor κB; PAH, polycyclic aromatic hydrocarbons; PFT-α, pifithrin-α; PI, propidium iodide; PI-3, phosphatidyl-inositol-3; PMSF, phenylmethyl-sulfonyl fluoride; SAPK/JNK, stress activated protein kinase/jun N-terminal kinase.

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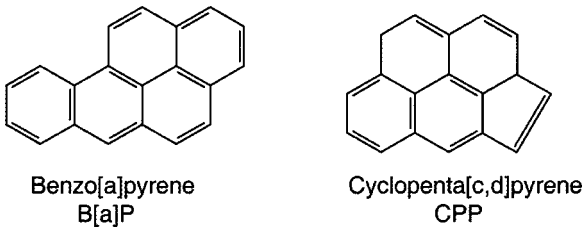

Fig. 1. Chemical structures of benzo[a]pyrene (B[a]P) and cyclopenta[c,d]pyrene (CPP).

B[a]P, CPP is also activated by CYP1A1 to mutagenic and carcinogenic metabolites [Eisenstadt and Gold, 1978; Kwon et al., 1992].

Several PAH and CP-PAH compounds have also been shown to induce apoptosis in vitro [Burchiel et al., 1993; Rorke et al., 1998; Salas and Burchiel, 1998; Kwon et al., 2002; Chen et al., 2003; Solhaug et al., 2004]. The onset of apoptosis by genotoxic chemicals like B[a]P may be viewed to proceed in three major steps. The first step often requires a cell-specific activation of the chemical to reactive metabolites that covalently bind to macromolecules. In the second step, the damage is recognised. The third step is defined by a number of cellular events that starts from the recognition of the primary cellular damage and ends up with cell death. Various electrophilic metabolites may be formed from the same parent compound, and different macromolecules may be damaged by identical reactive molecules. Furthermore, the DNA molecule may be damaged at a number of different places, and even similar damage often initiates very different responses at the same time. Both cell survival signals involving the DNA repair system and components in the cell cycle machinery, as well as apoptotic signals may be triggered. Interestingly, in our recent study [Solhaug et al., 2004] we found indications that the unmetabolised B[a]P and CPP compounds initiated cell survival signals, measured as increased phosphorylation of Bad.

Several cellular signalling pathways, including mitogen activated protein kinases (MAPKs) pathways, have been proposed to have a role in cellular events following exposure to carcinogenic chemicals, including apoptosis. In mammalian systems, there are three subgroups of MAPKs: extracellular signal-regulated kinases (ERKs), SAPK/JNK (stress activated protein kinase/jun N-terminal kinase), and p38 MAPK. In general ERK is activated in response to mitogen or growth factor stimulation, and has been suggested to give important surviving signals modulating receptor-induced apoptosis [Wilson et al., 1999; Tran et al., 2001]. A variety of cellular stress initiators have been shown to activate JNK and p38 MAPK [Lei et al., 1998; Assefa et al., 2000; Kwon et al., 2002; Chen et al., 2003]. MAPK pathways are considered to transmit environmental signals from the cell membrane to the nucleus through phosphorylation cascades, resulting in the activation of transcription factors, which in turn regulate gene expression. Among these transcription factors is the tumour suppressor protein p53 [Hu et al., 1997; She et al., 2000; Kwon et al., 2002], which is considered to play an important role in the genotoxic stress responses. Following DNA damage p53 is often stabilised and activated by phosphorylation, translocated to the nucleus where it may lead to the induction of a set of target genes involved in cell cycle arrest, DNA repair, or apoptosis [Vousden, 2000; Mitchell et al., 2003]. The response depends on the type and strength of the signal as well as the cell type. Although it is still somewhat unclear how toxic exposure results in p53 phosphorylation, various protein kinases, such as DNA-dependent protein kinase (DNA-PK), ataxia-telangiectasia mutated (ATM) kinase, and ATM related kinase (ATR) are often considered to be important sensors of DNA damage and stress related to DNA synthesis and replication (reviewed in [Yang et al., 2003]).

We have earlier reported that B[a]P and several CP-PAH compounds induce expression of CYP1A1 in Hepa1c1c7 cells, which corresponded well with their relative apoptotic responses. Both mitochondria as well as receptor-mediated pathways seemed to be involved [Solhaug et al., 2004]. The aims of the present study were to further characterise the cellular stress responses by B[a]P and CPP in Hepa1c1c7 cells, and to explore the possible role of cell signalling in the apoptotic process.

MATERIALS AND METHODS

Chemicals and Cell Culture Media

CPP was obtained from National Cancer Institute, Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO). B[a]P, α-naphthoflavone (αNF), ponceau S, dimethyl sulfoxide (DMSO), propidium iodide (PI), Nonidet P-40, RNase A (R5000), phenylmethylsulfonyl fluoride (PMSF), Hoechst

33258, Hoechst 33342, aprotinin, and proteinase K (KP0390) were obtained from Sigma Chemical Company (St. Louis, MO). Pepstatin A was from Calbiochem (La Jolla, CA) and leupeptin from Amersham Biosciences (Buchinghamshire, UK). Wortmannin, PD169316, pifithrin-α (PFT-α), PD98059, SB202190 were from Calbiochem (Cambridge, MA), and U0126 from Cell Signaling, (Beverly, MA). D-JNKI1 was from Alexis Biochemicals (Lausen, Switzerland). SeaKem GTG agarose and Gel star nucleic acid gel stains were obtained from FMC Bioproducts (Rockland, ME) and the Bio-Rad DC protein assay from Bio-Rad Laboratories, Inc. (Hercules, CA). MEM alpha medium with L-glutamine, without ribonucleosides and deoxyribonucleosides, foetal bovine serum (FBS) and gentamycin were from Gibco BRL (Paisley, Scotland, UK). All other chemicals were purchased from commercial sources and were of analytical grade.

Antibodies

Antibodies against: cleaved caspase-3, phospho-Bad (Ser112), phospho-Bad (Ser115), Bad, phospho-p38 MAPK, p38 MAPK, phospho-JNK and JNK, phospho-Akt (Ser473), Akt and phospho-p53 (Ser15) were obtained from Cell Signaling; phospho-ERK1/2, ERK2, and murine double minute 2 (MDM2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); p53 (CM5) from Novocastra Laboratories Ltd., (Newcastle, UK) and β-actin from Sigma Chemical Company. As secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Sigma Chemical Company) and HRP-conjugated rabbit anti-goat or rabbit anti-mouse IgG from Dako (Glostrup, Denmark) were applied.

Cell Cultures and Treatments

Mouse hepatoma cell line Hepa1c1c7 (purchased from European Collection of Cell Culture; ECACC) was maintained in MEM alpha medium with L-glutamine, without ribonucleosides and deoxyribonucleosides, and supplemented with 10% heat-inactivated FBS and 0.1 mg/ml gentamicin in 5% CO_2 at 37°C. The cells were routinely kept in logarithmic growth phase at $1.0-9.0 \times 10^6$ cells/75 cm^2 by splitting the cells twice a week. The cells were seeded near confluence the day before treatment. The medium was replaced with fresh medium before treating the cells with inhibitors and/or B[a]P/CPP. For treatment with inhibitors, cells were pre-incubated with the inhibitor for 1 h, followed by B[a]P/CPP treatment for the time indicated. The inhibitors and B[a]P/CPP were dissolved in DMSO (final concentration of DMSO in cell culture was 0.5%) and used at concentrations and time points previously found to be effective by others. Appropriate controls containing the same solvent were included in each experiment.

Flow Cytometry

The percentage of apoptotic cells and cells in different phases of the cell cycle were determined by flow cytometry. After treatment, the cells were prepared for flow cytometry with Triton X-100 (0.1%) and Hoechst 33258 (1.0 μg/ml) for the staining of cellular DNA. The histograms were recorded on a Skatron Argus 100 flow cytometer and analysed using the Multiplus Program (Phoenix Flow Systems, San Diego, CA). The different cell phases as well as apoptotic cells/bodies and secondary necrotic cells were distinguished on the basis of their DNA content (Hoechst fluorescence; channel number) and cell size (forward light scatter; channel number) [Gorczyca et al., 1993; Wiger et al., 1997]. Apoptotic index was determined as the percentage of signals between the G_1 peak and the channel positioned at 20% of the G_1 peak. When inhibitors are used, the apoptosis induced by B[a]P and CPP alone are normalised to 100%, except in Figure 1, which shows the absolute frequency of apoptosis.

Microscopic Characterisation of Cells

Plasma membrane damage and changes in nuclear morphology associated with necrosis and apoptosis, were determined after staining cells (approximately 0.5×10^6 cells) with PI (10 μg/ml) and Hoechst 33342 (5 μg/m) for 30 min, as previously described [Solhaug et al., 2004].

DNA Fragmentation Assay

DNA fragmentation assay was performed according to the method of Gorczyca et al. [1993], with minor modifications [Wiger et al., 1997]. Briefly, cells were washed, resuspended, and incubated in buffer containing RNase. Then proteinase K was added and the samples were further incubated prior to addition of loading buffer. The samples, containing DNA from 0.1×10^6 cells were run on an agarose gel (1.5%).

Cell Lysis and Western Blotting

After treatment the cells were washed twice in ice-cold phosphate-buffered saline (PBS) and incubated with ice-cold lysis buffer (20 mM Tris; pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) on ice for 5 min, scraped, sonicated, and clarified by centrifugation. Western blot analysis was performed as previously described [Solhaug et al., 2004]. The blots were stripped by incubating in stripping-buffer (62.5 mM Tris; pH 6.8, 10% SDS, 100 mM β-mercaptoethanol) for 0.5 h at 60°C.

RESULTS

Metabolic Activation of B[a]P and CPP

In order to elucidate a possible role of reactive electrophilic metabolites in B[a]P-/CPP-induced apoptosis, we preincubated the cells with the CYP1A1 inhibitor, αNF. αNF markedly reduced both B[a]P- and CPP-induced apoptosis, determined by flow cytometric analyses (Fig. 2A), DNA fragmentation assay (Fig. 2B), and fluorescence microscopy (data not shown). Analyses by flow cytometry revealed that B[a]P and CPP also caused a marked accumulation of cells in S phase, which could be reduced by the addition of αNF (Fig. 2A).

Role of p53 in B[a]P-/CPP-Induced Apoptosis

An increase in the number of apoptotic cells was observed starting at 10–15 h after exposure to B[a]P (20 μM) and CPP (30 μM) [Solhaug et al., 2004], (data not shown). To explore a possible role of p53 in B[a]P-/CPP-induced apoptosis, we analysed whole cell extracts from Hepa1c1c7 cells exposed to various concentrations of B[a]P or CPP for 25 h by Western blotting. Both protein level and phosphorylation (Ser15) of p53 increased markedly after treatment with high, apoptotic concentrations of B[a]P or CPP, whereas at lower concentrations no changes were seen (Fig. 3A). PFT-α is a chemical inhibitor of p53, that has been demonstrated to effectively block p53-dependent transcriptional activation and apoptosis [Komarov et al., 1999; Lorenzo et al., 2002; Proietti et al., 2003]. Accumulation of p53 caused by B[a]P and in particular CPP, was markedly reduced upon

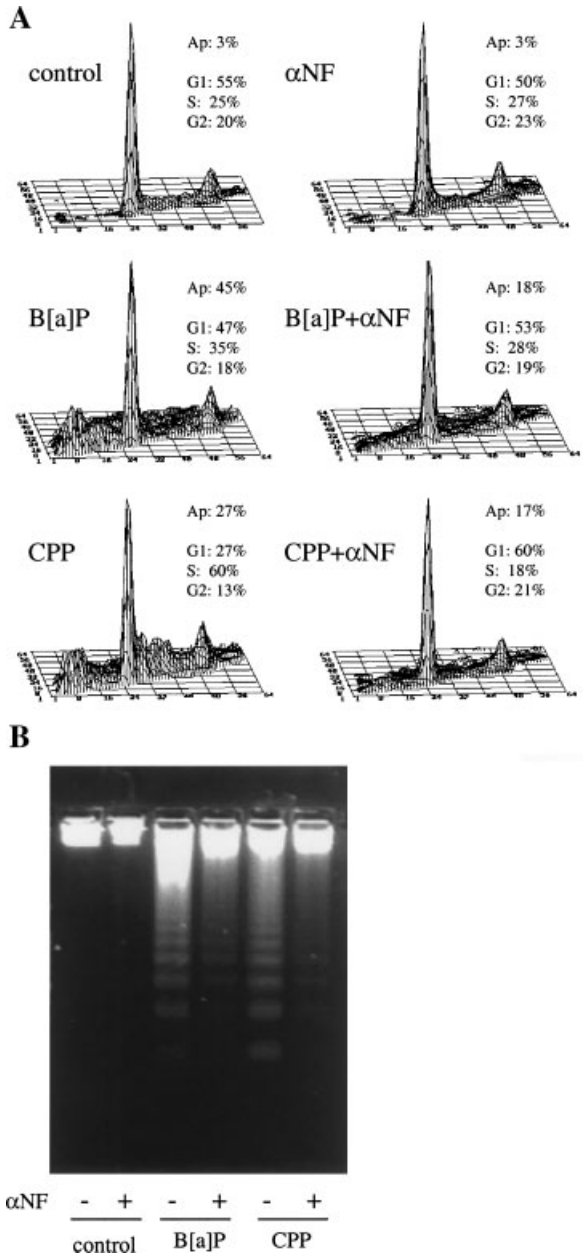

Fig. 2. Effects of α-naphthoflavone (αNF) on B[a]P-/CPP-induced apoptosis and accumulation of cells in S phase. Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) for 25 h in the presence or absence of αNF (25 μM) and analysed by flow cytometry (**A**) and by DNA fragmentation assay (**B**) for effects on apoptosis and cell cycle as described in "Materials and Methods." Computer-drawn two parameter histograms of the result from flow cytometry (cell size vs. DNA content; channel number) representing light scatter versus Hoechst 333258 fluorescence are shown. In general, apoptotic cells (Ap) have cell size and DNA content less than G_1 cells, whereas necrotic cells often have an increased cell size. Results from one representative experiment out of three are shown.

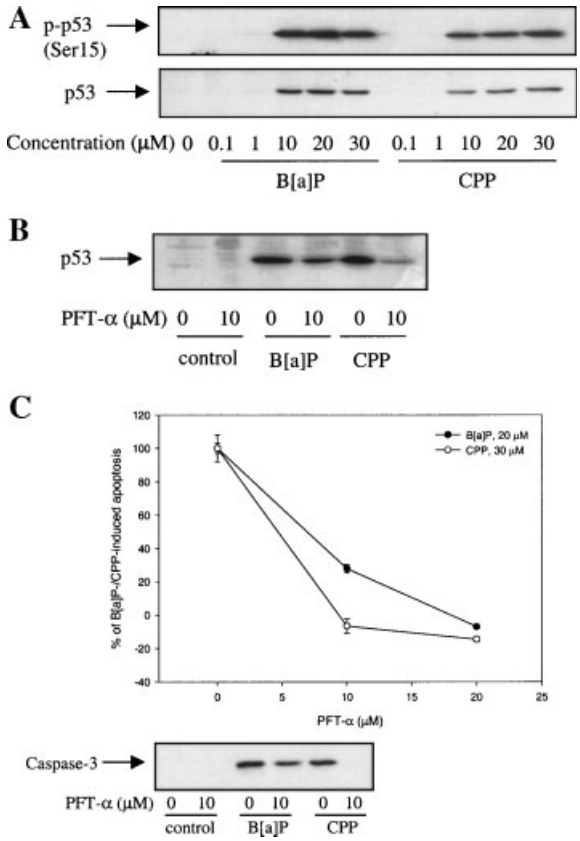

Fig. 3. p53 in B[a]P-/CPP-induced apoptosis. **A**: Expression of p53 and p53 phosphorylated at Ser15 in response to B[a]P and CPP at different concentrations (0.1–30 μM) after 25 h exposure of Hepa1c1c7 cells, analysed by Western blot. **B**, **C**: The effect of the p53 inhibitor, pifithrin-α (PFT-α) on B[a]P-/CPP-induced p53 expression and apoptosis. Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) in the presence or absence of PFT-α (10 or 20 μM) for 25 h and analysed for p53 expression by Western blotting (B), for apoptotic cells by flow cytometry and for cleavage of caspase-3 by Western blotting, using specific antibody to the active form of caspase-3 (17 kDa) (C). The effect of PFT-α is presented in percent of the B[a]P-/CPP-induced apoptosis, respectively. The absolute frequency of apoptosis induced by B[a]P and CPP are shown in Figure 2A. Results are representative of 2–3 experiments (Western blotting), or data from one of three independent experiments, expressed as mean ± SE of three parallels (flow cytometry).

treatment with PFT-α (Fig. 3B). PFT-α also resulted in an almost complete blockade of the B[a]P-/CPP-induced apoptosis and activation of caspase-3 (Fig. 3C). A similar reduction of the necrosis was also seen (data not shown).

Involvement of Phosphatidyl-Inositol-3 (PI-3) Kinases in B[a]P-/CPP-Induced Apoptosis

Wortmannin, an inhibitor of kinases of the PI-3 kinase family members such as DNA-PK, ATM, and ATR, reduced the B[a]P- and CPP-induced accumulation of p53 (Fig. 4A). Pretreatment with wortmannin in combination with B[a]P resulted in an increase of apoptosis, whereas in combination with CPP, apoptosis was reduced (Fig. 4B). Similar findings were observed looking at the activation of caspase-3 (Fig. 4B). Akt is a serine/threonine kinase that mediates the PI-3 kinase-related cell survival signalling pathway in the cytosol. To elucidate the possible involvement of this PI-3 kinase, we examined the effect of B[a]P and CPP on Akt phosphorylation (activation) by Western blot analysis with antibodies that recognise the activated phosphorylated form of Akt (Ser473). As shown in Figure 5A, both B[a]P and CPP induced phosphorylation of Akt. Already after

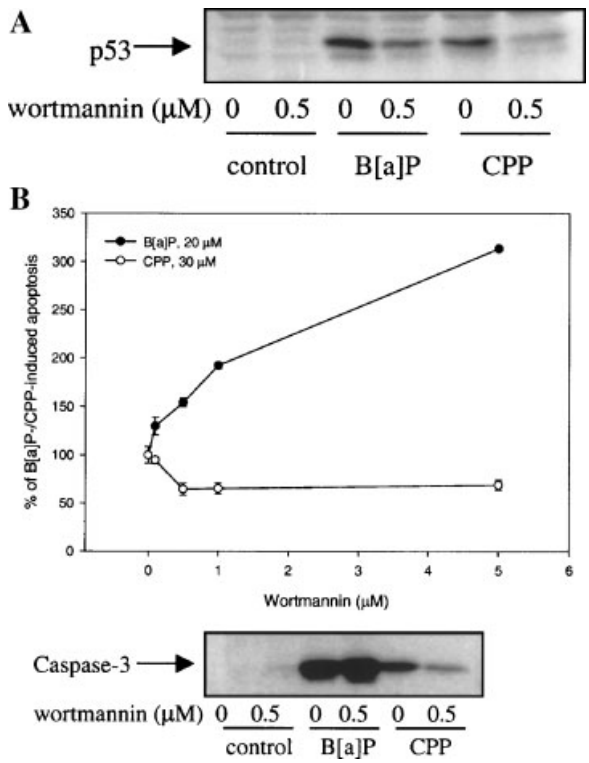

Fig. 4. Role of phosphatidyl-inositol-3 (PI-3) kinases in B[a]P-/CPP-induced apoptosis. The effect of wortmannin on p53 expression and apoptosis induced by B[a]P or CPP. Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) in the presence or absence of the PI-3 kinase inhibitor, wortmannin (0.1–5 μM) and analysed for p53 expression by Western blotting after 20 h exposure (**A**), for apoptotic cells by flow cytometry after 25 h exposure, and for cleavage of caspase-3 by Western blotting after 20 h exposure (**B**). The effect of wortmannin is presented in percent of the B[a]P-/CPP-induced apoptosis, respectively. Results are representative of 2–3 independent experiments (Western blotting), or data from one of four independent experiments expressed as mean ± SE of three parallels (flow cytometry).

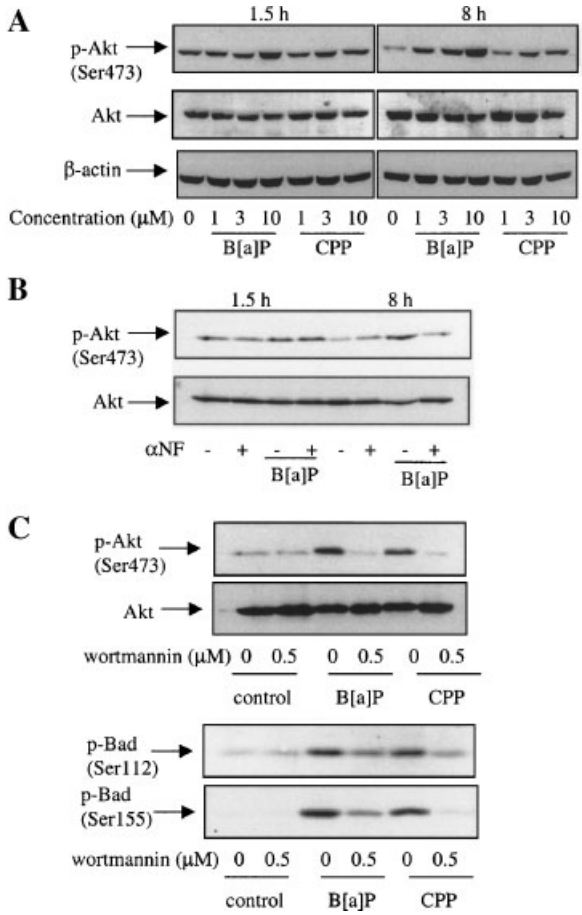

Fig. 5. Survival signals in response to B[a]P and CPP. **A**: Expression of Akt and Akt phosphorylated at Ser473 at different concentrations (1–10 μM) in B[a]P-/CPP-treated Hepa1c1c7 cells after 1.5 or 8 h exposure as analysed by Western blot analysis. **B**: The effect of αNF on expression and phosphorylation (Ser437) of Akt. Hepa1c1c7 cells were incubated with B[a]P (10 μM) for 1.5 or 8 h in the presence or absence of αNF (25 μM) and processed as described in A. **C**: The effect of wortmannin on the phosphorylation of Akt (Ser437) and Bad phosphorylated at Ser112 or Ser155. Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) for 20 h in the presence or absence of wortmannin (0.5 μM) and analysed by Western blotting. Results from one representative of 2–3 experiments are shown.

1.5 h B[a]P induced a marked phosphorylation of Akt, while CPP-induced Akt phosphorylation seemed to be more delayed. At the time-points and concentrations where Akt was activated by B[a]P or CPP, no apoptosis could be seen (data not shown). B[a]P-induced phosphorylation of Akt could only be inhibited by αNF after 8 h exposure (Fig. 5B). We have recently identified phosphorylation of Bad at Ser112 and Ser155 as a survival signal induced by B[a]P and CPP in Hepa1c1c7 cells [Solhaug et al., 2004]. To examine a possible link between Akt and Bad, we studied the effect of wortmannin on the phosphorylation of these proteins. As seen in Figure 5C, wortmannin clearly reduced both B[a]P- and CPP-induced phosphorylation of Akt (Ser437) and Bad (both at Ser112 and Ser155).

Involvement of p38 MAPK, ERK1/2, and JNK in B[a]P-/CPP-Induced Apoptosis?

To elucidate any possible role of ERK, p38 MAPK, and JNK in B[a]P- and CPP-induced apoptosis, we analysed the relative levels of their respective activated/phosphorylated forms. B[a]P exposure resulted in a small and transient increased phosphorylation of ERK1/2 first seen at 8 h after start of exposure (data from earlier time points not shown), while for CPP no effects on ERK1/2 were observed (Fig. 6). In contrast, B[a]P and CPP exposure, resulted in a marked and sustained increase of phosho-p38, first observed after 8 and 20 h respectively. B[a]P gave a strong phosphorylation of JNK at 8 h, with a subsequent time-dependent decline. CPP also enhanced the level of phosphorylated JNK, but this increase was first observed at 20 h exposure (Fig. 6). In order to study the mechanisms involved in stress-induced MAPKs, we treated the cells with αNF (1.5 and 8 h) and wortmannin (20 h) in

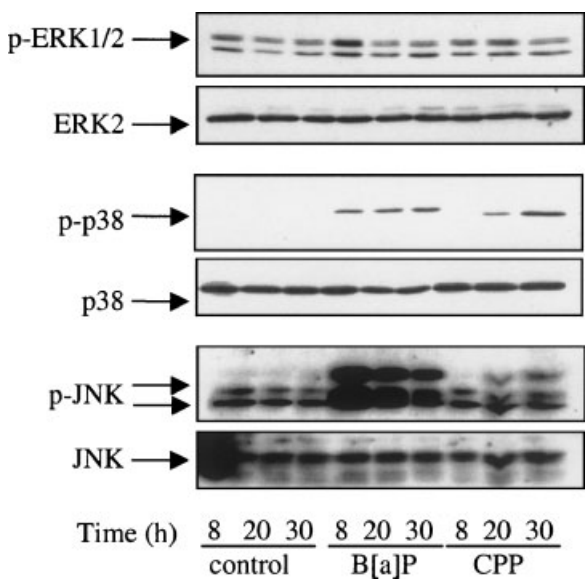

Fig. 6. Expression of JNK, ERK1/2 and p38 MAPK following B[a]P or CPP exposure. Hepa1c1c7 cells were incubated with B[a]P or CPP (30 μM) for 8, 20, and 30 h. The cell lysates were analysed for the phosphorylated forms of JNK, ERK1/2, or p38 MAPK by Western blotting. The blots were stripped and reprobed with antibodies to the respective proteins. Results from one representative of 2–3 experiments are shown.

combination with B[a]P or CPP and analysed the activation of JNK. An increased level of phospho-JNK was observed after 8 h, which could be inhibited by αNF as well as by wortmannin (Fig. 7).

Next, we examined a possible role of ERK1/2, p38 MAPK, and JNK in B[a]P-/CPP-induced apoptosis, using specific inhibitors of these MAP kinases. Pre-treatment with PD98059, an inhibitor of ERK1/2, reduced B[a]P-/CPP-induced apoptosis as measured with flow cytometry. In contrast, another inhibitor of ERK1/2 activity, U0126, increased the induced apoptosis (Fig. 8A). This discrepancy is most probably explained by a marked inhibitory effect of PD98059 on the metabolism of B[a]P to reactive metabolites (data not shown). Pre-treatment with the p38 MAPK inhibitors, SB202190 or PD169316 [Kummer et al., 1997], reduced both B[a]P- as well as CPP-induced apoptosis (Fig. 8B). Accordingly, the ERK1/2 inhibitor PD98059 and the p38 MAPK inhibitors also reduced the chemical-induced activation of caspase-3. The other ERK1/2 inhibitor U0126, caused an increased activation of caspase-3 after exposure with B[a]P, whereas no changes with CPP. The JNK inhibitor D-JNKI1 [Bonny et al., 2001] (2 μM) had no marked effect on B[a]P (20 μM)-/CPP (30 μM)-induced apoptosis after 25 h exposure (B[a]P, 100% ± 10%; B[a]P + JNK1, 103% ± 1%; CPP, 100% ± 13%; CPP + JNK1, 82% ± 4%).

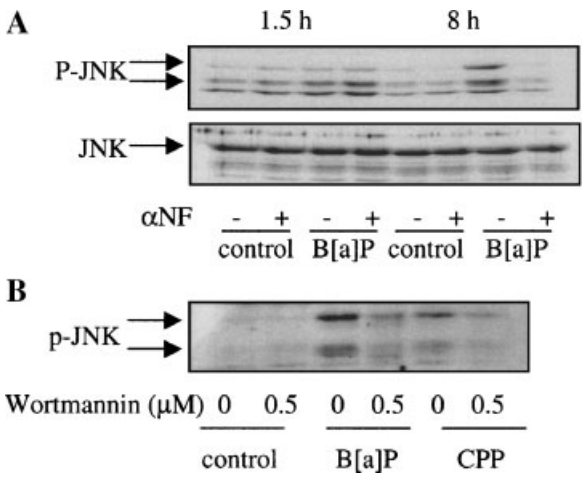

Fig. 7. Role of PI-3 kinases in the phosphorylation of JNK **A**: The effect of αNF on phosphorylation of JNK. Hepa1c1c7 cells were incubated with B[a]P (10 μM) for 1.5 or 8 h in the presence or absence of αNF (25 μM) and analysed by Western blotting. **B**: The effect of wortmannin on the phosphorylation of JNK. Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) for 20 h in the presence or absence of wortmannin (0.5 μM) and analysed by Western blotting. Results from one representative of 2–3 experiments are shown.

Relationship Between p38 MAPK Activation and the Level of p53 in B[a]P-/CPP-Induced Apoptosis

To evaluate the importance of reactive metabolites in B[a]P-/CPP-induced activation of p38 MAPK, we examined the effect of αNF on p38 phosphorylation. αNF reduced both B[a]P- and CPP-induced phosphorylation of p38 MAPK (Fig. 9A). We then tested the involvement of p38 MAPK in the accumulation of p53 induced by B[a]P and CPP. The B[a]P-/CPP-induced accumulations of p53 were reduced by inhibitors of p38 MAPK activation.

DISCUSSION

The overall cellular response to genotoxic carcinogens, not only the DNA repair process, but also modulation of cell survival and cell death pathways, may be of great importance for the mutation frequency and are thus a central part of the cancer development. In a recent study, we reported that B[a]P and CPP induce both apoptotic as well as anti-apoptotic signals in Hepa1c1c7 cells and suggested that the final results may be an increased mutation frequency and probability to survive DNA damage [Solhaug et al., 2004]. In the present study, we further elucidate molecular signalling events that could be involved in these processes.

The CYP1A1 inhibitor αNF reduced both B[a]P- and CPP-induced apoptosis in Hepa1c1c7 cells as measured by flow cytometry and DNA fragmentation (Fig. 2), illustrating that the formation of reactive electrophilic metabolites seems to be an important initiating step. p53 is considered to play a major role in regulating the response of mammalian cells to cellular stress and DNA-damage. Here, we show that B[a]P and CPP induce a concentration-dependent accumulation and phosphorylation at (Ser15) of p53 (Fig. 3A). The increase could be seen at earlier time points (8 h), but at concentrations that later resulted in apoptosis [Solhaug et al., 2004]. As also previously reported [Solhaug et al., 2004] this accumulation appears to depend on the generation of electrophilic metabolites. Activation of p53 is most often a result of DNA damage, although there are exceptions

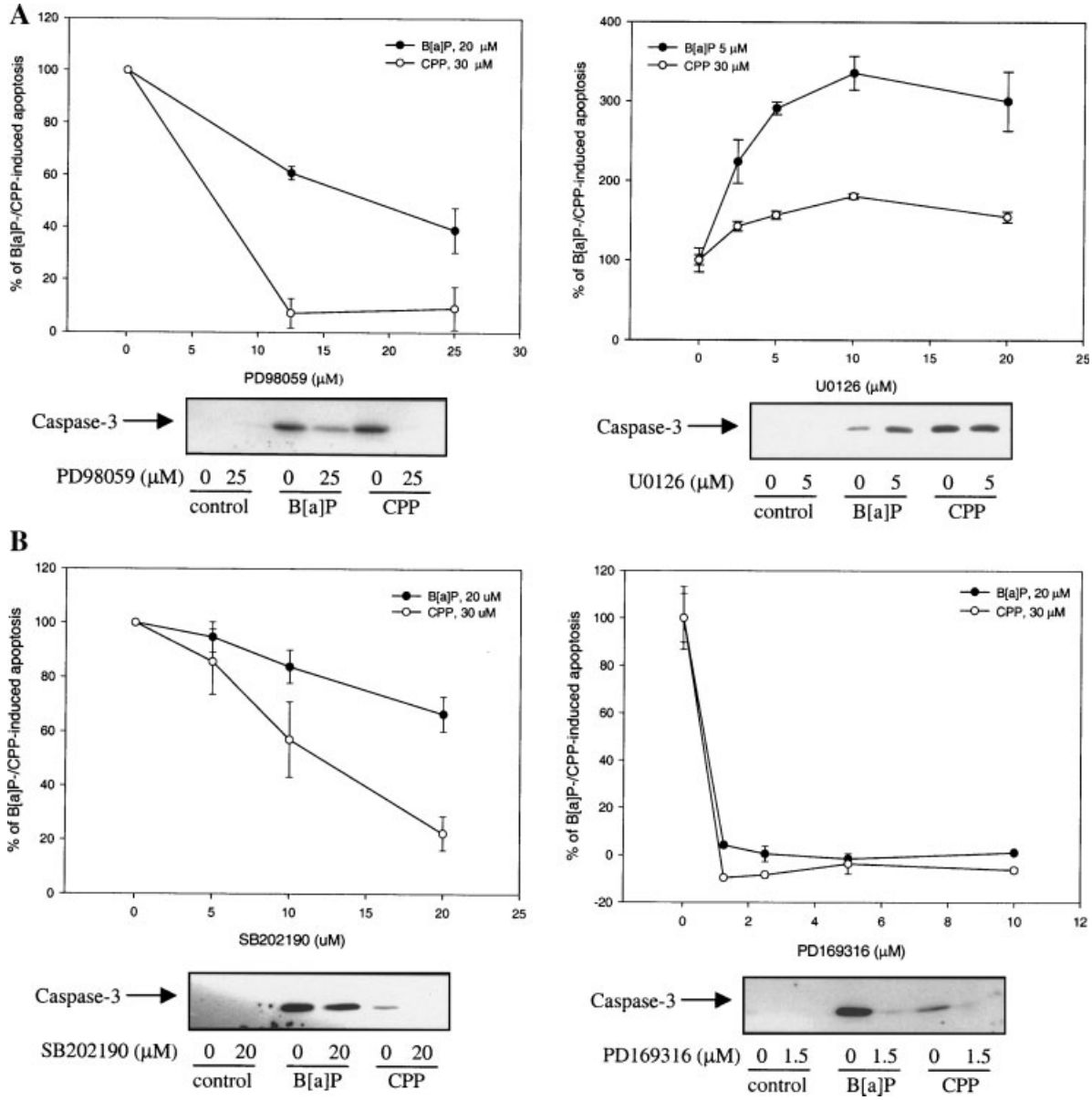

Fig. 8. Role of ERK and p38 MAPK in B[a]P-/CPP-induced apoptosis Hepa1c1c7 cells were incubated with B[a]P (20 or 5 μM) or CPP (30 μM) for 25 h in the presence or absence of (**A**) ERK inhibitors (PD98059 or U0126) or (**B**) p38 MAPK inhibitors (SB202190 or PD169316) and analysed for apoptotic cells by flow cytometry. The effect of the inhibitors are presented in percent of the B[a]P-/CPP-induced apoptosis, respectively. The data are mean ± SE of three parallels and are representative of at least 2–3 experiments. Caspase-3 cleavage were analysed by Western blot, using specific antibody to the active form of caspase-3 (17 kDa), after the cells had been incubated with B[a]P (20 or 5 μM) or CPP (30 μM) for 25 h in the presence or absence of (A) ERK inhibitor (PD98059 25 μM or U0126 10 μM) and (B) p38 inhibitor (SB202190 20 μM or PD169316 1.5 μM). In combination with U0126, the cells were exposed to B[a]P 5 μM, otherwise 20 μM B[a]P were used. The caspase blots are representative of two experiments.

[Pluquet and Hainaut, 2001]. Furthermore, metabolites of B[a]P and CPP are well known to bind to DNA, and the induced accumulation of p53 was inhibited by the PI-3 kinase inhibitor, wortmannin [Sarkaria et al., 1998] (Fig. 4A). It has been reported that the nuclear PI-3 kinases, DNA-PK, and ATM are more sensitive to wortmannin than ATR [Sarkaria et al., 1998]. Nevertheless, it is difficult to conclude from these experiments which of the PI-3 kinases that are causing the up-regulation of p53. However, some of the nuclear PI-3 kinases

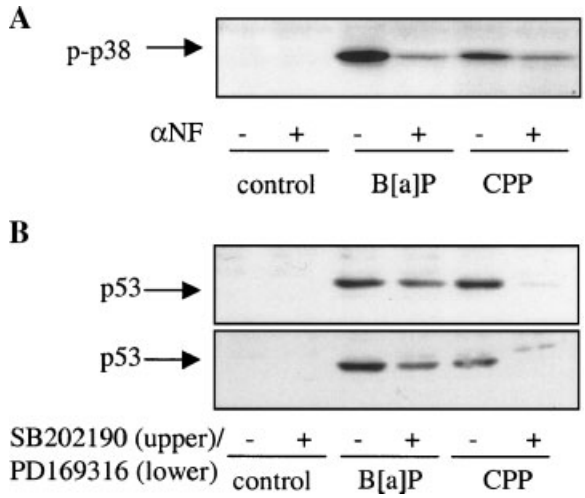

Fig. 9. Relationship between activation of p38 MAPK and p53 Hepa1c1c7 cells were incubated with B[a]P (20 μM) or CPP (30 μM) for 25 h in the presence or absence of (**A**) the CYP1A1 inhibitor, αNF (25 μM) or (**B**) the p38 MAPK inhibitors SB202190 (20 μM) and PD169316 (1.5 μM) and analysed by Western blotting. The blots are representative of 2–4 experiments.

seem to be important in the damage-recognition and activation of p53 [Yang et al., 2003].

The p53 inhibitor PFT-α clearly inhibited both B[a]P- and CPP-induced accumulation of p53, apoptosis and cleavage of caspase-3 (Fig. 3B,C). This indicates that p53 could be mediating B[a]P-/CPP-induced apoptosis via activation of the caspase pathway, in response to DNA damage generated by reactive metabolites. This suggestion is supported by experiments showing that B[a]P induces translocation of p53 to the nucleus and Bax to the mitochondria, as well as activation of caspase-8 and Bid [Solhaug et al., 2004].

MDM2 has been shown to play a role in allowing export of p53 from the nucleus to the cytoplasm, and degradation of p53 by the ubiquitin–proteasome pathway. MDM2 is also a transcriptional target of p53, thereby creating an autoregulatory feedback loop [Alarcon-Vargas and Ronai, 2002]. Both B[a]P and CPP treatment led to a down-regulation of MDM2 expression (data not shown), which has also been seen in response to UV radiation and treatment with the DNA damaging agents mitomycin C and methylmethane sulfonate in RKO cells [Inoue et al., 2001]. Down-regulation of MDM2 expression has therefore been suggested to be a secondary mechanism, in addition to increased phosphorylation of p53, that can led to stabilisation of p53 [Maki, 1999], and may thereby also have a role in the apoptotic process caused by B[a]P and CPP.

Interestingly, wortmannin enhanced B[a]P-induced apoptosis while it had the opposite effect on CPP-induced apoptosis (Fig. 4B). A possible explanation to this could be that wortmannin-sensitive cell survival signals in the cytosol, such as the PI-3 kinase Akt [Datta et al., 1999; Tomita et al., 2003], are more important in the B[a]P-induced than in the CPP-induced apoptotic process. This suggestion is in accordance with the findings that B[a]P induced phosphorylation of Akt and Bad to somewhat larger extent than CPP (Fig. 5). Interestingly, it has been demonstrated that B[a]P can mimic signalling through the insulin-like growth factor-I receptor and increase cell survival through PI-3 kinase activation in human mammary epithelial cells [Tannheimer et al., 1998]. Viewed in the light of these results, we suggest that B[a]P induces cell survival through activation of Akt, and that this occur to a lesser extent by CPP.

Akt has been found to suppress apoptosis through phosphorylation (inactivation) of Bad, caspase-9, the forkhead transcription factor, and IκB kinases [Datta et al., 1999]. The Bcl-2 protein Bad is considered to be a cell-death promoter because it can bind Bcl-2 and Bcl-xl and inhibit their anti-apoptotic effects. Phosphorylation of Bad at either Ser112 or Ser136 reduces its ability to form heterodimers with Bcl-2 or Bcl-xl, thereby promoting cell survival [Zha et al., 1996]. Both B[a]P and CPP induced phosphorylation of Bad at Ser112 and Ser155, which could be inhibited by wortmannin (Fig. 5C). Neither B[a]P nor CPP induced phosphorylation of Bad at Ser136 (data not shown). The effect of wortmannin indicates that Akt may act upstream of Bad as a survival signal. Interestingly, activation of Akt is an early event after B[a]P/CPP exposure that occurs at low concentrations compared to what causing apoptosis. We have previously reported that the up-regulation of phosphorylated Bad seems to be independent of reactive electrophilic metabolites, since αNF had no effect on the phosphorylation of Bad and the effect could be seen already after 1/2–2 h after start of exposure [Solhaug et al., 2004]. This suggestion is further supported by an early, αNF-insensitive up-regulation of Akt. Only the Ser136 phosphorylation site at Bad has so far been demonstrated to be a direct target of Akt [Datta et al.,

1997]. Although, the signalling cascades regulating Ser112 and Ser155 phosphorylation are not yet clearly understood, both ERK and protein kinase A have been reported to participate in the regulation of Bad-phosphorylation, respectively, at Ser112 and Ser155 [Scheid and Duronio, 1998; Lizcano et al., 2000]. The fact that wortmannin reduced phosphorylation of Bad at these sites, implicates that a PI-3 kinase also is involved as a cell surviving signal in response to B[a]P and CPP exposure.

JNK has been reported both as an apoptotic signal [Levresse et al., 2000; Yoshii et al., 2001; Tomita et al., 2003], to support in cell survival [Potapova et al., 1997], as well as to be of minor importance in the apoptotic process [Lei et al., 1998; Wilson et al., 1999]. We found that B[a]P-/CPP-induced phosphorylation of JNK was inhibited by αNF (8 h) as well as wortmannin (20 h) (Fig. 7), suggesting that this activation is mediated through DNA-damage and ATM/ATR/DNA-PK. However, the apparent lack of effect of the JNK inhibitor D-JNKI1 [Bonny et al., 2001] on apoptosis indicates that neither the induction of JNK nor the JNK activity are of major importance for the induction of apoptosis. This suggestion is also in accordance with results from previous studies [Lei et al., 1998] and present studies (data not shown) showing that B[a]P induced JNK at low concentrations which did not result in apoptosis.

In addition to JNK, both ERK and p38 MAPK have been suggested to be involved in PAH-induced apoptosis [Chin et al., 1998; Kwon et al., 2002; Chen et al., 2003]. Several studies have found that ERK acts as an anti-apoptotic mediator [Wilson et al., 1999; Tran et al., 2001], but it has also been reported that ERK mediates apoptotic effects in response to DNA damage [Chen et al., 2003]. In our study, phospho-ERK is somewhat up-regulated in response to B[a]P (and benz[j]aceanthrylene, data not shown) after 8 h exposure, while no such effect could be seen after treatment with CPP (Fig. 6). Treatment with U0126, markedly increased B[a]P-/CPP-induced apoptosis (Fig. 8A). These findings indicate that ERK acts as an anti-apoptotic factor in response to both B[a]P and CPP, but that the effect is most prominent for B[a]P.

In the present study, we also found that B[a]P and CPP induced a phosphorylation of p38 MAPK, which seemed to be dependent on the generation of reactive electrophilic metabolites (Fig. 9A). Combined with the finding that pretreatment with p38 MAPK-specific inhibitors, SB202190 or PD169316 reduced both B[a]P- and CPP-induced apoptosis (Fig. 8B), this indicates a role of p38 MAPK in the apoptotic process. Interestingly, we found that inhibitors against p38 MAPK activity reduced the accumulation of p53 (Fig. 9B), which indicates that p38 MAPK activity is needed for p53 accumulation. In agreement with our findings, others have also reported that p38 MAPK seems to act upstream of p53. In addition to phosphorylating p53 at Ser15 [She et al., 2000; Kim et al., 2002; Kwon et al., 2002], p38 MAPK may also activate nuclear factor κB (NFκB) resulting in increased transcriptional expression of p53 [Kim et al., 2002; Shimada et al., 2003]. Although NFκB is known to protect cells from apoptosis in most cases, it is also known to contribute to apoptosis depending on cell types and extracellular stimuli [Foo and Nolan, 1999].

However, care should be taken when basing the suggestions on induced activities and on use of inhibitors. Induced activities may be parallel events and the inhibitors may have unspecific effects such as seen with PD98059. Such studies should therefore be followed up with other studies using, for example, using reactive metabolites, cells with gene knockouts, or siRNA techniques.

Overall, the present data suggest that both Akt and ERK act as anti-apoptotic signals at least partly by increasing p-Bad. JNK is induced, but is apparently not important in the apoptotic process. In contrast DNA-PK, ATM and/or ATR, p53, and p38 MAPK seem to have roles in the pro-apoptotic signalling pathway(s) induced by B[a]P and CPP (Fig. 10).

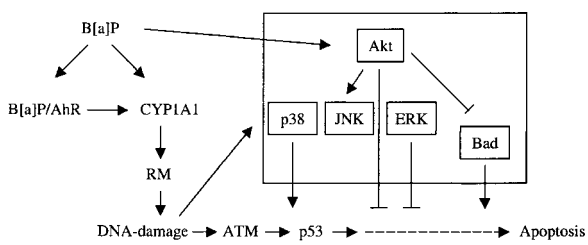

Fig. 10. A model for B[a]P-/CPP-induced signalling pathway involved in apoptosis. B[a]P/CPP induce expression and activation of CYP1A1, which leads to the generation of reactive metabolites (RM) and DNA-damage. The DNA-damage leads to induction of apoptosis, activation of PI-3 kinases, p53, and MAPK. Survival signals, such as activation of Akt, seem to be induced by the unmetabolised compounds.

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