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GENOTOXICITY AND CARCINOGENICITY

Role and interaction of p53, BAX and the stress-activated protein kinases p38 and JNK in benzo(a)pyrene-diolepoxide induced apoptosis in human colon carcinoma cells

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Abstract Polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants formed during incomplete combustion of organic material. For example benzo[a]-pyrene (B[a]P) is a constituent and contaminant of cigarette smoke, automobile exhaust, industrial waste and even food products. B[a]P is carcinogenic to rodents and humans. B[a]P induces its own metabolism, which generates different metabolites such as the highly reactive electrophilic genotoxin and ultimal carcinogen B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can bind to nucleophilic macromolecules such as proteins and DNA and causes mutations. Multiple defence mechanisms have evolved to protect the cell from DNA damage. Specific signalling pathways operate to detect and repair different kinds of lesions. In case, the damage is poorly removed expansion of damaged cells can be counteracted, e.g., by the inhibition of proliferation or triggering apoptosis. Examples of damage sensors and transducers are stress-activated protein kinases (SAPKs) and the tumour suppressor protein p53. Here, we studied the role of p53 and the pro-apoptotic protein BAX in BPDE-induced cell death by using wild-type- or knock-out-human colon carcinoma cells. As reported previously, we could reconfirm a critical role of p53 in BPDE-induced apoptosis. Furthermore, induced levels of total p53 and its transcriptional target p21 declined at higher BPDE concentrations correlating with reduced rates of apoptosis. Interestingly, increased phosphorylation of p53 at serine 15 remained elevated at higher BPDE concentrations thus disconnecting p53 phosphorylation from downstream apoptosis. Hence, phosphorylation of p53 seems not only to be a more sensitive biomarker of BPDE exposure but might serve other functions unrelated to apoptosis. In addition, we identify BAX as a novel and essential factor to trigger the intrinsic pathway of apoptosis in response to BPDE. Furthermore, BPDE in parallel activates the SAPKs p38 and JNK, which are as well involved in apoptosis. Although several routes of mutual regulation of p53 and SAPK have been described, we present evidence that the SAPK pathway in response to genotoxic stress can unexpectedly operate independently of p53 and controls apoptosis by a novel mechanism possibly downstream of caspases.

Keywords Polycyclic aromatic hydrocarbons · p53 · BAX · Stress-activated protein kinases · Apoptosis

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread organic pollutants. Epidemiological studies link PAH exposure to lung and bowel cancer (de Kok and van Maanen 2000; Zhang et al. 2009). PAHs are created during incomplete combustion, which occurs either naturally (Edwards 1983) or manmade in, e.g., cigarette fume, automobile exhaust and industrial processes. In ambient air, PAHs are mostly bound to particulate matter, which can deposit on soil and bodies of water, but also on plants where they can coalesce with the waxy plant surface. They have thus been found in vegetables and crops (Guillen et al. 1997). Even more severely contaminated are barbecued or smoked meat or fish

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with up to 320 μg/kg (Phillips 1999). Thus, the main route of uptake of PAHs for humans is via the gastrointestinal tract (Hattemer-Frey Ha 1992; Ramesh et al. 2004).

Once ingested, PAH excretion is hindered by their lipophilic properties. To prevent their accumulation, xenobiotic metabolism renders them more hydrophilic to be excreted. This conversion proceeds via several enzymatic steps and might produce metabolic intermediates, some of which are carcinogenic. One of the best-studied PAHs is benzo[a]-pyrene, which due to metabolism could give rise to the ultimate carcinogen benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE). If not detoxified, BPDE can bind via its reactive epoxide group not only to proteins but also to DNA where it forms adducts with adenine and especially guanine. Improper removal of this so-called bulky adducts leads to mutations and subsequently cancer (Rubin 2001).

Aside from DNA repair and cell cycle arrest, one mechanism to prevent cancer formation is to kill the mutated cell, i.e., via programmed cell death (apoptosis) or necrosis. Apoptosis is a strictly regulated process, in which, under energy consumption, the cell follows a programmed path to cell death. Hallmarks of apoptosis are, e.g., loss of cellular adhesion, disintegration of the cell membrane (blebbing), formation of apoptotic bodies, condensation and cleavage of DNA and nuclear fragmentation. This process includes enzymatic digestion of cellular components by, e.g., cysteine-dependent aspartyl-specific proteases (caspases). Activation of caspases involves cleavage of pro-caspases, which themselves activate downstream caspases to form an enzymatic cascade (Pop and Salvesen 2009). There are two major caspase-dependent apoptotic pathways differing in the initiator caspases and their activation mechanisms. The extrinsic pathway is activated by extracellular signals acting on membrane receptors (i.e., Fas), leading to activation of the initiator caspase 8, while the intrinsic pathway is activated via intracellular signals and involves initiator caspase 9. In the latter pathway, members of the Bcl-2 protein family (i.e., BAX) play an important pro- or anti-apoptotic role. Intracellular cues that trigger the intrinsic pathway are DNA damage, oxidative or oncogenic stress to name a few. Downstream of the initiator caspases are the so-called effector caspases such as caspases 3 or 7, leading to proteolysis of structural proteins like actin and activation of nucleases leading to DNA degradation. In contrast to the highly regulated cell death through apoptosis, necrosis constitutes an alternative way of cell death. Necrosis is characterized by cell rupture, resulting in the release of cellular content eliciting for example inflammatory responses.

Important regulators of cell death are the tumour suppressor protein p53 and the stress-activated protein kinases (SAPK) p38 and c-Jun N-terminal kinase (JNK) (Herrlich et al. 2008). p53 regulates cell growth, cell cycle progression and apoptosis. p53 can act as transcription factor in the nucleus and induces gene expression of cell cycle regulators such as p21 or directly interacts with other cytoplasmic proteins to induce apoptosis (Lee et al. 2010; Menendez et al. 2009; Vaseva and Moll 2009). SAPKs are activated by phosphorylation via upstream kinases in response to stress factors such as osmotic shock, oxidative stress or UV irradiation (Herrlich et al. 2008). Once phosphorylated, they translocate into the nucleus where they activate as proline-directed serine/threonine kinases transcription factors like AP-1 and thus regulate expression of target genes (Schreck et al. 2011; Weiss and Bohmann 2004; Weiss et al. 2003).

Several studies addressed the role of p53 and SAPKs in BPDE-triggered cell death in different cell types mostly liver cells from different species (Chen et al. 2003; Solhaug et al. 2004b). More recently, human colon cancer cells with a defined deletion of the p53 gene due to homologous recombination were used to assess more precisely the role of p53 in BPDE-induced apoptosis and gene regulation (Hockley et al. 2008). To clarify the role of p53 and in addition of the pro-apoptotic Bcl-2 family member BAX as well as the relevance of the SAPK pathway for BPDE-induced cell death, we explored wild-type human colon cancer cells (Hct116) and their isogenic derivatives that differ only in the presence or absence of the p53 or *BAX* gene, respectively (Zhang et al. 2000). Furthermore, a possible interaction between the p53 and SAPK pathway in BPDE-induced cell death was investigated.

Materials and methods

Material

Anti-PCNA (PC-10), -PARP (H-250), -p53 (FL-393), -p21 (SX-118) and -laminB antibodies were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. Antibodies against cleaved caspase 7 (Asp198),8 (1C12),9 (Asp315) and for the detection of phosphorylated proteins [p53 (Ser15), p38 (Thr180/Tyr182), JNK (Thr183/Tyr185)] were purchased from Cell Signaling Technology, Frankfurt am Main, Germany. zVAD, SB203580 and SP600125 inhibitors were purchased from Enzo Life Sciences, Lörrach, Germany. Synthesis of *anti*-dihydrodiol epoxides of benzo[*a*]pyrene has been described previously (Schreck et al. 2009).

Cell culture

Hct116 wt, p53−/− and BAX−/− cells (kindly provided by B. Vogelstein, John Hopkins University, Baltimore, USA) were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS),

penicillin and streptomycin (each 100 U/ml). Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 2 days. Toxicant treatment was performed as previously described (Schreck et al. 2009). Control cells were treated with 0.1% DMSO, the solvent used for the investigated chemical compounds.

Western blot

Whole cell extracts were separated by SDS-PAGE using the Mini-Protean system from Bio-Rad Laboratories (Germany) and electroblotted 1 h/100 V onto Immobilon-P membranes (Millipore, Germany). Membranes were blocked for 1 h with 5% dry low-fat milk powder/TBS/0,1% Tween20 (5% MP/TBST), washed two times with TBST and incubated with appropriate primary antibodies (details on dilutions are listed in supplementary table 1) for 1 h at RT or o/n at 4°C. After two washing steps with 1× TBST, membranes were incubated with horseradish-peroxidase-conjugated antibody (DAKO, Germany; dilution 1:5000 in 5% MP/TBST) for 1 h. Enhanced chemiluminescence (ECL) (Amersham ECL Plus, GE Healthcare Life Sciences, Germany, or Pierce ECL Western Blotting Substrate, Thermo Scientific, Germany; details in supplementary table 1) detection was performed according to the manufacturer's instructions. To detect PCNA (36 kDa), which served as loading control, the lower part of the gels was separated prior to blotting and handled as described above. When proteins of similar size to PCNA were analysed, the same extracts were loaded onto a separate gel and PCNA was detected individually.

Microscopy-based determination of cell counts, apoptosis and necrosis

For the microscopic assays, 8,000 cells/well were plated on a 96-well plate and toxicant treatment was performed as described above. After incubation, cell nuclei were stained with 0.3 μg/ml Hoechst 33342 and 0.5 μg/ml Propidiumiodid (PI) for 15 min at 37°C and 4 pictures per well were taken with an Olympus IX81 microscope. The acquired pictures were analysed using the Scan® Analysis software from Olympus. Cell number was determined by counting the nuclei. Apoptotic cells were distinguished from live cells via the intensity of the nuclear Hoechst staining. Compared to live cells apoptotic cells show a brighter staining due to the condensed chromatin. Necrotic cells were determined via the PI staining.

Caspase 3/7 assay

Caspase 3/7 activity was determined using the caspase-Glo 3/7 Assay Kit (Promega, Germany) following the manufacturer's instructions.

Results

Anti-benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide (BPDE) induces apoptosis in human colon cancer Hct116 cells

Treatment of Hct116 cells with increasing concentrations of BPDE for 24 h reduced the number of viable cells to approximately 50% compared to the solvent control–treated cultures (Fig. 1a). As in murine cells benzo[a]pyrene (B[a]P) triggers preferentially apoptosis but not necrosis (Solhaug et al. 2005), we analysed the percentage of apoptotic and necrotic cells after exposure to BPDE. Indeed, also in Hct116 cells, the B[a]P metabolite BPDE selectively induced apoptosis and not necrosis dependent on dose (Fig. 1b). Next, we investigated the role of caspases in BPDE-induced apoptosis. As shown previously in (Hockley et al. 2008), we also detected activation of caspase 3 and 7 after BPDE treatment of Hct116 cells (Fig. 1c). Interestingly, induction of apoptosis and caspase activity seems to decrease again at the highest dose of BPDE (Fig. 1b, c). Thus, increased levels of BPDE progressively stimulate apoptosis up to a maximal dose beyond which the rate of apoptosis again decreases. To explore the two main apoptotic pathways regulated by caspases, we analysed cleavage of the initiator caspase 9 as representative of the intrinsic way, the initiator caspase 8 as representative of the extrinsic way and the effector caspase 7. Similar to the dose-response observed before, BPDE induces activation of both pathways (Fig. 1d). As a consequence of caspase activation, also the cleavage of poly (ADP-ribose) polymerase (PARP) was detected (Beneke and Burkle 2007). To proof the importance of caspases in BPDE-induced cell death, cells were pre-treated with the pan-caspase-inhibitor zVAD before exposure to BPDE and subsequently apoptosis was monitored. Clearly, for all tested BPDE doses, zVAD abolished initiation of apoptosis (Fig. 1e).

p53 is activated by BPDE and regulates the activation of the intrinsic, but not extrinsic, apoptotic pathway

A central mediator in response to genotoxic stress is the tumour suppressor protein p53 (Aylon and Oren 2007). Knock-out of p53 in Hct116 cells inhibits the expression of target genes involved in apoptosis and enhances the percentage of viable cells after BPDE exposure (Hockley et al. 2008). The activity of p53 is regulated at the level of protein stabilization and by a variety of post-translational modifications such as phosphorylation. To examine p53 activation by BPDE, we analysed both, the p53 level and phosphorylation of p53 at serine 15 (Ser15) a site targeted by multiple kinases (Toledo and Wahl 2006). Furthermore, as a functional read-out of p53 activity, we studied induction of its target p21. p53 is stabilized and phosphorylated after BPDE

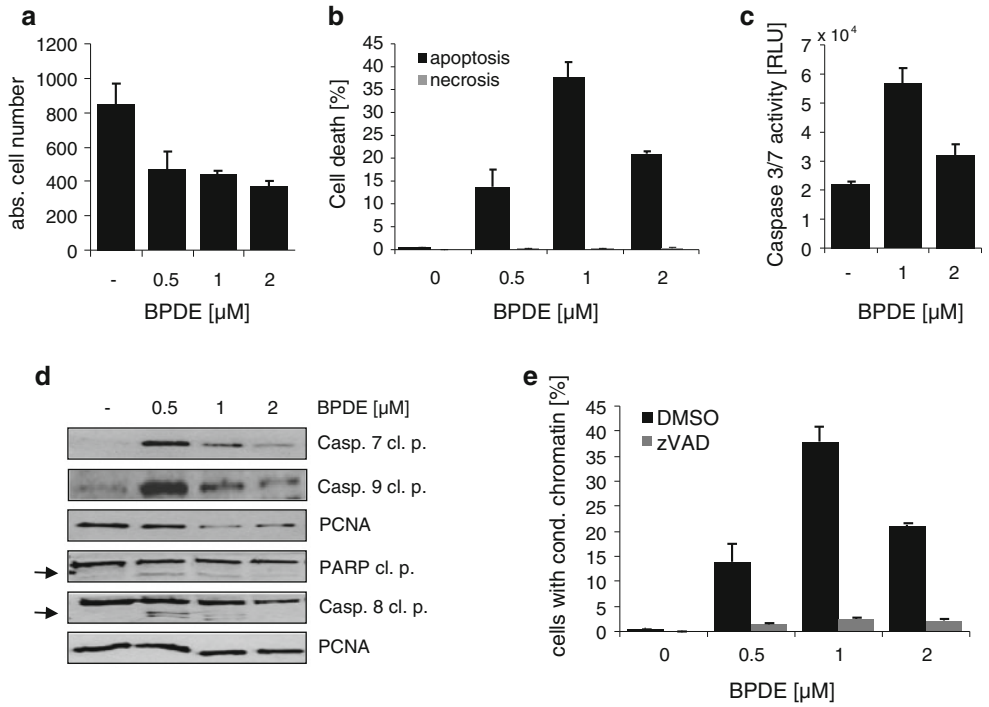

Fig. 1 BPDE induces caspase-dependent apoptosis in Hct116 human colon cancer cells. **a**, **b** BPDE reduces cell numbers and induce apoptosis. 8000 cells/well were seeded onto a 96-well plate and the following day treated with the indicated BPDE concentrations. After 24 h, **a** the cell numbers, **b** percentage of apoptotic and necrotic cells were determined. **c**, **d** BPDE activates caspases 7, 8, and 9. Proliferating Hct116 cells were treated with the indicated BPDE doses for 16 h. Apoptosis was detected via measurement of caspase 3/7 activity (**c**) and Western blotting (**d**) using specific antibodies against cleaved caspases and PARP. PCNA was used as loading control. **e** BPDE-induced apoptosis is mediated by caspases. Prior to BPDE exposure, cells were pre-treated with the caspase inhibitor zVAD and analysed as described in **a** one representative out of three independent experiments is shown. *cl. p.* cleavage product

exposure (Fig. 2a). The dose-response of p53 accumulation and of its target p21 is similar to those observed for the induction of apoptosis and caspase activation. However, elevated phosphorylation of p53 is also detectable at higher doses of BPDE despite declining p53 protein levels. Possibly, the pool of phosphorylated p53 only marginally contributes to the overall increase in total p53 and seems not to be sufficient to promote p21 expression and apoptosis at high BPDE concentrations. Vice versa, the absence of increased p53 and p21 levels at high doses of BPDE would have indicated an absence of the p53 response if we would not have analysed phosphorylation in parallel. At present, the functional consequences of the phosphorylated pool of p53 remain unknown. Induction of p21 by BPDE is clearly p53-dependent as in p53 knock-out Hct116 cells BPDE does not induce p21 expression (Fig. 2b). To test whether p53 is also involved in the onset of apoptosis in response to BPDE, Hct116 wild-type and p53 knock-out cells were investigated. The percentage of apoptotic cells was drastically diminished in the absence of p53 thus demonstrating its central role in BPDE-provoked cell death (Fig. 2c). As expected the activity of caspase 3 and 7 was found to be dependent on p53 as well (Fig. 2d), which is in accordance with previous findings (Hockley et al. 2008). p53-dependent apoptosis can occur both via the intrinsic and the extrinsic pathway (Pietsch et al. 2008). To further explore the role of p53 in BPDE-dependent caspase activation, the dose-dependent cleavage of caspases was analysed in p53 proficient and deficient cells. Whereas activation of the effector caspases 7 and 9 is severely impaired in p53 knock-out cells, there is no major difference for the cleavage of caspase 8 (Fig. 2e). Thus, p53 specifically regulates the intrinsic pathway of apoptosis in response to BPDE.

BPDE-induced apoptosis is in part BAX dependent

In murine hepatoma cells, B[a]P leads to accumulation and phosphorylation of Bad, suggesting that members of the Bcl-2 family, which are key components of the intrinsic death pathway, might play a role in apoptosis in response to polycyclic aromatic hydrocarbons (Solhaug et al. 2004b). An important pro-apoptotic Bcl-2 protein acting downstream of p53 is BAX, which translocates from the cytosol into the mitochondria and leads to mitochondria

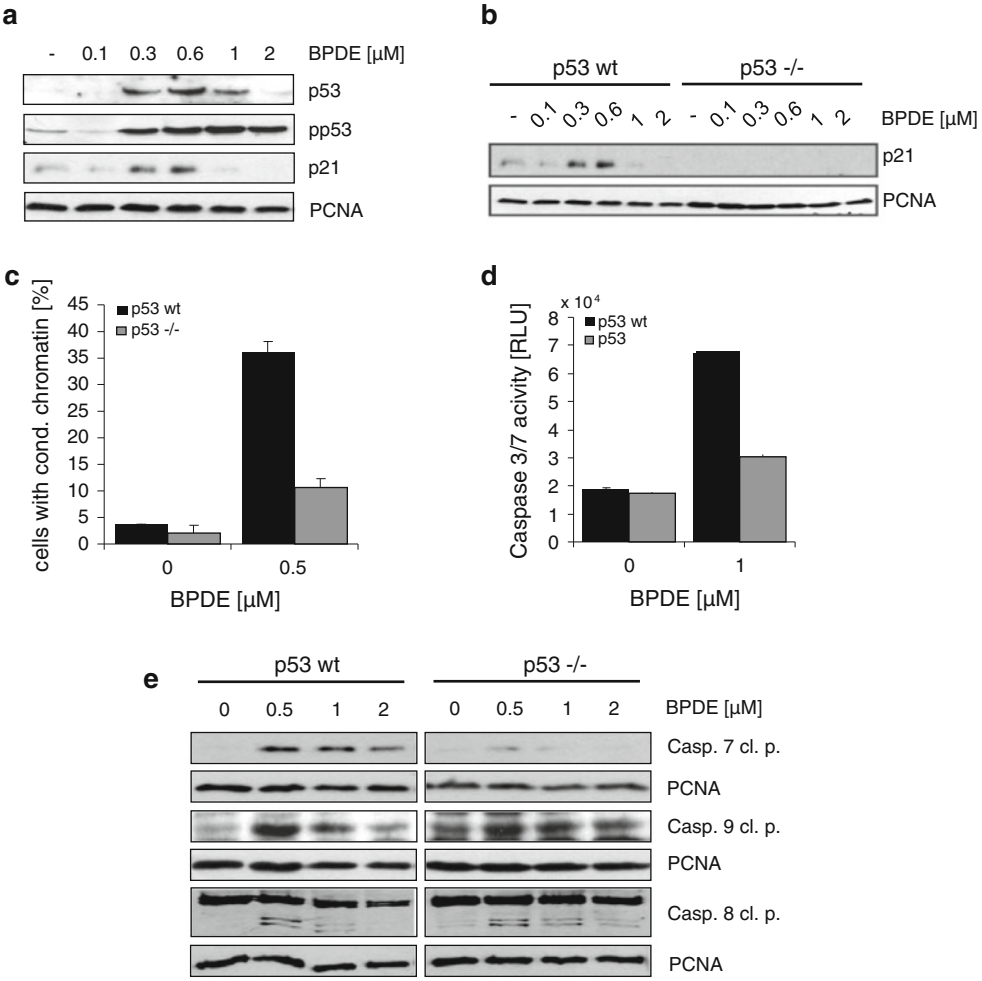

Fig. 2 p53 is activated by BPDE and regulates the activation of the intrinsic, but not extrinsic, apoptotic pathway. **a** BPDE induces p53 phosphorylation and enhances total protein levels of p53 and its target p21. Proliferating Hct116 cells were treated with the indicated BPDE concentrations for 16 h. Whole cell lysates were analysed by Western blotting using specific antibodies to detect p21, p53 and its phosphorylation at serine 15. **b** Induction of p21 by BPDE depends on p53. Wild-type (wt)- and p53 knock-out (−/−) cells were treated and analysed as described before. **c**–**e** Induction of apoptosis and activation of caspases 3, 7 and 9 by BPDE are dependent on p53. **c** Apoptosis, **d** caspase 3/7 activity and **e** cleavage of caspases were determined as described in Fig. 1. One representative out of three independent experiments is shown

outer membrane permeabilization (MOMP). To examine whether BAX contributes to BPDE-induced apoptosis, BAX knock-out Hct116 cells were compared with the parental wild-type cells after treatment with BPDE. In the absence of BAX, the rate of apoptosis triggered by BPDE was 50% less when compared to wild-type cells (Fig. 3a). To get an insight at which level BAX is required for BPDE-induced apoptosis, the cleavage of caspases and PARP was evaluated. In BAX knock-out cells, the cleavage of caspase 3 and 7 and PARP was almost absent (Fig. 3b). However, differences in the cleavage of caspase 8 were less pronounced in wild-type and BAX knock-out cells. Taken together, BAX and p53 knock-out cells are both impaired to fully activate the intrinsic pathway in response to BPDE. When we analysed the p53 response in BAX-deficient cells, we found a decreased accumulation of total p53, indicating a pivotal role of BAX to promote p53 stabilization (Fig. 3c). However, p53 phoshorylation was unaffected, and p21 was still inducible again, suggesting that the phosphorylated pool of p53 is a more sensitive biomarker for genotoxin exposure.

The stress-activated protein kinases p38 and JNK are activated after BPDE treatment and contribute to BPDE-induced apoptosis independent of caspase and p53 activation

Genotoxins such as ultraviolet radiation (UVR) but also BPDE can activate stress-activated protein kinases (SAPK) (Herrlich et al. 2008; Li et al. 2004a, b). Therefore, BPDE-induced SAPK phosphorylation was detected by Western blotting using phospho-specific antibodies (Fig. 4a). A dose-dependent activation of both, JNK and p38, is visible after BPDE exposure. As SAPK are known regulators of apoptosis (Dhanasekaran and Reddy 2008; Herrlich et al. 2008; Junttila et al. 2008), cells were pre-treated with specific inhibitors of either p38 or JNK. Inhibition of p38 and, although less pronounced, JNK suppressed apoptosis induced by BPDE (Fig. 4b). Surprisingly, inhibition of p38 or JNK did not significantly affect cleavage of caspase 7, 9 and 8 (Fig. 4c), suggesting that SAPKs do not act upstream of caspases. To study the role of SAPKs in p53 activation by BPDE, we investigated the level of the total p53 protein

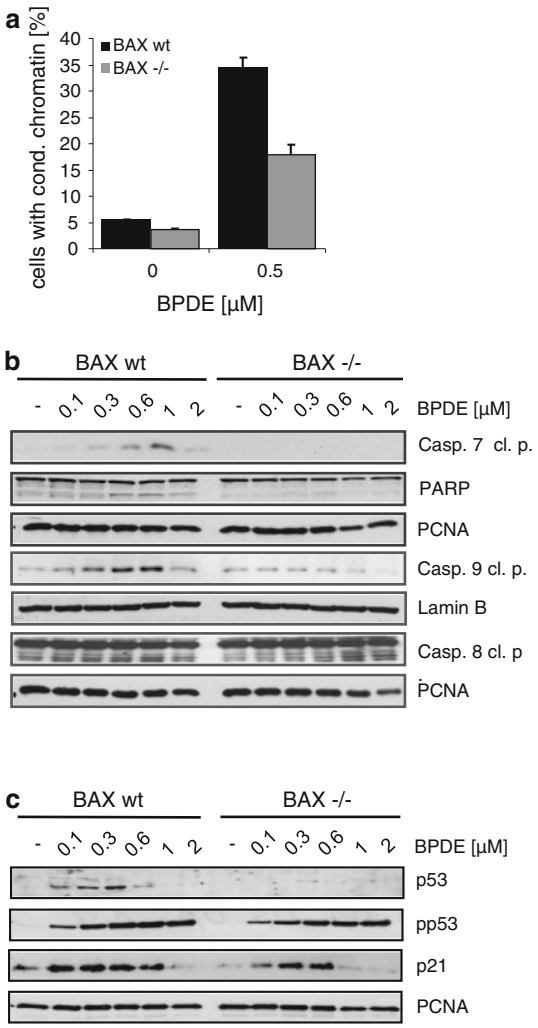

Fig. 3 BPDE-induced apoptosis is in part Bax dependent. **a**, **b** Induction of apoptosis and activation of caspases 7 and 9 by BPDE are dependent on BAX. **c** Induction of total p53 protein levels but not p53 phosphorylation by BPDE depends on BAX. Wild-type (wt)- and BAX knock-out (−/−) cells were treated and analysed as in Fig. 2. One representative out of three independent experiments is shown

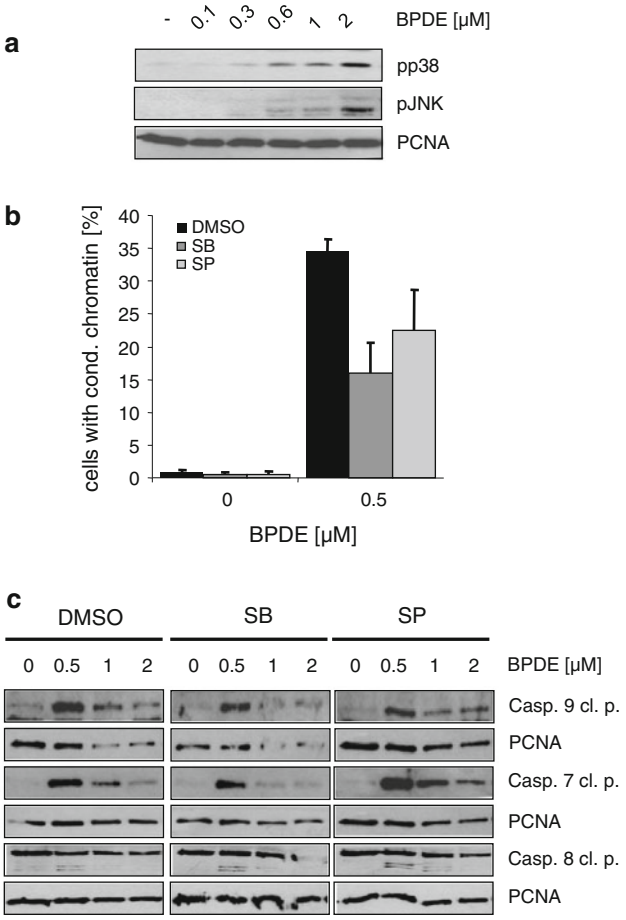

Fig. 4 p38 and JNK are activated after BPDE treatment and contribute to BPDE-induced apoptosis independent of caspase activation. **a** Hct116 cells were incubated with the indicated concentrations of BPDE for 16 h, and whole cell lysates were analysed by Western blotting using phospho-specific antibodies against p38 and JNK. PCNA was used as loading control. **b**, **c** Cells were treated with the solvent control DMSO or specific SAPK inhibitors (SB: p38; SP: JNK) prior to 24-h treatment with 0.5 µM BPDE. Apoptosis rates (**b**) and caspase cleavage (**c**) were determined as described in Fig. 1. One representative out of three independent experiments is shown

as well as p53 phosphorylation. In contrast to previous reports, which identified p53 stabilization and phosphorylation as a downstream consequence of SAPK activity in response to, e.g., UVR (Herrlich et al. 2008), we could not detect any effect on the BPDE-triggered p53 response when SAPK were inhibited (Fig. 5a). Conversely, we addressed whether SAPK activation by BPDE is controlled by p53 or BAX and thus a consequence of initiation of apoptotic signalling. However, neither the knock-out of p53 nor BAX abrogated SAPK activation by BPDE (Fig. 5b, c). Hence, activation of SAPK is not only independent of the p53 pathway but also contributes to apoptosis independent of p53 presumably downstream of caspases.

Discussion

Cells are constantly exposed to mutagenic insults from both endogenous and exogenous sources. To protect the organism, highly conserved mechanisms have evolved to counteract cell damage or even malignant transformation. Metabolic enzymes can detoxify potential carcinogens before they induce DNA damage. DNA repair mechanisms can remove DNA lesions, and even if DNA repair fails, damaged cells can still be eliminated from the organism through different forms of cell death including apoptosis, necrosis and autophagy.

BPDE induces cell death mainly by apoptosis in Hct116 cells as indicated by the condensation of DNA and nuclear

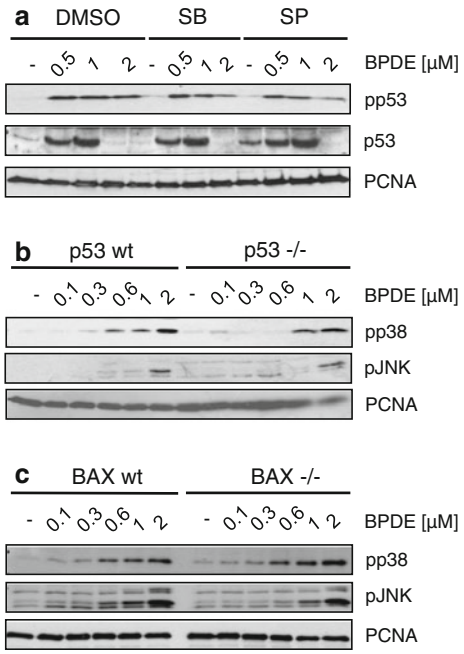

Fig. 5 BPDE activates p53 and SAPKs independent of each other. **a** p53 activation in Hct116 cells by BPDE is independent of SAPKs. Prior to BPDE treatment, cells were treated with the solvent control DMSO or specific SAPK inhibitors (SB: p38; SP: JNK). **b**, **c** SAPK activation does not require p53 or BAX. Wild-type (wt)- and **b** p53 or **c** BAX knock-out (−/−) cells were treated and analysed as in Fig. 2. One representative out of three independent experiments is shown

fragmentation. This is in accordance with the parallel activation of caspases 3 and 7, which has been also reported previously by others (Hockley et al. 2008). Their substrates include structural proteins as well as cleavage-activated proteins with apoptotic functions. The cleavage of PARP, a substrate of caspases, and inhibition of apoptosis by the caspase inhibitor zVAD demonstrate the central role of caspases in BPDE-induced cell death. BPDE triggered both, the extrinsic and intrinsic pathway of apoptosis as evidenced by cleavage of caspase 8 and 9, respectively. Similarly, in murine hepatoma cells, BPDE induces caspase 8 cleavage and apoptosis (Solhaug et al. 2004b). The classical pathway leading to caspase 8 activation is via membrane receptors. Also, in murine pre-B-cells, PAH-induced caspase 8 cleavage could be detected (Page et al. 2002), but was independent of death receptor activation (Ryu et al. 2005). Indeed, other pathways independent of death receptors exist, which link caspase 3 to caspase 8 in human B-cells after exposure to the PAH 7,12-dimethylbenzo[a]anthracene (Ryu et al. 2005). Activation of the intrinsic apoptotic pathway after BPDE has been documented in several different cell lines. In embryonic mouse fibroblasts, BPDE induces apoptosis associated with cytochrome c release into the cytosol (Chen et al. 2003). Members of the Bcl-2 family induce pore formation in the mitochondria (mitochondria outer membrane permeabilization, MOMP), which promotes cytochrome c regression (Brunelle and Letai 2009). MOMP represents therefore the key element of intrinsic apoptosis. It is regulated by the relative levels of pro- and anti-apoptotic members of the Bcl-2 family of proteins. One member of this protein family is BAX, which is directly involved in pore formation at the mitochondrial membrane. Under normal conditions, BAX resides in the cytosol and is bound to anti-apoptotic members of the Bcl-2 family. Following an apoptotic trigger, BAX and/or other pro-apoptotic Bcl-2 members are upregulated at the protein level. The latter bind to anti-apoptotic Bcl-2 counterparts and induce the release of BAX, which can then directly bind to the mitochondrial membrane. There, several BAX proteins form oligomers and thus build pores to promote MOMP. In the human lung cancer cell line, H460 BPDE induces apoptosis and changes as described above the balance between members of the Bcl-2 family (Xiao and Singh 2007). In Hct116, no changes in BAX levels could be detected after BPDE treatment (data not shown). However, a role of BAX in BPDE-induced apoptosis could nonetheless be shown based on the comparison of apoptosis rates in BAX wild-type- and knock-out-cells. Possibly, a release of BAX, as described above via the upregulation of other pro-apoptotic Bcl-2 members, might induce BPDE-provoked apoptosis in Hct116 cells. Following cytochrome c release, the so-called apoptosome catalyses caspase 9 cleavage and thus the initiation of the intrinsic caspase cascade. Caspase 9 activates effector caspase 7, whose cleavage product as well as cleaved caspase 9 could not be detected in Bax-deficient Hct116 cells after BPDE treatment. Thus, Bax is an essential component of the intrinsic apoptotic pathway stimulated by BPDE.

A well-known transcription factor able to regulate the expression of different Bcl-2 family members is p53 (Bellamy 1997; Vaseva and Moll 2009). p53 plays an important role in BPDE-induced changes of gene expression patterns and apoptosis in Hct116 cells (Hockley et al. 2008). Downregulation of p53 also lead to a reduction in BPDE-induced apoptosis in human lung cancer cells (Xiao and Singh 2007). We reconfirmed an essential role of p53 in BPDE-induced apoptosis. BPDE increased the total protein level and phosphorylation of p53. Knock-out of p53 selectively interfered with the activation of the intrinsic pathway by BPDE. One important role of p53 is to regulate target genes as a transcription factor in the nucleus, as shown here for p21. Aside from its role as transcription factor in the nucleus p53 can also interact with members of the Bcl-2 family in the cytosol. Here, it can interact directly with BAX to promote MOMP. On the other hand, p53 can bind anti-apoptotic Bcl-2 family members leading to a release of their pro-apoptotic-binding partners, allowing

the latter to activate BAX (Chipuk and Green 2006). In this context, we have preliminary results indicating a translocation of p53 into the mitochondria in response to BPDE (data not shown), which also could be shown by other groups with other stimuli (Vaseva and Moll 2009). This might indicate a dual role for p53 in BPDE-induced apoptosis: as a transcription factor in the nucleus and at mitochondria. Surprisingly, we detected a reduced p53 stabilization and p21 induction, but not p53 phosphorylation in BAX-deficient cells. This has so far not been reported. The lack of p53 stabilization correlates well with the reduced rate of apoptosis in BAX knock-out cells. However, as p53 phosphorylation was not diminished, it serves probably a different function unrelated to apoptosis. Similarly, at high BPDE concentration, we also observed a reduced stabilization of p53 accompanied by a lower percentage of apoptotic cells. Interestingly, phosphorylation of p53 did not decline thus again disconnecting p53 phosphorylation from downstream apoptosis.

Important stress sensors are the stress-activated protein kinases (SAPK) p38 and JNK (Herrlich et al. 2008). BPDE also leads to p38 and JNK activation in epidermal mouse cells (Li et al. 2004a, b). Phosphorylation of p38 could also be monitored in human hepatoma (HepG2) cells and murine fibroblasts (Chen et al. 2003). Chemical inhibition of p38 in HepG2 cells or knock-out of p38 in fibroblasts reduced BPDE-induced PARP cleavage and apoptosis. Also, in murine hepatoma cells, inhibition of p38 but not of JNK decreased apoptosis (Solhaug et al. 2004a). In the present study, we could also confirm a role of p38 in BPDE-induced apoptosis in Hct116 cells. However, also inhibition of JNK interfered with initiation of apoptosis, suggesting species or cell-type-specific differences for the role of JNK in BPDE-triggered cell death. Interestingly, although the inhibition of p38 and JNK reduced apoptosis, there was no parallel decrease in the activation of caspases 7, 8 and 9. Both p38 and JNK are able to phosphorylate and activate p53 to control cell cycle regulation and death (Herrlich et al. 2008). Yet, increased phosphorylation and stabilization of p53 in response to BPDE is not abrogated by downregulation of p38 and JNK activity in Hct116 cells. Vice versa, knock-out of p53 or BAX also does not prevent the activation of p38 and JNK by BPDE, indicating that both pathways operate independently of each other. As p38 and JNK seem not to regulate caspase cleavage, they might regulate caspase-independent effectors of apoptosis such as the nuclease EndoG or apoptosis-inducing factor (AIF). In healthy cells, they are located in the mitochondrial intermembrane space and translocate in response to apoptotic signals into the nucleus, where they cause chromatin fragmentation and condensation (Lorenzo and Susin 2004; Mathiasen and Jäättelä 2002). As we were unable to detect such a nuclear translocation of EndoG or AIF (data not shown), the exact role of SAPK in BPDE-induced apoptosis needs to be further explored. Indeed a more direct role of JNK in the induction of chromatin condensation downstream of caspases has been postulated, which unfortunately was not further elucidated (Ura et al. 2007).

In summary, we could show that BPDE induces via BAX and p53 apoptosis in Hct116 cells. BAX and p53 are essential for BPDE to activate the intrinsic but not the extrinsic apoptotic pathway. Furthermore, the SAPKs p38 and JNK are also mediators of BPDE-induced apoptosis and act presumably downstream of caspase activation. Interestingly, the p53 and SAPK pathway are induced by BPDE independently of each other. Primary initiators of BPDE-induced signalling leading to p53 activation are, e.g., the DNA damage responsive kinases ATM/ATR, which might also be involved in SAPK activation.

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