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Effects of nitrated-polycyclic aromatic hydrocarbons and diesel exhaust particle extracts on cell signalling related to apoptosis: Possible implications for their mutagenic and carcinogenic effects

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Abstract

Nitrated-polycyclic aromatic hydrocarbons (nitro-PAHs) and diesel exhaust particle extracts (DEPE) induced apoptosis in Hepa1c1c7 cells with the following potency: 1,3-dinitropyrene (1,3-DNP)>1-nitropyrene (1-NP) ≫ DEPE ≫ 1,8-dinitropyrene (1,8-DNP). The compounds induced cyp1a1, and activated various intracellular signalling pathways related to apoptosis. The CYP inhibitor α-naphthoflavone strongly reduced 1,3-DNP-induced cell death, whereas cell death induced by 1-NP was rather increased. Toxic 1,3-DNP and 1-NP were found to induce a concentration-dependent lipid peroxidation. 1,3-DNP caused pro-apoptotic events, including increased phosphorylation and accumulation of p53 in the nucleus, cleavage of bid and of caspases 8 and 3, down-regulation of bcl-x_L and phosphorylation of p38 and JNK MAPK. Furthermore, 1,3-DNP increased the activation of survival signals including phosphorylation of Akt and inactivation (phosphorylation) of pro-apoptotic bad. Although less potent, rather similar effects were observed following exposure to DEPE, compared to 1-NP. The most important finding was that the most mutagenic and carcinogenic compound tested, 1,8-DNP, induced little (if any) cell death, despite the fact that this compound seemed to give the most DNA damage as judged by DNA adduct formation, increased phosphorylation of p53 and accumulation of cells in S-phase. Immunocytochemical studies revealed that the p53 protein did not accumulate into the nucleus suggesting that 1,8-DNP inactivated the pro-apoptotic function of the p53 protein by a non-mutagenic event. These results suggest that after exposure to 1,8-DNP more cells may survive with DNA damage, thereby increasing its mutagenic and carcinogenic potential.

Keywords: Nitro-polycyclic aromatic hydrocarbons; Cell signaling; DNA damage; p53; Metabolism; Apoptosis

Abbreviations: α-NF, α-Naphthoflavone; AhR, Aryl hydrocarbon receptor; B[*a*]P, Benzo[*a*]pyrene; CP-PAHs, Cyclopenta-polycyclic aromatic hydrocarbons; CYP, Cytochrome P450; cyt *c*, Cytochrome *c*; DEP, Diesel exhaust particles; DEPE, Diesel exhaust particle extracts; DMSO, Dimethyl sulfoxide; DNP, Dinitropyrene; ERK, Extracellular signal-related kinase; JNK, c-jun N-terminal kinase; MAPK, Mitogen activated protein kinases; 1-NP, 1-Nitropyrene; nitro-PAHs, Nitrated-polycyclic aromatic hydrocarbons; NOS, Nitric oxide synthase; NR, Nitroreductases; PAHs, Polycyclic aromatic hydrocarbons; PI, Propidium iodide; PKA, Protein kinase A; ROS, Reactive oxygen species; TLC, Thin-layer chromatography

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants formed during incomplete combustion of organic material. Nitro-PAHs are an important subgroup of PAHs found on diesel exhaust particles (DEP) that have been suggested to be related to the development of lung cancer (Garshick et al., 2004; Pope et al., 2002; Vineis et al., 2004). Studies indicate that several nitro-PAHs are highly mutagenic in bacterial systems and that they are found to cause mutations and tumors in animal models (Tokiwa and Ohnishi, 1986).

Nitro-PAHs may be metabolized by ring oxidation, nitroreduction, as well as conjugation reactions. Some of these reactions may lead to reactive metabolites that can covalently bind to macromolecules including DNA. Reactive epoxides may be formed by various CYP enzymes (Yamazaki et al., 2000). CYP enzymes and/or CYP reductases may also reduce the nitro-group leading to the formation of nitroso, *N*-hydroxylamine and the corresponding amine form (Chae et al., 1999). However, a more important pathway is considered to be reduction of the nitro-PAHs to the corresponding amine by six electron reduction reactions catalyzed by nitroreductases like nitric oxide synthase (NOS), DT-diaphorase, xanthine oxidase and aldehyde oxidase, which can also result in the formation of reactive oxygen species (ROS) (Möller, 1994; Pluquet and Hainaut, 2001; Purohit and Basu, 2000). Further acetylation or sulphate conjugation of the *N*-hydroxylarylamine may result in the formation of reactive nitrenium ions that can form DNA adducts (Arlt, 2005). Furthermore, many of these drug-metabolising enzymes are induced, e.g. by the aryl hydrocarbon receptor (AhR) which is known to be activated by many aromatic compounds (Nebert et al., 2004).

DNA damage is considered to be an important part of the mutagenic and carcinogenic effects of PAHs. In general, however, there is often no simple correlation between initial DNA damage, mutations and cancer. DNA damage may be repaired, cause mutations or result in cell death like apoptosis depending both on the type and the extent of damage (Kim et al., 2005b; Roos and Kaina, 2006). Induced cell death may, in addition, also be an important part in the tumor development, stimulating proliferation of surrounding cells with possible fixation of their DNA damage. Furthermore, sustained DNA damage may cause a selection of preneoplastic cells that have become more resistant to chemical-induced cell death due to, e.g. mutations in the p53 gene. Thus, there is an increasing awareness that DNA induced cell death and/or lack of induced cell death are important parts of mutagenesis and cancer development. Accordingly, mechanisms and cell signalling pathways involved PAH-induced cell death or cell survival and proliferation have recently received much interest (Chen et al., 2005; Huc et al., 2003; Lei et al., 1998; Solhaug et al., 2004b; Tannheimer et al., 1998).

The p53 protein is considered to have a pivotal role in the process determining whether a damaged cell will live or whether the apoptotic program is triggered (Vousden, 2000). It is often activated by phosphorylation, as a result of cellular response to environmental and intracellular stress such as hypoxia, ultraviolet radiation irradiation and agents which cause DNA damage including genotoxic PAHs like benzo[*a*]pyrene (B[*a*]P). p53 may act as a transcription factor, thus inducing transcription of proteins involved in cell cycle arrest, DNA repair and cell death. It may also directly translocate to the mitochondria, increase the pro-apoptotic properties of bax or reduce the anti-apoptotic properties bcl-2 and bcl-x_L.

Other signalling pathways considered being important with regard to the decision between proliferation, survival and apoptosis are the mitogen activated protein kinase (MAPK) family (J. Yang et al., 2003a). One of the members, ERK, is often found to be a part of cell survival and cell proliferation signalling. The two others, stress activated kinases p38 and c-jun N-terminal kinase (JNK), are generally considered as being pro-apoptotic, although their precise roles in the apoptotic process are complicated and seem to partly depend on the experimental system used. Interestingly, PAHs have been found to activate several of these intracellular signalling pathways (Chen et al., 2005; Kim et al., 2005b; Roos and Kaina, 2006; Solhaug et al., 2005).

In previous studies (Solhaug et al., 2004a,b, 2005), we have used liver epithelial Hepa1c1c7 cells, which are metabolically competent and proven to be a good model to study PAH-induced toxicity. Exposure to B[*a*]P and several cyclopenta-PAHs (CP-PAHs) resulted in apoptosis, but at the same time also partly inhibited this process by triggering the cell survival signal Akt. In the present study, we further explore and extend these findings characterizing the apoptotic effects and cell signalling triggered by other PAHs including 1-nitropyrene (1-NP), 1,3-dinitropyrene (1,3-DNP) and 1,8-dinitropyrene (1,8-DNP), as well as diesel exhaust particle extracts (DEPE).

The most important finding is that the most mutagenic and carcinogenic compound tested, 1,8-DNP, induced little (if any) cell death despite the fact that this compound seemed to give the most DNA damage. Although markedly phosphorylated, the p53 protein did not accumulate in the nucleus suggesting that 1,8-DNP directly inactivated the pro-apoptotic function of the p53 protein by a non-mutagenic event. As DNA adduct formation

was observed for 1,8-DNP, in contrast to 1-NP and 1,3-DNP, we hypothesize that more cells may survive with DNA damage, thereby increasing the mutagenic and carcinogenic potential of 1,8-DNP.

2. Materials and methods

2.1. Chemicals

Benzo[*a*]pyrene (B[*a*]P), 1-nitropyrene (1-NP), 1,3-dinitropyrene (1,3-DNP), 1,8-dinitropyrene (1,8-DNP), α-naphthoflavone (α-NF), Triton X-100, protease (type1:crude P4630), DNase I (type III), 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–Aldrich Chemical Company (St. Louis, MO, USA). May-Grunwald and Giemsa were purchased from Merck Co, Inc. (NJ, USA). Standard reference material 1975 (SRM1975) containing diesel exhaust particle extract (DEPE) was purchased from the National Institute of Standards and Technology (Gaithersburg, USA). Pepstatin A was from Calbiochem (Cambridge, MA, CA, USA). Leupeptin from Amersham Biosciences (Uppsala, Sweden). SeaKem GTG agarose was from FMC Bioproducts (Rockland, ME, USA), SYBR®Green I nucleic acid gel stain was obtained from Cambrex Bio Science (Rockland, USA) and Bio-Rad DC protein assay from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). MEM alpha medium with L-glutamine, without ribonucleosides and deoxyribonucleosides, fetal bovine serum (FBS) and gentamycin were from Gibco BRL (Paisley, Scotland, UK). Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained from commercial sources as reported before (Arlt et al., 2002). All other chemicals were purchased from commercial sources and were of analytical grade.

2.2. Antibodies

Antibodies against: cleaved caspase-3, bcl-x_L, bad, bid, phospho-bad (Ser155), p38 MAPK, phospho-p38 MAPK, phospho-JNK, JNK and phospho-p53 (Ser15) were obtained from Cell Signaling (Beverly, MA, USA); CYP1A1 and CYP1A2 from Santa Cruz Biotechnology, Inc. (CA, USA); p53 (CM5) from Novocastra Laboratories Ltd. (Newcastle, UK). As secondary antibodies horseradish peroxidase-conjugated goat anti-rabbit (Sigma Chemical Company, St. Louis, MO, USA), horseradish peroxidase-conjugated rabbit anti-goat or rabbit anti-mouse IgG from Dako (Glostrup, Denmark) was applied.

2.3. Cell culture and exposure

The mouse hepatoma Hepa1c1c7 cell line was purchased from European Collection of Cell Culture (ECACC); the cell line expresses Ah-receptor and cyp1-enzymes can be induced. Cells were grown in alpha MEM medium with 2 mM L-glutamine, without ribonucleotides and deoxyribonucleotides, supplemented with 10% heat-inactivated FBS and 0.1 mg/mL gentamycin in 5% CO_2 at 37 °C. Cells were seeded in dishes or plates at a concentration of 90.000 cells/cm^2 1 day before exposure to test compounds. Fresh medium was added before exposure. When inhibitors were used, cells were pre-incubated with inhibitor for 1 h before adding test substances. Cells were exposed to various concentrations of 1-NP, 1,3-DNP, 1,8-DNP, B[*a*]P or DEPE (1, 3, 10, 30 or 60 μM of nitro-PAH; and 10, 20, 30 50 or 70 μg/mL of DEPE) dissolved in DMSO mostly for 24 h. Total amount of DMSO added to cells was ≤0.5%, except when cells were exposed to 70 μg/mL DEPE, then the DMSO amount was 0.7%.

2.4. Fluorescence microscopy

Plasma membrane damage and changes in nuclear morphology associated with apoptosis and necrosis were determined after trypsination and staining of cells with Hoechst 33342 and PI as previously described (Solhaug et al., 2004b). At least 300 cells were counted per microscopic slide.

2.5. Flow cytometry

After treatment, cells were trypsinized and prepared for flow cytometry as previously described (Wiger et al., 1997). Briefly, DNA was stained with Hoechst 33258 and fluorescence was measured using an Argus 100 Flow cytometer (Skatron, Lier, Norway). Different cell phases as well as apoptotic cells/bodies were distinguished on the basis of their DNA content (Hoechst fluorescence) and cell size (forward light scatter). Percentages of cells in different phases of the cell cycle as well as apoptotic cells were estimated from DNA histogram using the Multicycle Program (Phoenix Flow system, San Diego, CA, USA). 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 (sub-G_1 population).

2.6. DNA fragmentation assay

DNA fragmentation assay was performed according to the method by Gorczyca et al. (1993), with minor modifications (Bjelogrlic et al., 1994). Briefly, approximately 2×10^6 cells were resuspended in 0.25 mL of TBE (45 mM Tris borate buffer, 1 mM EDTA; pH 8) containing 0.25% Nonidet P-40, and 0.1 mg/mL RNase and incubated at 37 °C for 30 min. Proteinase K (1 mg/mL final concentration) was added and samples were incubated for an additional 30 min. Then, to each sample 50 μL loading buffer (1 mL containing: 0.01 mL 1 M Tris, pH 7.5; 0.04 mL 0.5 M EDTA, pH 7.5; 0.5 mL glycerol (85%); 0.8 mg bromophenol blue; 0.45 mL H_2O) was added and incubated at 65 °C for 10 min just prior to application to the gel. Horizontal 1.5% agarose gels were run for at least 3 h at room temperature at 2 V/cm. DNA bands (laddering) were visualized under UV light in gels run with SYBR®Green I nucleic acid gel stain.

2.7. DNA isolation and ^{32}P-Postlabelling analysis

DNA from cells was isolated by the phenol extraction method as described previously (Arlt et al., 2001b). ^{32}P-Postlabelling analysis using butanol enrichment was carried out essentially as described with minor modifications (Arlt et al., 2002). Briefly, DNA samples (4 μg) were digested with micrococcal nuclease (120 mU, Sigma Chemical Co., Poole, UK) and calf spleen phosphodiesterase (40 mU, Calbiochem, Nottingham, UK) as reported. Resolution of ^{32}P-labelled adducts was carried out by thin-layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) sheets (10 cm × 20 cm, Macherey-Nagel, Düren, Germany) using the following solvents (Gallagher et al., 1991): D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M lithium formate, 7 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL, USA) and DNA adduct levels (RAL, relative adduct labelling) were calculated from the adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. For the preparation of reference DNA salmon testis DNA (500 μg) was incubated with 1-NP, 1,3-DNP or 1,8-DNP (300 μM) activated by xanthine oxidase essentially as described previously (Arlt et al., 2001a).

2.8. Lipid peroxidation

Lipid peroxidation was measured using the fluoroprobe C_{11}-BODIPY$^{581/591}$ (4,4-difluoro-5-[4-phenyl-1,3-butadienyl]-4-bora-3a,4a-diaza-*s*-indacine-3-undecanoic acid; Molecular Probes). After 24 h of treatment with 1-NP, 1,3-DNP, 1,8-DNP or B[*a*]P, the medium was removed and cells were incubated for 1 h at 37 °C in medium-diluted probe at a final concentration of 10 μM. After washing in PBS, the oxidation of C_{11}-BODIPY was measured by spectrofluorimetry (Spectramax Gemini, Molecular Devices), using two wavelengths, which allows measurement of the amount of oxidized probe (λ_{exc} = 485 nm; λ_{em} = 535 nm) and non-oxidized probe (λ_{exc} = 590 nm; λ_{em} = 635 nm). Lipid peroxidation was quantified by calculating the ratio [oxidized probe/total probe], which is independent of protein content (Pap et al., 1999).

2.9. Cell lysis and Western blotting

Cells were lysed in 20 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerol phosphate, 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. After cell lysis, solutions were sonicated and centrifuged. Supernatants were collected and protein concentration was measured using the Bio-Rad DC protein assay kit. Samples were adjusted to an equal protein concentration with lysis buffer. 5× SDS-PAGE sample buffer (0.312 M Tris–HCl, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.05% bromophenol blue, 10% glycerol) was added. Samples were boiled for 5 min and subjected to SDS-PAGE. Proteins were transferred by electroblotting to nitrocellulose membrane and stained with ponceau S. Blots that contained equal amount of protein loading were probed with specific primary antibodies according to the manufacturer's recommendations. Afterwards blots were incubated with horseradish peroxidase-conjugated secondary antibodies. Western blots were developed using the ECL chemiluminescence system according to the manufacturer's instructions (Amersham Pharmacia, Little Chalfont, UK). Results from one representative experiment out of three are shown.

2.10. Immunocytochemistry

After treatment, cells were fixed in cold ethanol (100%) for 10 min and permeabilized in PBS with 0.75% Triton X-100 for 30 min. Avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to quench the endogenous biotin–avidin. After 1 h in 5% dried milk, cells were incubated for 30 min with primary rabbit antibody. Cells were then incubated with biotin-conjugated secondary antibody for 10 min followed by fluorescein–avidin DCS for 5 min. Between each step cells were washed with PBS. Cells were finally mounted in Vectashield mounting medium (Vector Laboratories).

2.11. Statistical method

Statistical comparisons were carried out using a Student's *t*-test for unpaired two-tailed comparisons. *P*-values of less than 0.05 were considered significant.

3. Results

3.1. Induction of apoptosis

Hepa1c1c7 hepatoma cells were treated with different nitro-PAHs and DEPE for 24 h and B[*a*]P was used as a positive control. As illustrated in Figs. 1 and 2A and B all compounds except 1,8-DNP induced apoptosis and necrosis, as measured by fluorescence microscopy. 1,3-DNP was the most potent compound to induce cell death with a significant change detected at 3 μM. The highest increase in apoptosis was 31% obtained with 30 μM of 1,3-DNP, along with 14% necrotic cells. Regarding 1-NP, both apoptosis and necrosis were also observed, starting at 10 μM and giving maximal responses at 60 μM (17 and 23%, respectively). In contrast, 1,8-DNP did not induce cell death whatever the concentrations (10, 30 and 60 μM) tested. Furthermore, DEPE did not cause any marked increase in apoptosis (30 and 70 μg/mL). However, an increased amount of necrotic cells was measured at concentrations of DEPE above 30 μg/mL, the highest concentration tested (70 μg/mL) yielding 11% necrosis.

Flow cytometric analyses were also used to measure apoptosis. Data in Fig. 2C show that 1,3-DNP

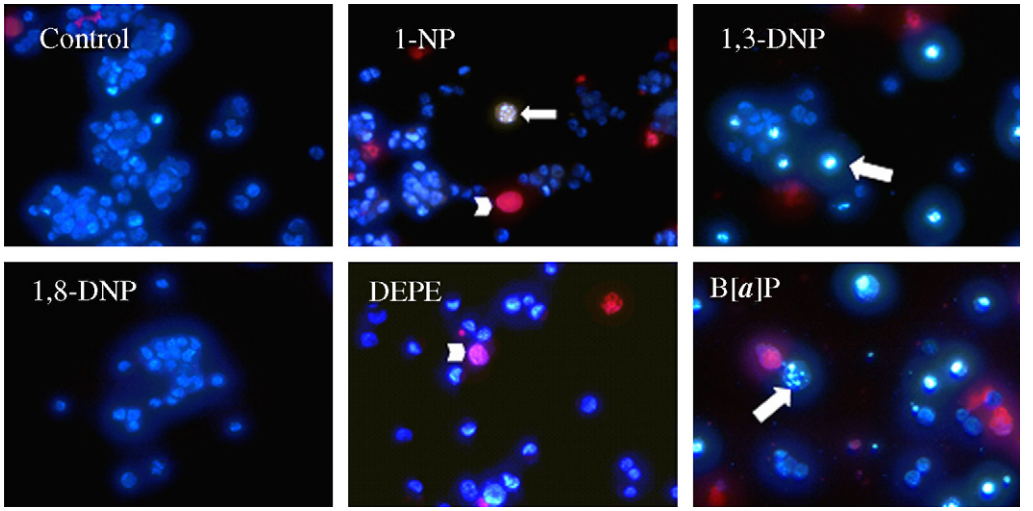

Fig. 1. Effects of nitro-PAHs on cell morphology. Hepa1c1c7 cells were treated with or without B[*a*]P, 1-NP, 1,3-DNP, 1,8-DNP and DEPE for 24 h. The concentrations used were 30 μM for nitro-PAHs and B[*a*]P and 70 μg/mL for DEPE. Arrows indicate apoptotic cells and arrow-head indicate necrotic cells. Cells were stained with Hoechst 33342 and propidium iodide (PI). Original magnification 400×.

was the most potent compound followed by 1-NP. No increase in apoptosis was seen upon exposure to 1,8-DNP, whereas DEPE resulted in a minor increase. The results from these studies correlated well with findings from the microscopic analyses. When looking at the time course of cell death appearance at a concentration of 30 μM, flow cytometric analysis revealed that apoptosis was significantly induced following 16 h of exposure to 1-NP and 1,3-DNP, with a more marked increase after 24 h of exposure. 1,8-DNP resulted only in a minor increase in apoptosis after 40 h. At a concentration of 70 μg/mL DEPE induced 6% apoptosis after 24 h, increasing to 18% following 48 h of incubation (data not shown).

3.2. Cleavage of caspases and DNA-fragmentation

Caspase 3 is an effector caspase that is activated by the initiator caspases 8 and 9 (Bratton and Cohen, 2001). Western blot analysis of cell lysates following 24 h of exposure showed increased amounts of cleaved caspase 3 with regard to all the nitro-PAHs tested except 1,8-DNP (Fig. 3A). 1,3-DNP was again the most potent starting at 10 μM; an increase was already seen after 16 h at a concentration of 30 μM (data not shown). In comparison, 1-NP exposure resulted in a slight increase only at the highest concentration tested (30 μM). DEPE also caused a minor activation at the two highest concentrations (50 and 70 μg/mL; Fig. 3A). Furthermore, the level of procaspase 8 was reduced when cells were exposed to the highest concentration tested (30 μM) for both 1-NP and 1,3-DNP. No change was observed with 1,8-DNP (data not shown).

The cleaved caspases activate DNA endonucleases resulting in DNA fragmentation. The gel presented in Fig. 3B show that 1,3-DNP induced DNA fragmentation following exposure to 10 and 30 μM, while no marked fragmentation was observed with the other nitro-PAHs and DEPE.

3.3. Metabolism

CYP1-enzymes are central in the activation of PAHs to reactive molecules that may bind covalently to macromolecules like DNA. To examine the relative potency of the nitro-PAH compounds and DEPE to induce the different cyp1-enzymes, we analysed protein levels after 8 or 24 h of exposure. The results showed that all compounds increased the level of cyp1a1 compared to control following 8 h-exposures (Fig. 4A). 1,3-DNP, 1,8-DNP and DEPE enhanced cyp1a1 level at the lowest concentration tested (i.e. 1 μM for 1.3-DNP/1.8-DNP and 1 μg/mL for DEPE), whereas a higher concentration of 1-NP (10 μM) was required. No induction of cyp1a2 was detected (data not shown). To further analyse the possible involvement of cyp1a1 in the bioactivation of the compounds, we used the CYP inhibitor α-naphthoflavone (α-NF) and assessed the induced toxicity. Compounds tested were 1,3-DNP and 1-NP, since the most marked effects on apoptosis were seen after exposure to these compounds. Fig. 4B shows that the toxicity of 1,3-DNP was almost completely blocked by α-NF. Interestingly,

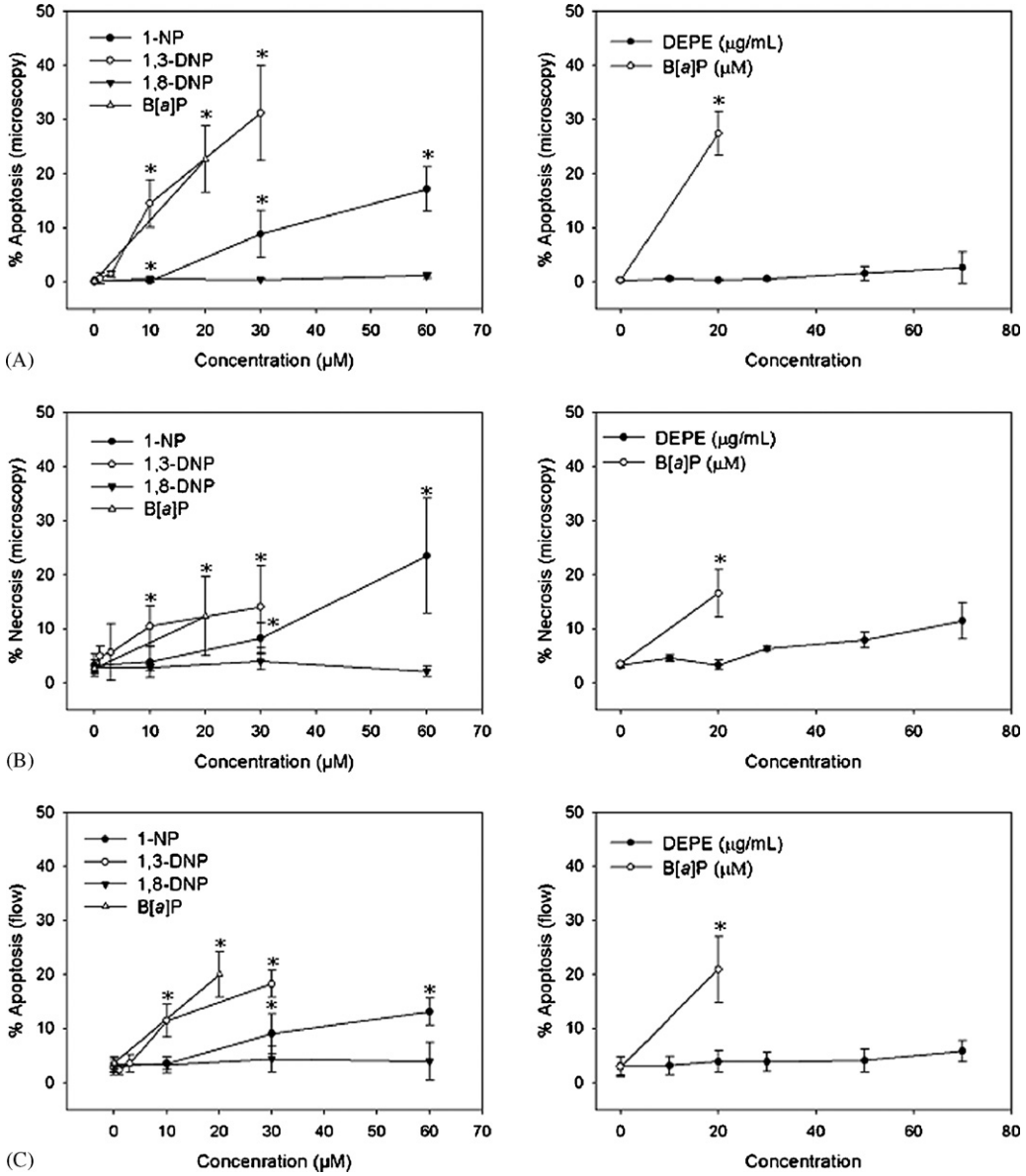

Fig. 2. Concentration-dependent effects of nitro-PAHs and DEPE on cell death. Hepa1c1c7 cells were treated for 24 h. The results are from fluorescence microscopic (A and B) and flow cytometric (C) analysis and presented as % apoptotic cells (A and C) and necrotic cells (B). Values represent means ± S.D. of ≥3 separate experiments. Asterisks (*) indicate a significant increase from control ($P < 0.05$).

α-NF rather increased the level of apoptosis in cells exposed to 1-NP.

DNA binding by 1-NP, 1,3-DNP and 1,8-DNP was evaluated by the ^{32}P-postlabelling method after 24 h of exposure at non-cytotoxic concentrations (Fig. 5). DNA adduct formation was only observed at 10 μM 1,8-DNP (Table 1), the highest concentration tested. In contrast, no DNA adducts were detectable for 1-NP at concentrations of up to 10 μM and for 1,3-DNP at concentrations of up to 3 μM, the highest concentration tested for 1,3-DNP due to increased cytotoxicity (Table 1). No DNA adduct formation was observed in control samples (data not shown). *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene (dG-C8-1-amino-8-NP) has previously been characterized and found to be the major adduct formed in vitro and in vivo following exposure to 1,8-DNP (Carmichael et al., 1996). Here we show that the major DNA adduct detected in Hepa1c1c7 cells after treatment with 1,8-DNP had similar chromatographic characteristics as the major adduct found when incu-

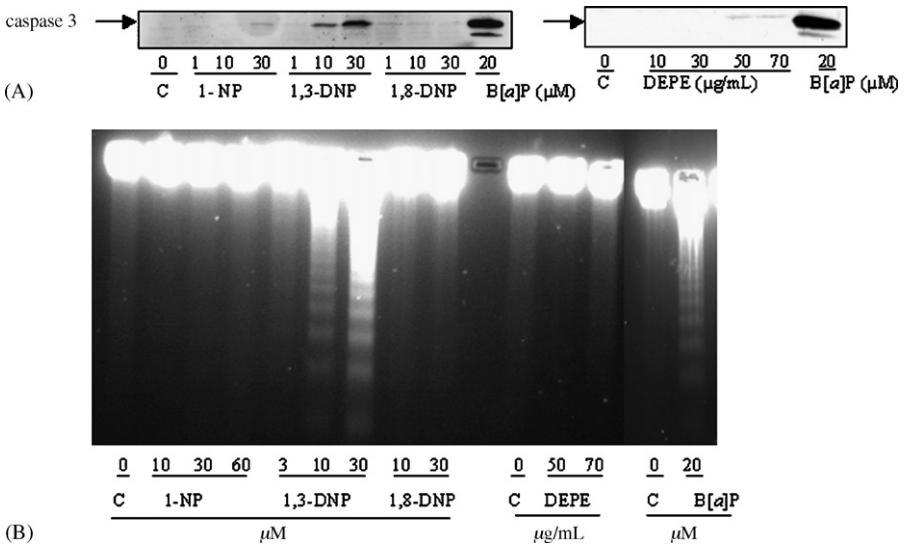

Fig. 3. Effects of nitro-PAHs and DEPE on the cleavage of caspase 3 and DNA fragmentation. Hepa1c1c7 cells were exposed to various concentrations of test compounds for 24 h. (A) Cleaved caspase 3 analysed by Western blotting. (B) Agarose gel electrophoresis of DNA. Typical experiments are shown.

bating DNA with 1,8-DNP in the presence of xanthine oxidase (data not shown), suggesting the formation of dG-C8-1-amino-8-NP. Similarly, reference DNA modified in vitro with 1-NP and 1,3-DNP resulted in the formation of one major DNA adduct, respectively (data not shown).

PAHs and nitro-PAHs are known to induce oxidative stress resulting in lipid peroxidation (Elbekai et al., 2004). The increased ROS production may be a result of the one electron metabolism of these compounds as well as due to secondary mitochondrial damage/changes as a part of the cytotoxic process. It was, however, not

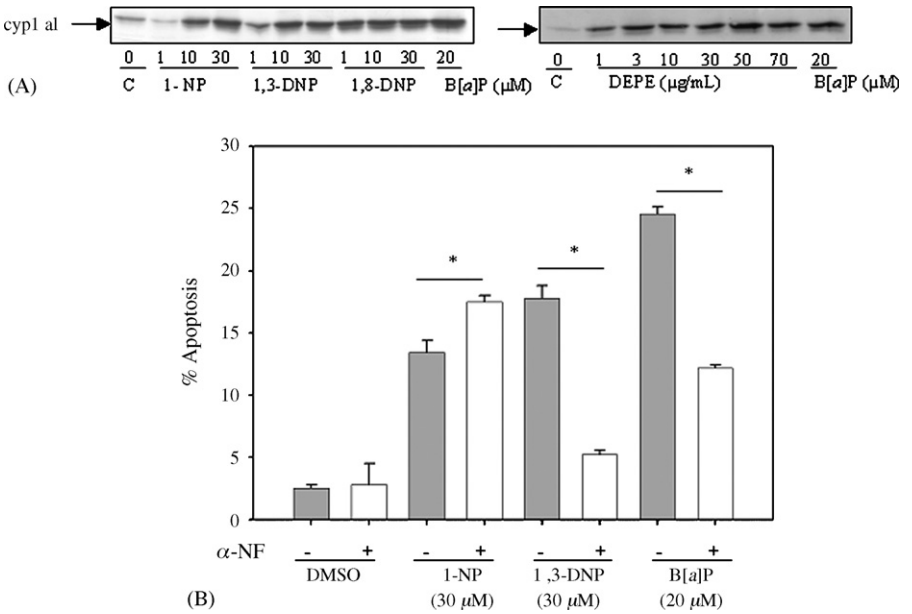

Fig. 4. Effects of nitro-PAHs and DEPE on CYP. (A) Effects of nitro-PAHs and DEPE on cyp1a1 analysed by Western blotting after 8 h of exposure. (B) Effect of α-NF on 1-NP- and 1,3-DNP-induced apoptosis analysed by flow cytometry. Cells were exposed for 24 h and data represent means ± S.D. of three incubations from one representative experiment. Asterisks (*) indicate a significant change from respective exposure without α-NF addition ($P < 0.05$).

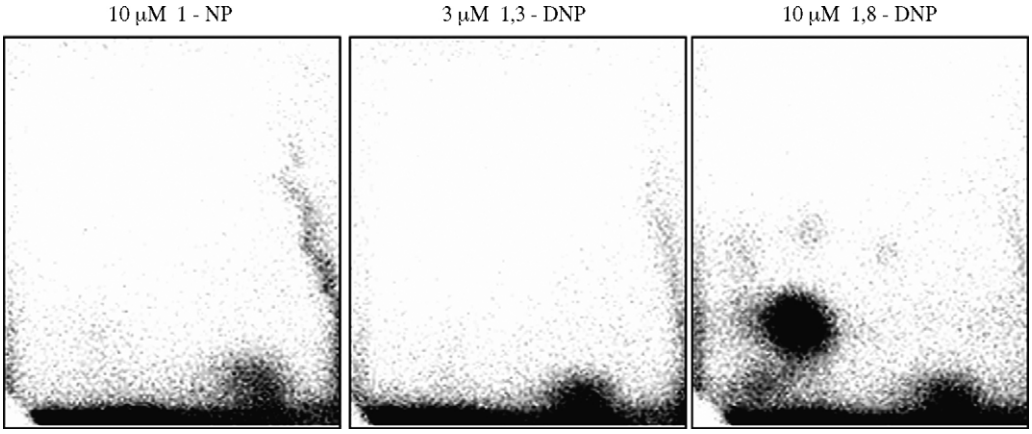

Fig. 5. ^{32}P-post labelling analysis of DNA from cells exposed to nitro-PAHs. DNA adduct pofiles measured in Hepa1c1c7 cells after exposure to the highest concentrations of 1-NP (10 μM), 1,3-DNP (3 μM) or 1,8-DNP (10 μM) tested for 24 h using the butanol enrichment version of the ^{32}P-post labelling assay.

possible to measure ROS in our system, due to interference of the nitro-PAHs with the fluorochrome used in the assay (data not shown). Thus, we decided to measure the effects of nitro-PAHs on lipid peroxidation as an indicator of ROS formation (Girotti, 1998; Y. Yang et al., 2003b). No significant increase in lipid peroxidation was observed after 8 and 12 h (data not shown), whereas following 24 h exposure, B[*a*]P elicited a significant lipid peroxidation at the highest concentration used (20 μM; Fig. 6). Regarding nitro-PAHs, a significant increase in lipid peroxidation was observed with both 1-NP and 1,3-DNP after 24 h, starting at 10 μM and stronger at higher concentrations. 1,3-DNP appeared to be somewhat more potent than 1-NP, especially at 30 μM. No marked effect on lipid peroxidation was found with 1,8-DNP.

Table 1
Quantitative DNA adducts analysis by ^{32}P-postlabelling

Nitro-PAH	RAL per 10^8 nucleotides[a]
1-NP—1 μM	ND[b]
1-NP—3 μM	ND
1-NP—10 μM	ND
1,3-DNP—0.3 μM	ND
1,3-DNP—1 μM	ND
1,3-DNP—3 μM	ND
1,8-DNP—1 μM	ND
1,8-DNP—3 μM	ND
1,8-DNP—10 μM	42.5 ± 13.7

Hepa1c1c7 cells were exposed to 1-NP, 1,3-DNP or 1,8-DNP for 24 h.
[a] Values (RAL, relative adduct labelling) represent mean ± S.D. of three separate incubations each determined by two separate postlabelling analyses.
[b] ND, not detected.

3.4. Accumulation of cells in S-phase and p53 phosphorylation

Using flow cytometry, 1-NP, 1,3-DNP and 1,8-DNP were found to increase the amount of cells in S-phase (Fig. 7A), whereas no changes were observed with DEPE (data not shown). 1,8-DNP induced a concentration-dependent S-phase accumulation starting at 3 μM (data not shown), with the highest increase at 10 μM (45% cells in S-phase). With B[*a*]P, an increase in S-phase was observed at 3 μM, as previously reported (Solhaug et al., 2005). DNA-reactive compounds including B[*a*]P are also found to phosphorylate p53. Western blot analysis of cell lysates after 24 h of exposure showed that all compounds gave an increased amount of phosphorylated p53 (p-p53) at serine 15. 1,3-DNP and 1,8-DNP phosphorylated p53 at 1 μM (which was the lowest

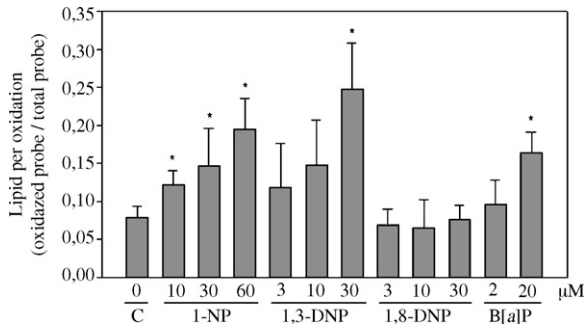

Fig. 6. Effect of nitro-PAH exposure on lipid peroxidation. Hepa1c1c7 cells were exposed for 24 h and lipid peroxidation was measured using the fluoroprobe C_{11}-BODIPY$^{581/591}$ as described in Section 2. Results represent means ± S.D. of three separate experiments. Asterisks (*) indicate significant change from control ($P < 0.05$).

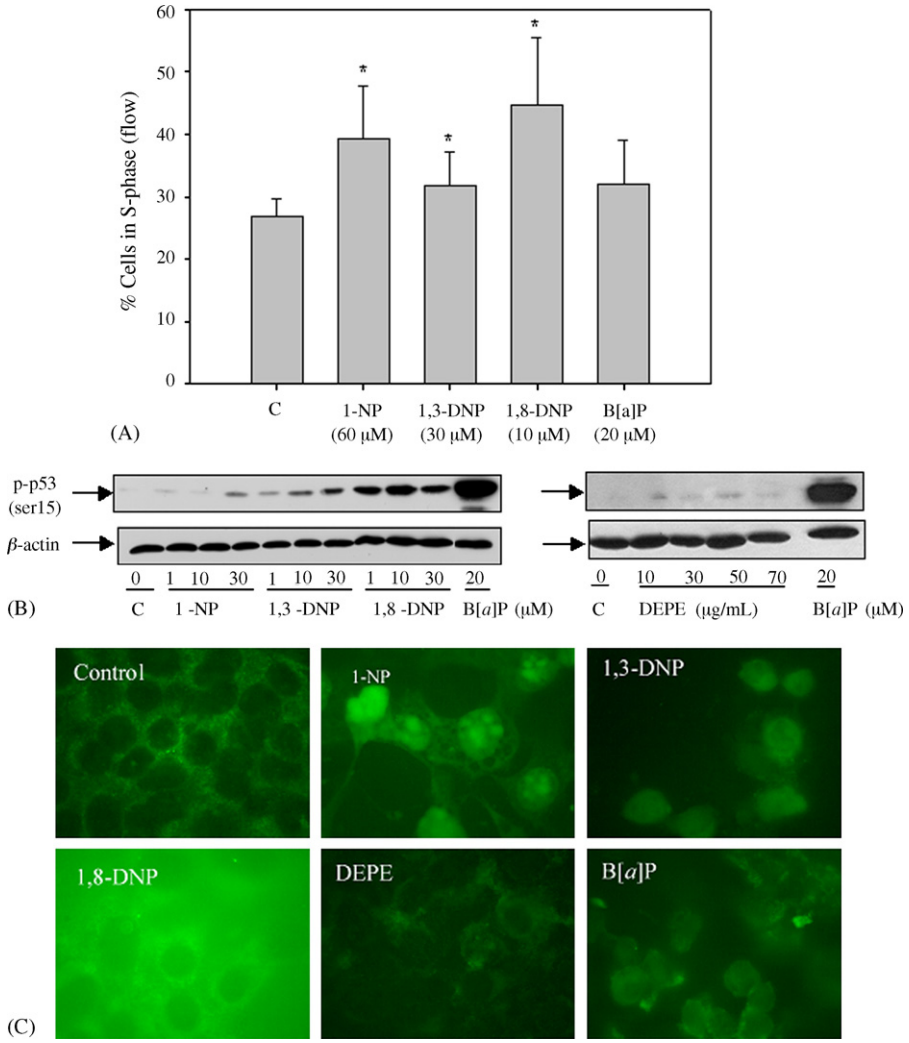

Fig. 7. Effects of nitro-PAHs and DEPE on the accumulation of cells in S-phase and on the p53 protein. (A) The accumulation of Hepa1c1c7 cells in S-phase was measured by flow cytometry after 24 h exposure to 60 μM 1-NP, 30 μM 1,3-DNP, 10 μM 1,8-DNP or 20 μM B[*a*]P. The concentrations shown for 1-NP and 1,3-DNP were the highest tested and the only concentration to give a significant increase. 1,8-DNP gave an increase in cells in S-phase at 3 μM (data not shown), the highest increase being at 10 μM. Data represent means ± S.D. of ≥3 separate experiments. Asterisks (*) indicate a significant increase from control ($P < 0.05$). (B) Increase in phosphorylation of p53 at serine 15 analysed by Western blotting after a 24 h-exposure to various concentrations of test compounds. (C) Localisation of p53 in cells, analysed by immunocytochemistry after 24 h of exposure. Concentrations used were 30 μM for nitro-PAHs, 50 μg/mL of DEPE and 20 μM for B[*a*]P. Original magnification 1000×.

concentration tested) with 1,8-DNP exerting the greatest effect. 1-NP exposure resulted in an increased level of p-p53 only at the highest concentration tested (30 μM), whereas 10 μg/mL of DEPE caused a slight increase in p53 phosphorylation (Fig. 7B). Microscopic analysis of cells after immunocytochemical staining for p53, showed that p53 was translocated to the nucleus following exposure to 1-NP, 1,3-DNP as well as B[*a*]P. Interestingly, no such p53 accumulation in the nucleus was observed after exposure to 1,8-DNP (Fig. 7C).

3.5. Bcl-proteins

Bcl-proteins can either be pro-apoptotic or anti-apoptotic (Gross et al., 1999). Anti-apoptotic bcl-x_L was markedly decreased following exposure to 10 μM 1-NP and 1 μM 1,3-DNP. DEPE gave a slight decrease in bcl-x_L levels at the highest concentration tested (70 μg/mL), whereas no changes were observed with 1,8-DNP (Fig. 8). Pro-apoptotic bid showed a reduced level after exposure to 30 μM 1,3-DNP. Interestingly, 1-NP, 1,8-DNP and DEPE seemed to rather increase the

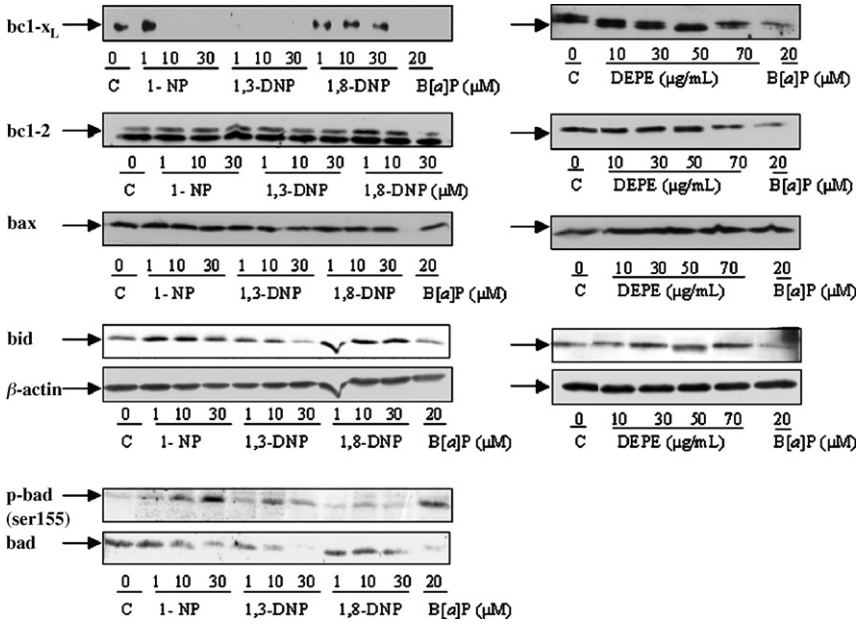

Fig. 8. Effects of nitro-PAHs and DEPE on bcl-proteins. Protein samples from Hepa1c1c7 cells were analysed by Western blotting following 24 h of exposure to various concentrations of test compounds using antibodies for anti-apoptotic bcl-x_L and bcl-2, pro-apoptotic bax and bid, phosphorylated bad at ser155 and total bad.

level of bid at several concentrations (1, 10 and 30 μM 1,8-DNP, 1 and 10 μM 1-NP and 30 and 70 μg/mL DEPE; Fig. 8). Furthermore, phosphorylation of bad (anti-apoptotic) increased following exposure to 10 and 30 μM of 1-NP and 10 μM of 1,3-DNP, whereas 30 μM 1,3-DNP decreased phosphorylation (Fig. 8). There were no changes in the level of pro-apoptotic bax and anti-apoptotic bcl-2 from nitro-PAHs-treated cells after 24 h.

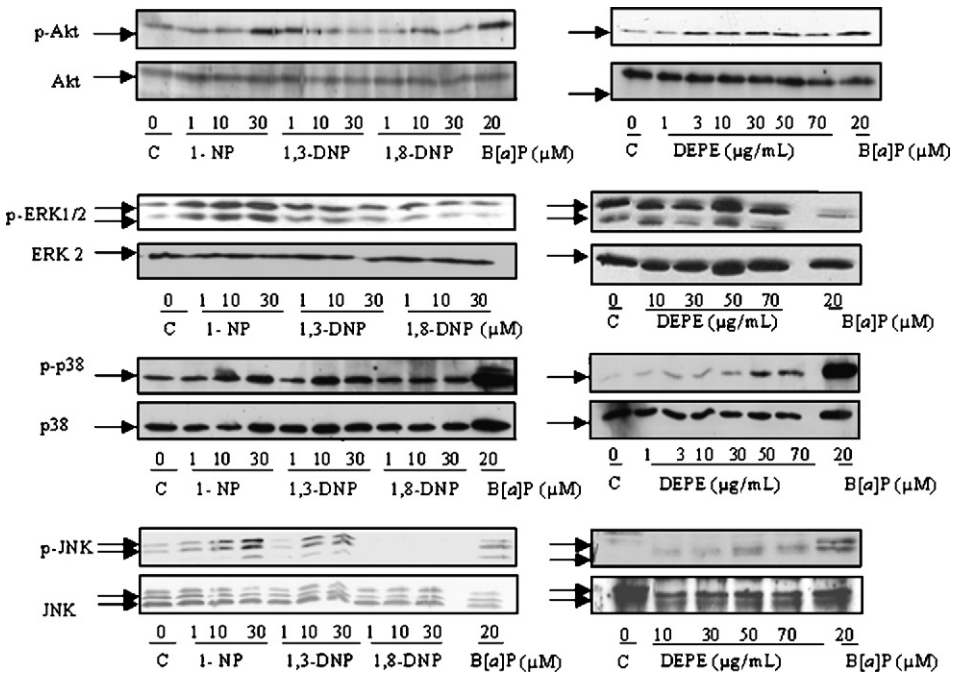

Fig. 9. Effects of nitro-PAHs and DEPE on Akt phosphorylation and MAPK. Protein samples from Hepa1c1c7 cells were analysed by Western blotting at various exposure times to test substance: p-Akt and Akt (24 h), p-ERK1/2 and ERK2 (24 h), p-p38 and p38 (8 h) and p-JNK and JNK (24 h).

DEPE gave a decrease in bcl-2 at 70 μg/mL, but no change in bax was observed (Fig. 8).

3.6. Akt phosphorylation

Akt is a protein kinase known to promote growth factor-mediated cell survival and to inhibit apoptosis (Song et al., 2005). Effects of test substances on Akt were analysed by Western blotting following 24 h of exposure. 1-NP gave an increased phosphorylation (activation) of Akt at 30 μM (Fig. 9), which was the highest concentration tested. 1,3-DNP caused an elevated level of p-Akt at the lowest concentration tested (1 μM). Increased levels of p-Akt was seen when cells were exposed to 1,8-DNP (10 μM). In the range of 3–50 μg/mL DEPE also markedly enhanced phosphorylation of Akt (Fig. 9).

3.7. MAPK phosphorylation

MAPKs are often found to be involved in the apoptotic signalling pathways. Exposure to 1-NP gave a higher level of phosphorylated ERK1/2 following 24 h-treatments (all concentrations), while 1,3-DNP, 1,8-DNP and DEPE did not seem to cause any marked change in the level of p-ERK1/2 (Fig. 9). 1-NP and 1,3-DNP increased phosphorylation of p38 following an 8 h-exposure to 10 μM, whereas DEPE elicited a similar response at a concentration of 3 μg/mL. 1,8-DNP remained ineffective whatever the concentration tested (Fig. 9). 1-NP, 1,3-DNP and DEPE enhanced the level of phosphorylated JNK after 24 h of exposure. 1-NP resulted in a concentration-dependent increase, starting at 1 μM, whereas 1,3-DNP and DEPE activated JNK at 10 μM and 10 μg/mL and higher, respectively. No activation of JNK was observed with 1,8-DNP (Fig. 9).

4. Discussion

1-NP, 1,3-DNP and 1,8-DNP have been reported to induce tumors in rats, and 1,8-DNP is generally considered to be the most potent genotoxic and carcinogenic compound of these nitro-PAHs (Imaida et al., 1991, 1995; Ohgaki et al., 1984; Takayama et al., 1983). In this study, we report that 1-NP and 1,3-DNP induced both apoptosis characterized by several parameters as well as necrosis in Hepa1c1c7 cells. Longer exposure times and higher concentrations were needed to observe a slight increase in cell death following 1,8-DNP and DEPE exposure.

The various compounds tested induced cell death in the following order: 1,3-DNP > 1-NP ≫ DEPE ≫ 1,8-DNP. These variations may be due to differences with regard to the constitutive rate of metabolic activation of the parent compound, the ability of the parent compound to induce its own activation, as well as the nature of the reactive metabolites formed. Although all nitro-PAHs tested as well as DEPE induced cyp1a1, the induction did not directly reflect the induced toxicity, which is in line with the notion that CYP metabolism does not represent a major step in the activation of many nitro-PAHs. However, α-NF almost completely reduced 1,3-DNP toxicity, suggesting that the formation of reactive metabolites was depending on CYP metabolism. In contrast, this inhibitor rather increased 1-NP-induced cell death, suggesting that CYP enzymes in this case primarily were involved in its detoxification.

Metabolic activation of nitropyrenes to reactive DNA binding species occurs predominately by reduction of the nitro group to *N*-hydroxyarylamines intermediates which can bind to DNA (Purohit and Basu, 2000). Moreover, in contrast to 1-NP, which is only *N*-acetylated, 1,3- and 1,8-DNP can be converted into highly reactive *O*-acetyl metabolites by transacetylases which bind to DNA to even greater extent (Beland and Marques, 1994). The major DNA adduct formed from 1-NP is *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP) and *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene (dG-C8-1-amino-8-NP) from 1,8-DNP (Heflich et al., 1986; Howard et al., 1983; Kinouchi et al., 1992; Silvers et al., 1997). This is in line with the detection of one major DNA adduct, most probably dG-C8-1-amino-8-NP, in Hepa1c1c7 cells after exposure to 1,8-DNP, whereas no adduct formation was observed with 1-NP and 1,3-DNP. Previously it was shown that in rat liver cytosolic incubations, 1-NP and 1,3-DNP were reduced to much lesser extent than 1,8-DNP, which suggests that there may be fundamental differences in the reduction pathways between these nitropyrenes (Djuric et al., 1986). As opposed to bacteria, most mammalian cell lines were only weakly mutated or not mutated by 1-NP or 1,3-DNP, but were highly sensitive to 1,8-DNP (Eddy et al., 1986, 1987). Induction of cyp1a1 in Hepa1c1c7 cells by the nitro-PAHs tested including 1-NP did not reflect the induced toxicity, but it may have resulted in a metabolic switch from nitroreduction to C-oxidation (Silvers et al., 1997) explaining why dG-C8-AP was not detected by ^{32}P-postlabelling at the concentration used. The enzymatic complement of nitroreductases in Hepa1c1c7 cells may be one basis for the different responses of nitropyrenes to DNA adduct formation. Whereas 1-NP and 1,3-DNP are reduced by a nitroreductase that transfers a single electron, 1,8-DNP are reduced by an enzyme that transfers 2 electrons (Eddy et al., 1986). This differential sensitivity could reflect

the relative balance between the activities of these two enzymes in different mammalian cell systems including Hepa1c1c7 cells (Busby et al., 1994). Thus, possibly cytosolic one and two electron nitroreductases catalyze the formation of different reactive metabolites of 1-NP, 1,3-DNP and 1,8-DNP, which may also result in differences with regard to ROS formation. ROS formation can give rise to oxidative DNA damage (Kim et al., 2005c; Nagy et al., 2005).

In general, differences in ROS production, may change the relative damage of various cell constituents and hence the cellular responses (Behrend et al., 2003; Esposito et al., 2004; Haddad, 2004). Several studies have demonstrated that metabolic activation and toxicity of several PAHs (Elbekai et al., 2004; Huc et al., 2003, 2004) and nitro-PAHs (Kim et al., 2005a; Nagy et al., 2005) have been associated to ROS production. However, it is also important to note that ROS production is often found to be a common part of the cytotoxic response due to secondary mitochondrial damage. In the present study, we only detected effects of ROS formation measured as increased lipid peroxidation following long-time exposure (24 h) to, B[*a*]P, 1-NP and 1,3-DNP suggesting that ROS formation seems to be an important part of their toxic response. No lipid peroxidation was observed following 1,8-DNP exposure, which corresponds with its lack of toxicity. Possible roles for ROS in the initiation of DNA damage with regard to 1-NP and 1,3-DNP toxicity are currently under further investigation, looking at the formation of oxidative DNA damage, including 8-oxo-guanine.

Mutations in the tumor suppressor *p53* gene are known to be one of the most frequently observed alterations in cancers, and reflects the advantage developing tumor cells thereby receives. Often these mutants are found to make the cells more resistant towards chemical-induced apoptosis. Some tumor-derived p53 mutants are even known to exhibit defect in inducing apoptosis but not to cell cycle arrest, indicating an important role of apoptosis in tumor suppression by p53 (Hussain and Harris, 1999). It is believed that low levels of DNA damage will activate a minor fraction of p53 resulting in cell cycle arrest and have anti-apoptotic functions. Following higher levels of DNA damage, p53 will accumulate above a certain threshold and activate pro-apoptotic genes including bax, PUMA and FAS receptor. Thus, the degree of ATM-ATR-p53 activation is important in DNA damage triggered apoptosis (Roos and Kaina, 2006). P53 protein may initiate apoptosis both as a transcription factor and by non-transcriptional events. Possible mechanism for non-transcriptional p53 apoptosis are translocation of the p53 protein itself to the mitochondria, while other report a direct activation of the pro-apoptotic protein bax (Marchenko et al., 2000; Moll and Zaika, 2001). In the present study all the nitro-PAHs and DEPE induced phosphorylation of p53 at serine 15, suggesting that the induced DNA damage results in an accumulation of phosporylated p53 and triggers the apoptotic signal, as previously reported for other PAHs (Solhaug et al., 2004a,b). However, significant DNA damage measured by DNA adducts was only observed after 1,8-DNP. Furthermore, we also found that 1,8-DNP which resulted in the largest increase in phosphorylated p53 and accumulation of cells in S-phase, only caused a minor increase in cell death. Interestingly, fluorescence microscopy revealed that 1,8-DNP did not result in the "classical" accumulation of p53 in the nucleus (often used as a marker of a functional p53), suggesting that it directly reduced the apoptotic function of p53 through a non-mutagenic event. Possible p53 post-translational modifications include site-specific phosphorylations, acetylations and ubiquitination (Appella and Anderson, 2001). However, 1,8-DNP exposure may also have effects on other proteins known to control stress-induced p53 nuclear accumulation such as MDM2 (Inoue et al., 2005).

Furthermore, it is interesting to note that 1,8-DNP is the more potent mutagen and carcinogen of the nitro-PAHs tested (Imaida et al., 1991; Takayama et al., 1983). It is possible that 1,8-DNP induces DNA damage that "normally" should result in cell death, but at the same time inhibits the triggering of the apoptotic process. It is hypothesized that a possible implication could be that more cells survive the DNA damage, but with an increased probability of having mutations and chromosomal aberrations. Such properties may contribute to the explanation to why 1,8-DNP are particular mutagenic and carcinogenic. Somewhat similar observations and ideas with other compounds have previously been reported. Khan et al. (1998) found that some PAHs induce p53 phosphorylation without causing G_1-arrest, which result in accumulation of cells in S-phase. Dipple et al. (1999) described this as "stealth properties" and suggested that it could increase the risk of mutations and thereby contribute to the carcinogenic potential of the compounds. Other compounds such as Cd(II) have been reported to suppress the p53-mediated cell cycle arrest by changing its transcriptional activity (Hartwig et al., 2002). Thus, the hypothesis that some carcinogens may cause post-translational changes in the p53 protein or proteins interacting with p53, thereby reducing its activity, should be further explored.

Despite no apparent DNA damage, 1-NP and 1,3-DNP induced phosphorylation and translocation of p53

to the nucleus, suggesting that p53 may nevertheless be involved in the induced apoptosis. Oxidative DNA damage should not be excluded (Kim et al., 2005c; Nagy et al., 2005), but the p53 protein has also been found to be phosphorylated by other ways than through DNA damage (Pluquet and Hainaut, 2001). Furthermore, other targets for the reactive metabolites triggering apoptosis that not necessarily involve p53 should also be considered. Regarding chemical-induced apoptosis, the mitochondria-dependent apoptotic pathway, including changed levels and translocation of various bcl-2 proteins, is considered to be important (Marchenko et al., 2000; Oren, 2003; Slee et al., 2004). In the present study, the nitro-PAHs and DEPE did not change the levels of the bcl-2 family members bax and bcl-2; however, a down regulation of bcl-x_L was seen. Similar findings have also been reported with CP-PAHs and B[*a*]P (Solhaug et al., 2004b). Further studies revealed that B[*a*]P induced a translocation of bax from the cytosol to the mitochondria as often seen with pro-apoptotic members following activation of death signals. A similar translocation of bax has also been found following exposure to 1,3-DNP (Landvik et al., unpublished data). Furthermore, we found that 1,3-DNP resulted in decreased levels of pro-caspase 8 (data not shown) and bid, suggesting a role of death receptors in 1,3-DNP-induced apoptosis. Effects of PAHs and nitro-PAHs on receptors/proteins in the plasma membrane are currently under investigation.

An interesting finding was that several cell signalling pathways seemed to be activated following exposure to the nitro-PAHs and DEPE similar to what has been previously reported following exposure to B[*a*]P (Solhaug et al., 2004b). 1-NP and 1,3-DNP exposure decreased the levels of bad and increased levels of phosphorylated bad at serine 155, a cell survival signal which has been found to be phosphorylated by protein kinase A (PKA) (Lizcano et al., 2000). Bad may also be phosphorylated at other sites by other cell survival pathways including Akt. Here, we report that 1-NP, 1,3-DNP and DEPE all increased the phosporylation/activation of Akt known to be a central kinase which is triggered by growth factors as well as insulin (Song et al., 2005). In previous studies, activation of the MAPKs has been associated with B[*a*]P (Li et al., 2004; Solhaug et al., 2005) and DEPE (Bonvallot et al., 2001; Hiura et al., 1999) exposure. Here, only 1-NP caused an activation of ERK, which is most often found to be an important cell survival signal (Roux and Blenis, 2004). 1-NP, 1,3-DNP and DEPE all induced phosphorylation of the stress MAPKs JNK and p38, which are generally considered to be pro-apoptotic (Sumbayev and Yasinska, 2005). However, in Hepa1c1c7 cells the increase in these MAPK following exposure to high concentrations of B[*a*]P and/or its reactive metabolite B[*a*]P-7,8-diol-epoxide, did not seem to have major effects on the apoptotic process (Solhaug et al., 2004a).

DEP is a major component of traffic-derived PM_{10} pollutants and consists of a vast number of PAHs as well as nitro-PAHs. In the standard reference material (SRM) 2975 for DEP (NIST, 2000b) there is about 700 times more 1-NP (36 mg/kg) than the well known carcinogenic compound B[*a*]P (0.05 mg/kg). DNP are generally considered to be more potent than 1-NP, but the SRM1975 for DEPE (NIST, 2000a) used in this study, contains more than 10 times the amount of 1-NP (16.4 mg/kg) than the DNP (1,3-DNP 0.6 mg/kg and 1,8-DNP 1.55 mg/kg, respectively). In the present study, when looking at p53 phosphorylation, the nitro-PAHs seemed to be able to induce DNA damage, however, DNA adduct formation was only detected after exposure to 1,8-DNP. DEPE increased the level of cleaved caspase 3 and phosphorylated p53, but to a lower degree than the nitro-PAHs on the mass basis. This was to be expected since the concentrations of the nitro-PAHs were higher when exposed as single components than in DEPE. DEPE gave a marked increase in cyp1a1 level, which reflects its high level of PAHs. The nitro-PAHs seem to contribute to the toxic effects of DEPE observed in Hepa1c1c7 cells; furthermore, when considering the relative amounts of 1-NP and DNP, the effects of 1-NP will be possibly higher or the same as the DNP. However, the diesel engines are continuously improving and correspondingly the levels of many PAHs and nitro-PAHs decreasing.

In summary, we found that the most mutagenic and carcinogenic compound tested, 1,8-DNP, induced little (if any) cell death, despite the fact that this compound seemed to give the most DNA damage as judged by DNA adduct formation, increased phosphorylation of p53 and accumulation of cells in S-phase. Immunocytochemical studies revealed that the p53 protein did not accumulate into the nucleus suggesting that 1,8-DNP inactivated the pro-apoptotic function of the p53 protein by a non-mutagenic event. These results suggest that after exposure to 1,8-DNP more cells may survive with DNA damage, thereby increasing its mutagenic and carcinogenic potential.

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