Effekter av dieseleksosekstrakt og nitrosubstituerte polysykliske aromatiske hydrokarboner på cellesignalisering og apoptose i Hepa1c1c7 celler.

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FORORD

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Nina E. Landvik

SAMMENDRAG

Ulike epidemiologiske studier har assosiert luftforurensning og dieseleksos med økt risiko for lungekreft. Dieseleksos inneholder en rekke ulike polysykliske aromatiske hydrokarboner (PAH) og nitrosubstituerte-PAH (nitro-PAH), hvor enkelte er ansett som mutagene og kreftfremkallende. I dette studiet har vi undersøkt toksiske effekter de ulike nitro-PAH, 1-nitropyren (1-NP), 1,3-dinitropyren (1,3-DNP) og 1,8-dinitropyren (1,8-DNP), og sammenlignet med effekter etter eksponering for dieseleksospartikkelekstrakt (DEPE) på hepatoma-cellelinjen Hepa1c1c7. Forbindelsene førte til både apoptotisk og nekrotisk celledød i Hepa1c1c7 cellene. Flow cytometriske og mikroskopiske analyser viste at den relative potensen var: 1,3-DNP>1-NP>>DEPE>>1,8-DNP. Alle forbindelsene førte til økt nivå av cyp1a1 og cyp1b1 analysert ved western blotting. Mikroskopiske analyser viste at celler eksponert for 1-NP førte til økt dannelse av granuler eller cytoplasmiske vesikler, noe som indikerte at en annen type celledød enn apoptose og nekrose også kunne være involvert. Toksisiteten til 1,3-DNP ble nesten fullstendig hemmet ved bruk av CYP-hemmeren α-naftaflavon (α-NF), mens toksisiteten til 1-NP økte ved bruk av hemmeren. 1,3-DNP initierte signaler som er assosiert med apoptose som fosforylering av p53 og dens translokasjon til kjernen, kløyving av caspase 8, bid og caspase 3, nedregulering av $bclx_L$ og fosforylering av p38 og JNK MAPK. Forbindelsen førte også til økt nivå av overlevelsessignaler som fosforylering av akt og inaktivering og nedregulering av bad. 1,8-DNP gav ingen markert økning i celledød, men var den mest potente forbindelsen til å fosforylere p53-protein og førte til akkumulering av cellene i S-fase. Dette kan tyde på at 1,8-DNP førte til DNA-skader selv om forbindelsen ikke førte til celledød. Immunocytokjemi viste at mangelen av celledød skyldes at 1,8-DNP ikke førte til en translokasjon av p53-protein til kjernen. Det at 1,8-DNP ser ut til å føre til DNA-skader, men at den apoptotiske prosessen er hemmet kan bidra til å forklare forbindelsens mutagene og kreftfremmende egenskaper. DEPE førte til endringer i genekspresjon og induksjon av apoptose, vi observerte fosforylering av p53 og dens translokasjon til kjernen, kløyving av caspase 3 og fosforylering av p38 og JNK MAPK. Celler eksponert for DEPE viste også økt nivå av overlevelsessignalet fosforylert akt. Funnet er i samsvar med at 1-NP, 1,3-DNP og 1,8-DNP bidrar til effektene en observerer ved DEPE eksponering. Ut i fra en vurdering av potens og mengder av de ulike nitro-PAH ser spesielt bidraget fra 1-NP ut til å være vesentlig.

FORKORTELSER

α-NF, α-naftaflavon; AhR, arylhydrokarbon reseptor; APAF-1, apoptose assosiertfaktor-1; ARNT, arylhydrokarbon nukleærtranslokator; ASK, apoptose signalregulerende-kinase; B[*a*]P, benzo[*a*]pyren; cdki, hemmer av cyklinavhengige-kinaser; CYP, cytokrom P450; 1,3-DNP, 1,3-dinitropyren; 1,8-DNP, 1,8-dinitropyren; ERK, ekstracellulær signalregulert-proteinkinase; JNK, c-JUN N-terminalkinase; MAPK, mitogenaktiverte proteinkinaser; MAPKK, mitogenaktiverte proteinkinase-kinaser; MAPKKK, mitogenaktiverte proteinkinase-kinase-kinaser; 1-NP, 1-nitropyren; nitro-PAH, nitrosubstituerte-polysykliske aromatiske hydrokarboner; NR, nitroreduktaser; PAH, polysykliske aromatiske hydrokarboner; ROS, reaktive oksygenforbindelser; TNF, tumornekrose faktor; XRE, xenobiotisk responselement;

BAKGRUNN

1. Luftforurensning og helse

Negative helseeffekter som en følge av luftforurensing er ikke et nytt fenomen, allerede i 1775 oppdaget Percivial Pott en sammenheng mellom testikkelkreft og soteksponering hos feiere (BROWN et al., 1957). I løpet av noen dager vinteren i 1930 økte dødeligheten i Meusey valley i Belgia markert. Svært mye industri vokste opp langs denne dalen etter starten av den industrielle revolusjonen, noe som førte til økt forurensning i området. Mellom 1. og 5. desember la det seg en tykk tåke over store deler av Belgia. Fra 3. Desember begynte hundrevis av mennesker i dalen og få respirasjonsproblem og plager. Mer enn 60 personer døde de neste tre dagene som en følge av denne tåken (Nemery et al., 2001). I 1952 var det en kraftig økning i luftforurensing i London kjent som ”den store London tåken”. Fra 5. til 9. desember var luften så dårlig at det er antatt at 4 000 mennesker døde disse dagene og de to neste ukene. De neste ti ukene døde ytterligere 8 000 mennesker som en følge av denne tåken (Black 2003; WILKINS 1954). Videre utover 1950 ble begrepet ”smog” opprettet, smog er en betegnelse som stammer fra ordene ”smoke” og ”fog”. Økningen i smog rundt om i verden, som f eks Los Angeles, førte til opprettelsen av ”The clean air act”. Denne opprettelsen har igjen gitt opphavet til USAs miljøvernsdepartement (USEPA), som først og fremst skulle beskytte folket mot skadelig utendørs luftforurensning (Casarett 2001). I dag indikerer ulike epidemiologiske undersøkelser at det er en sammenheng mellom lungekreft og økt luftforurensning. Pope og medarbeidere (Pope, III et al., 2002) konkluderer i sin kohortundersøkelse basert på 500 000 amerikanere at finpartikulær luftforurensning er en viktig risikofaktor i forhold til blant annet lungekreft. ”Six harvard studies” er en kohortundersøkelse som baseres på 8 111 personer fra seks amerikanske byer. Personene ble fulgt fra 1972 til 1989. Dataene indikerte en økning i antall lungekrefttilfeller med økt luftforurensning (Dockery et al., 1993; Vineis et al., 2004).

Luftforurensning består av ulike komponenter, de viktigste er svoveldioksid (SO_2), nitrogenoksider (NO_x), karbonmonoksid (CO), ozon (O_3) og svevestøv. Svevestøv vurderes som en viktig komponent av luftforurensningen med stor betydning for menneskers helse. De består av små partikler som holder seg svevende i luften i lengre tid. Store deler av svevestøvet er forbrenningspartikler. Disse partiklene er ofte bygd opp av ulike komponenter bundet til en karbonkjerne (Fig. 1). Forbrenningspartikler dannes ved ufullstendig forbrenning

av organisk materiale, og kan resultere i dannelsen av polysykliske aromatiske hydrokarboner (PAH) som ofte finnes på overflaten av partiklene.

Fig. 1. Forenklet bilde av en forbrenningspartikkel (Nasjonalt folkehelseinstitutt 2004)

Partikler er ulike når det gjelder størrelse og fasong, derfor beskrives de ut i fra deres aerodynamiske diameter. Aerodynamisk diameter er gitt ut i fra fallhastigheten til en dråpe med diameter 10 µm og tetthet 1 g/cm^3. En partikkel (fast stoff/væske/miks) med lik fallhastighet som dråpen defineres som PM_{10}. Ulik egenvekt gir ulik aerodynamisk diameter på partikler som geometrisk sett er identiske. Den aerodynamiske diameteren beskrives som PM_{10}, $PM_{2,5}$ og $PM_{0,1}$ som er henholdsvis grovfraksjon (2,5-10 µm), finfraksjon (≤ 2,5 µm) og ultrafinepartikler (≤ 0,1 µm) (Nasjonalt folkehelseinstitutt 2004; Ormstad et al., 2002). Størrelsen på partikkelen gjenspeiler i noen grad hvor i luftveiene de deponeres, større partikler avsettes høyere opp i luftveiene enn de som er mindre. Finfraksjon og ultrafinepartikler kan deponeres i lungeblærene. Fig. 2 illustrerer størrelsen på de ulike partikkelfraksjonene.

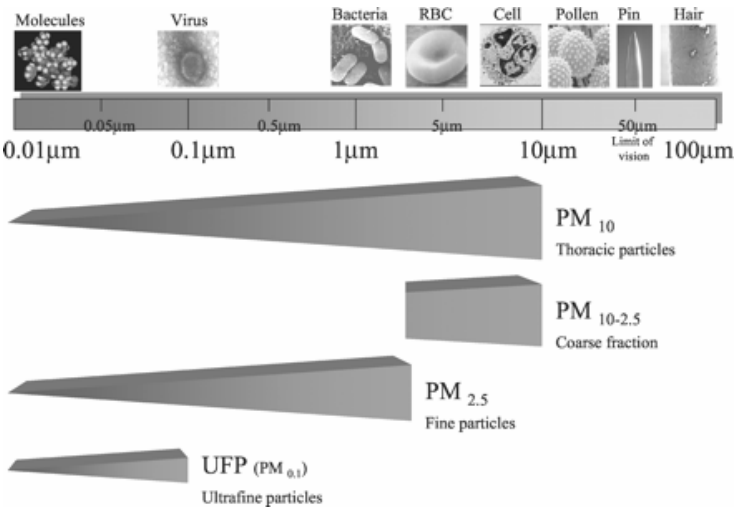


Fig.2. Størrelsesforhold til de ulike partikkelfraksjonene. Grovfrasksjonen (PM_{10}) tilsvarer størrelsen på en celle, finfraksjon ($PM_{2,5}$) en bakterie, de ultrafinepartiklene ($PM_{0,1}$) tilsvarer i størrelsesorden virus og molekyler. Figuren er hentet fra Brook og medarbeidere (Brook et al., 2004)

I en artikkel i Lancet i 2002 ble urban luftforurensning beskrevet som en av verdens store risikoer i forhold til mortalitet og morbiditet. Det ble antatt at nærmere 800 000 mennesker årlig dør p.g.a. dette (Ezzati et al., 2002). En rekke epidemiologiske undersøkelser indikerer en sammenheng mellom negative helseeffekter og luftforurensning. Sammenhengen er likevel ikke like klar i dagens studier som den var for 30 år siden. I den vestlige verden i dag er luftforurensningen generelt redusert og kildene er forandret. Partiklene kommer ikke først og fremst fra industrielle kilder, men fra biltrafikk. Partiklene har også forandret karakter ved at det er færre store. De viktigste kildene til svevestøv er dieseleksos og partikler fra veistøv og vedfyring (Ormstad et al., 2002).

2. Dieseleksos

Dieseleksospartikler er den største komponenten av finfraksjonen ($PM_{2,5}$) i urbane områder (Kawasaki et al., 2001) og har i befolkningsundersøkelser vært knyttet opp mot forverring av lungesykdommer som astma (Nicolai 1999; Segala et al., 1998). Dieseleksos kan også være en viktig kilde til økningen av risikoen for lungekreft som skyldes langtidseksponering av finpartikulær, forbrenningsrelatert luftforurensning (Dockery et al., 1993; Pope, III et al., 2002; Vineis et al., 2004). I andre undersøkelser er dieseleksos mer direkte knyttet opp mot økning i lungekreft. En studie som ble gjort på jernbanearbeidere etter innføringen av dieseldrevne lokomotiv i USA, viste økning i antall lungekrefttilfeller hos arbeidere som

jobbet i nærheten av jernbanen (Garshick et al., 2004). En svensk undersøkelse viser en økt forekomst av lungekreft hos yrkessjåfører i urbane områder, men ikke hos sjåfører i distriktene (Jakobsson et al., 1997; Vineis et al., 2004).

Dieseleksos inneholder en rekke ulike PAH og nitrosubstituerte-PAH (nitro-PAH). 1-nitropyren (1-NP) er en av de mutagene komponentene det er høyest nivå av, og forårsaker 25 % av den bakterielle mutagenisiteten i dieseleksos. Enkelte dinitropyrener (DNP) er ansett som mer mutagene, men forekommer i mye lavere konsentrasjon enn 1-NP (Purohit et al., 2000; Rosenkranz et al., 1980). Dieseleksos er klassifisert som gruppe 2A, sannsynligvis kreftfremkallende i mennesker av International Agency for Research on Cancer (IARC) (IARC 1989).

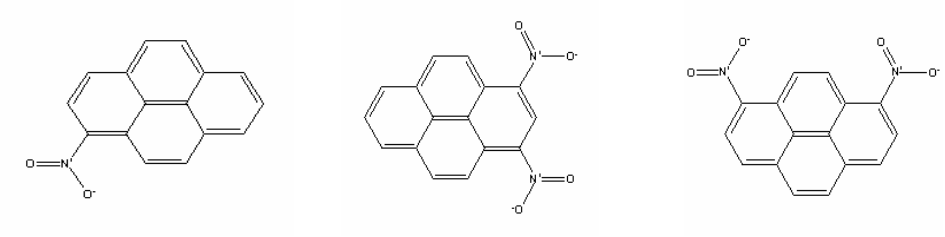
2.1. Polysykliske aromatiske hydrokarboner.

PAH dannes ved ufullstendig forbrenning av organisk materiale. Kildene kan være blant annet vedfyring, sigarettrøyk, grilling og dieseleksos (IARC 1989; Knize et al., 1999; Rosenkranz et al., 1980; Rosenkranz et al., 1983). PAH er en klasse strukturelt beslektede kjemikalier med to eller flere benzenringer (Yaffe et al., 2001). Disse ringene kan være substituert med ulike kjemiske grupper. Benzo[a]pyren (B[a]P) er den PAH det mest kunnskap om, og regnes som gruppe 2A av IARC (IARC 1987).

2.2. Nitro-PAH

Nitroarener er nitrosubstituerte PAH, disse inkluderer 1-NP, 1,3-DNP og 1,8-DNP (Purohit et al., 2000); Fig. 3). Enkelte nitro-PAH kan dannes ved ufullstendig forbrenning på samme måte som PAH, 1-NP er et eksempel på dette. De fleste dannes allikevel sekundert i atmosfæren fra PAH ved at PAH reagerer med nitrogenoksider (Yaffe et al., 2001).

1-NP og 1,8-DNP er klassifisert som gruppe 2B, mulige kreftfremkallende ovenfor mennesker, av IARC (IARC 1989). 1,3-DNP er klassifisert som gruppe 3, ikke kreftfremkallende i mennesker av IARC (IARC 1989).



1-Nitropyren 1,3-Dinitropyren 1,8-Dinitropyren

Fig. 3. Strukturformel og navn på ulike nitro-PAH

2.3. Metabolisme

PAH omdannes i kroppen via ulike enzymer til vannløselige forbindelser som lettere skilles ut av kroppen. Metabolisme av PAH via cytokrom P450 (CYP) enzymer kan midlertidig også resultere i dannelse av reaktive forbindelser som kovalent binder seg til makromolekyler som DNA. Dette kan igjen medføre DNA mutasjoner. B[a]P, er en kjent femringet PAH som er regnet som kreftfremkallende. B[a]P biotransformeres via CYP1A1 for å danne det ultimate karsinogen benzo[a]pyrene-7,8-dihydrodiol-9,10-epoksid. Nitro-PAH kan biotransformeres via CYP-enzymer, nitroreduktaser og fase II biotransformasjonsenzymer og gi opphav til reaktive metabolitter (Arlt et al., 2005; Chae et al., 1999; Guengerich 2000; Hatanaka et al., 2001), disse enzymene kan også føre til detoksikering av PAH. (Gelboin et al., 1981; Shimada et al., 1990). Hovedmengden PAH skilles ut via galle eller nyrer.

2.3.1. CYP1

Det er ulike CYP-enzymer som kan være involvert i metabolismen til PAH og nitro-PAH, hvorav CYP1A1, 1A2, 1B1 og 3A4 er noen viktige eksempler (Chae et al., 1999; Guengerich 2000; Moorthy et al., 2003). CYP-enzymene kan føre til oksidering av nitro-PAH slik at det dannes epoksider og/eller dioler, de kan også føre til en nitroreduksjon som kan gi opphav til arylhydroksylaminer.

CYP1 er ett av flere proteiner som induseres av arylhydrokarbon reseptoren (AhR). AhR aktiveres ved at kjemiske induktorer som B[a]P bindes slik at et kjernelokaliseringssignal blottlegges. AhR translokeres dermed til kjernen hvor den heterodimeriserer med arylhydrokarbon nukleærtranslokator (ARNT). Dette komplekset bindes til xenobiotisk responselement (XRE) som ligger oppstrøms for de aktuelle genene (Fig. 4). Minst syv ulike

gener involvert i metabolisme av PAH blir transkribert, fase 1 enzymer som CYP1A1, CYP1A2 og CYP1B1 samt fase 2 enzymer som, glutationtransferase (Gsta1, Ya), glukuronosyltransferase (Ugt1a6), NAD(P)H:kinon-oksidoreduktase (Nqo1) og cytosolisk aldehyd-dehydrogenase (Aldh3a1). Disse enzymene bidrar til å ytterligere gjøre enkelte stoffer mer vannløselig (Nebert et al., 2000; Nebert et al., 2004; Pascussi et al., 2004).

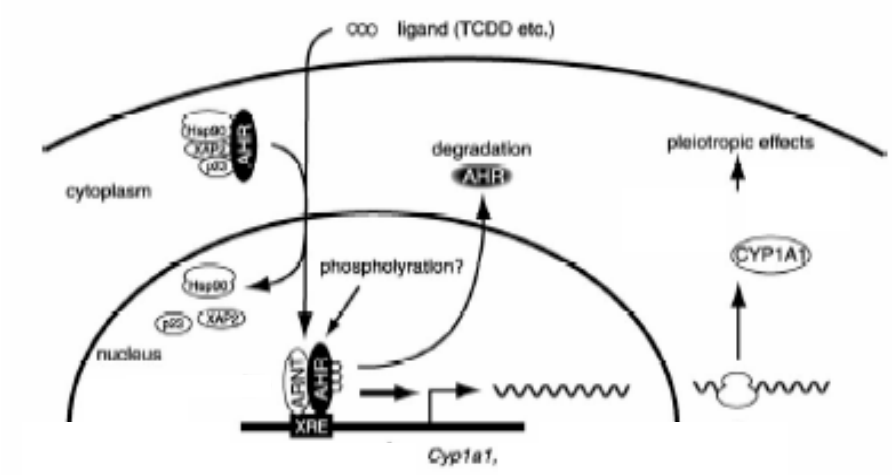


Fig. 4. CYP1 induksjon, modifisert fra Mimura og medarbeidere (Mimura et al., 2003)

2.3.2. Nitroreduktaser

Reduksjon av nitrosubstituerte aromatiske forbindelser kan skje via to typer nitroreduktaser (NR), type I NR som ikke krever tilstedeværelse av oksygen og type II NR som krever tilstedeværelse av oksygen. Reduksjon av nitroforbindelsen (R-NO_2) til det korresponderende amin (R-NH_2) gir en tilførsel på seks elektroner. Denne reaksjonen kan føre til dannelse av elektrofile reaktive mellomprodukter (nitroso og *N*-hydroksylamin) eller reaktive oksygenforbindelser (ROS). Hvilke produkter som dannes avhenger av hvilken NR som er involvert. Metabolisme av nitrosubstituerte-aromatiske forbindelser via type I NR gir først overføring av to elektroner slik at nitroso (R-NO) forbindelsen dannes. Nitroso derivatet kan videre reduseres med to elektroner til *N*-hydroksylamin (R-NHOH). Både nitroso og *N*-hydroksylamin derivater er reaktive forbindelser som kan bindes kovalent til makromolekyler som DNA. *N*-hydroksylamin kan videre reduseres med to elektroner til det korresponderende aminet. Ved tilstedeværelse av oksygen (type II NR) kan molekylært oksygen oksidere nitro-

anion ($R\text{-}NO_2^{\bullet -}$) radikalet. Dette fører til en redoks-syklus som gir regenerert nitro forbindelse og superoksidanion (Ask et al., 2003; Ask et al., 2004). Begge disse reaksjonene er illustrert i Fig. 5.

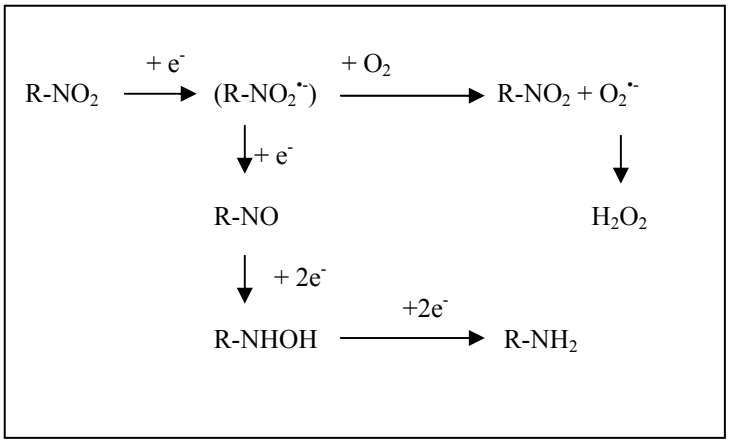


Fig. 5. Nitroreduksjon via oksygensensitive og ikke-sensitive nitroreduktaser. Begge de metabolske prosessene kan føre til dannelse av reaktive metabolitter

Ulike typer nitroreduktaser som kan være involvert er henholdsvis DT-diaphorase, xanthine oksidase, aldehyd oskidase og cytokrom c (P450) reduktase (P450R) (Arlt et al., 2003; Ask et al., 2003).

2.3.3. Metabolisme av 1-NP

Metabolisme av nitro-PAH via CYP1-enzymer og NR kan føre til dannelse av en rekke ulike metabolitter hvorav enkelte kan være reaktive. CYP-enzymene kan føre til detoksikering av 1-NP ved 3, 6 eller 8 hydroksylering til 3/6/8-OH-1-NP, de kan også føre til toksikering ved å danne 1-NP-4,5-epoksid. Både CYP-enzymer og NR kan føre til dannelse av *N*-hydroksy-1-aminopyren og 1-aminopyren. *N*-hydroksy-1-aminopyren kan videre metaboliseres av sulfotransferaser (SULT) og *N*-acetyltransferaser (NAT) (Arlt et al., 2005; Glatt 2000; Hein et al., 1997). Disse enzymene kan føre til dannelse av reaktive nitreniumioner som igjen kan reagere med DNA og gi skader (Fig. 6).

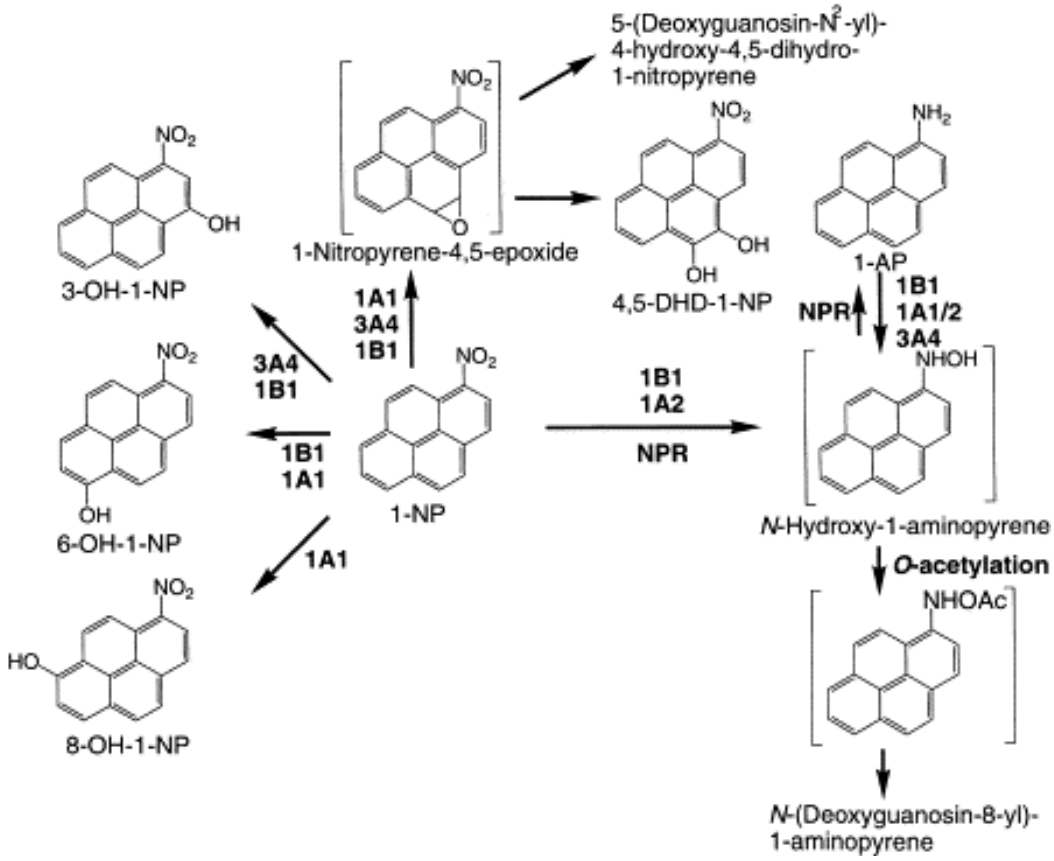


Fig. 6. Foreslått metabolisme av 1-NP (Hatanaka et al., 2001)

3. Kinaser

3.1. Mitogenaktiverte protein (MAP)-kinaser

MAP-kinase (MAPK) superfamilien består av hovedsakelig tre proteinkinase familier. Disse er, ekstracellulær signalregulert proteinkinase (ERK), c-JUN N-terminalkinase (JNK) og p38 kinase familien (p38). Disse familiene er involvert i et vidt spekter av signalveier som er tilknyttet vekstfaktormediert regulering og kan føre til celledeling, differensiering, permanent cellesyklusarrest og/eller apoptose (Cowan et al., 2003). Generelt aktiveres ERK av vekstfaktorer og knyttes til celledeling. JNK og p38 aktiveres av stresstimuli som osmotisk sjokk, ioniserendestråling og cytokiner. De knyttes ofte opp til celledød, permanent cellesyklusarrest og/eller apoptose. MAPK er serin/treonin kinaser som blir aktivert av MAPK-kinaser (MAPKK). MAPKK blir igjen aktivert av MAPKK-kinaser (MAPKKK). De ulike stimuliene nevnt over (vekstfaktorer, stress, cytokiner) aktiverer MAPKKK slik at kaskaden initieres (Fig. 7). Det er et stort antall ulike MAPKK og MAPKKK (Dent et al., 2003; Roux et al., 2004).

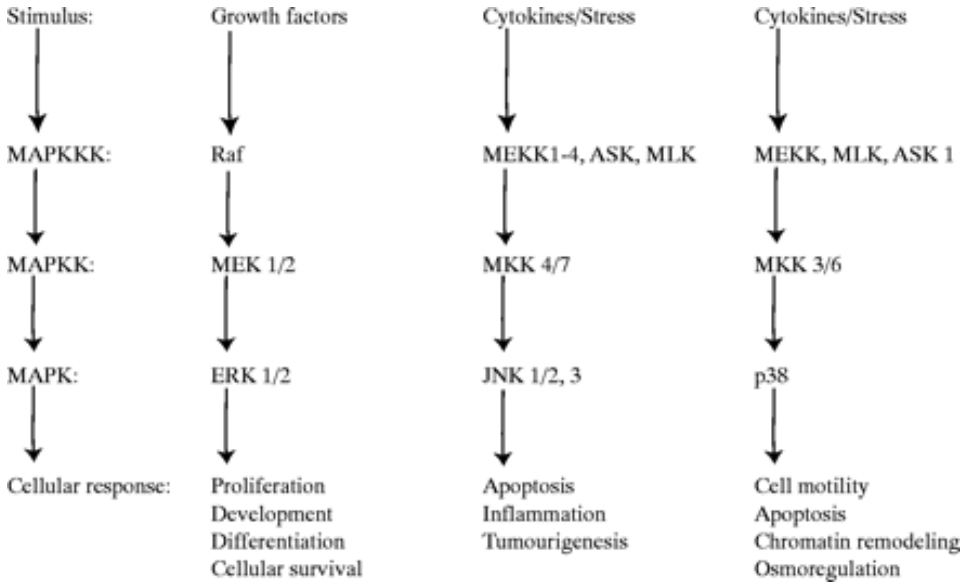


Fig. 7. Aktivering av de ulike MAPK-familiene (ERK, JNK og p38) (Cowan et al., 2003)

3.2. Akt/PKB proteinkinaser

Akt/PKB er en serin/treonin kinase og tilhørerer cAMP-avhengig proteinkinase A/G/C (AGC) superfamilien. Kinasen er sentral i signalveier som aktiveres av vekstfaktorer og insulin. Aktivering kan føre til cellevekst, regulering av transkripsjon og overlevelse. Vekstfaktorer stimulerer aktivering av fosfatidylinositol 3-kinase (PI3K) som videre fører til fosforylering av Akt/PKB. Akt/PKB aktiverer og inhiberer en rekke proteiner som er involvert i celleproliferasjon og celledød (Franke et al., 2003; Song et al., 2005). Akt/PKB fører til fosforylering og hemming av proapoptotisk bad (se punkt 4.4.2. for mer informasjon om bcl-2 familien). Videre vil Akt aktivering hemme caspase 9 som er viktig i apoptotisk celledød (se 4.4.2. for beskrivelse caspaser). Aktivering av akt fører også til hemming av apoptose signalregulerende-kinase (ASK). ASK er en MAPKKK som er oppstrøms for JNK og p38.

4. Celledød

Celledød inndeles ofte i apoptose og ”cellulær nekrose”. I den senere tid er det også vært fokus på andre måter å dø på som permanent cellesyklusarrest, mitotisk katastrofe og autophagy.

4.1. Permanent cellesyklusarrest (senescence)

Ved DNA-skade kan en celle reagere på ulike måter avhengig av hvor stor DNA-skaden er og om den kan repareres. Skaden vil ofte føre til cellesyklusarrest hvor den kan bli reparert og hvis den er stor vil cellen ofte dø. Cellesyklusarrest kan også bli vedvarende, og kalles da ofte permanent eller "levende celledød" ettersom cellene tilsynelatende lever, men ikke kan dele seg. p53-protein er en tumorsupressor som er svært sentral i permanent cellesyklusarrest. Det aktiveres ved enkelt- eller dobbelttrådbrudd i DNA. Aktivert p53-protein er fosforylert og kan enten virke som en transkripsjonsfaktor, eller direkte regulere ulike proteiner involvert i celledeling og/eller reparasjon (Fig. 8). Ved cellesyklusarrest aktiverer p53-protein ulike typer gener som hemmer cellesyklus, som *p21*. p21-protein er en hemmer av cyklinavhengige-kinaser (cdki), cyklinavhengigekinaser er sentrale i igangsetting av celledeling. Stopp i celledeling som gir tid til DNA reparasjon kan være med på å redusere risikoen for utvikling av kreft. Studier har vist at mus med mutasjoner som fører til at cellene ikke kan gå i permanent cellesyklusarrest er mer følsomme for utvikling av kreft (Okada et al., 2004).

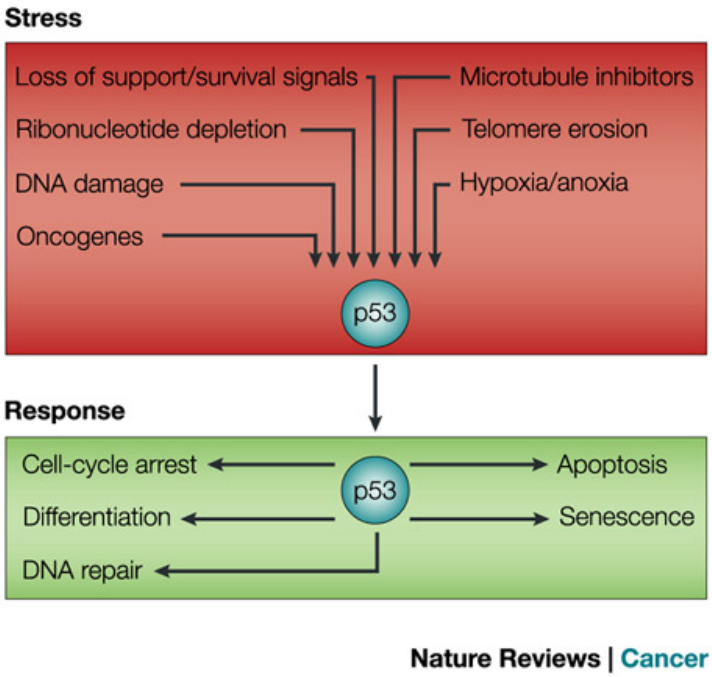


Fig. 8. DNA-skadeinduserte mekanismer kan resultere i enten reparasjon, apoptose eller cellesyklusarrest. p53 er et sentralt protein i disse intracellulære reaksjonsveiene (Vousden et al., 2002)

4.2. Mitotisk katastrofe

Mitotisk katastrofe ble beskrevet i 1986 og forklarer en type celledød observert hos gjærcellearten *Schizosaccharomyces pombe*. Cellene hadde overekspresjon av cdc2 som fører til aktivering av celledeling. Dette kan resultere i at cellene går for tidlig inn i mitosen og dør. Nyere studier har utvidet denne definisjonen, den brukes nå også på mammalsk celledød og skyldes abberasjoner i mitosen. Mitotisk katastrofe er karakterisert ved økt dannelse av multinukleære gigantceller som inneholder ukondenserte kromosomer og er morfologisk klart forskjellig fra apoptose, nekrose og autofagi (Okada et al., 2004).

4.3. Cellulær nekrose

Eksponering for stråling og kjemikalier kan føre til membranskader og redusert funksjon av ulike makromolekyler. Resultatet kan ofte være forstyrrelser i cellens ionebalanse som det intracellulære calsiumnivået og pH, ødeleggelse av mitokondrier og plasmamembran (Lockshin et al., 2004). Under denne cellulære nekrosen vil innholdet i cellen lekke ut til omgivelsene og derfor ofte resultere i inflammasjon. Denne type celledød kan også være forårsaket av en rekke andre forhold, som ulike former for infeksjoner.

4.4. Fysiologisk celledød

Celledød er viktig i utviklingen av flercellede organismer. For at cellene skal kunne degraderes og fjernes uten å gi noen form for inflammasjon har cellene egne kontrollerte ”dødsveier”. Disse dødsveiene kalles ofte fysiologisk celledød, som videre kan deles inn i autofagi og apoptose.

4.4.1. Autofagi

Autofagi er et forklarende navn for denne type celledød. Navnet har sin opprinnelse i gresk og betyr selvspisende. De fleste celler dør allikevel ikke av autofagi. Det er en velkjent fysiologisk prosess som er involvert i degradering av organeller. Prosessen starter ved dannelse av en membran rundt den aktuelle organellen. Denne kalles et autofagosom. Lysosom fusjonerer med autofagosomet og danner autolysosom og organellen degraderes (Fig. 9). I humane leverceller regner en med i størrelsesorden ett mitokondrium degraderes via autofagosomer hvert tiende minutt.

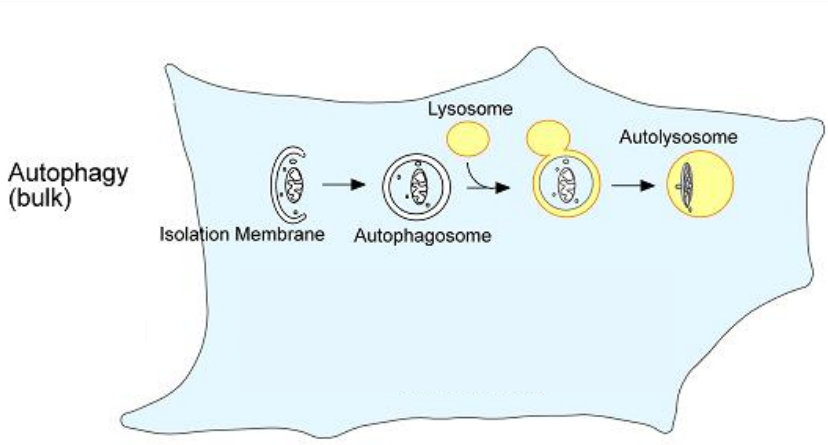


Fig. 9. Hvordan en celle degraderer organeller i cellen ved autofagi. Modifisert fra internet (The Tokyo Metropolitan Institute of Medical Science)

Autofagisk celledød karakteriseres ved celler som har ulike dobbelt- eller multimembran vesikler i cytoplasma. Disse består ofte av organeller som mitokondrium og endoplasmatisk reticulum. Vesiklene og innholdet i vesiklene blir ødelagt av det lysosomale systemet til cellen. Lysosomene har et surt miljø og innholder en rekke degraderingsenzymer som f.eks. proteaser. Ved en slik form for celledød dannes langt flere autofagosomer enn ved vanlig degradering av organeller. Konsekvensen er at cellen ”spiser” opp seg selv fra innsiden (Gozuacik et al., 2004).

4.4.2. Apoptose

Det er ulike morfologiske og biokjemiske kjennetegn ved apoptose. Cellen skrumper, men organellene ødelegges ikke så raskt siden den apoptotiske prosessen trenger energi. Det er en aktivering av caspaser som fører til oppkutting av DNA i spesifikke lengder (”DNA-laddering”) og tilslutt fragmentering av cellen. De apoptotiske cellene blir fagocytert av andre celler (naboceller eller immunceller; Fig. 10) og gir dermed ingen inflammasjon (Lockshin et al., 2004).

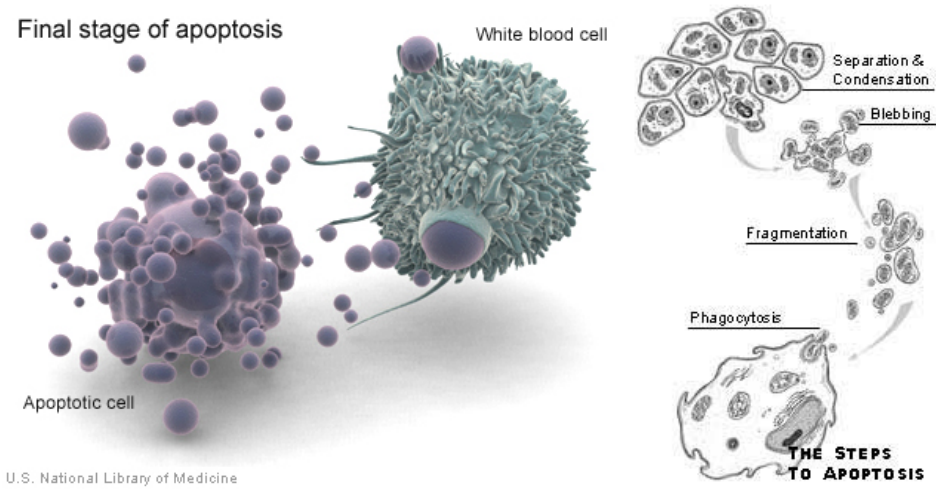


Fig. 10. Skrumping og fragmentering av cellen. Her vist hvordan en immuncelle fagocyterer en apoptotisk celle (Allstetter)

Apoptose også kalt programmert celledød er svært viktig for utviklingen av flercellede organismer. Hos mennesker fjernes blant annet "svømmehuden" mellom fingrene, og hos frosken forsvinner halen til rumpetrollet på denne måten. Denne typen celledød kan i tillegg initieres ved eksponering fra stråling eller kjemikalier ved at enkelte proteiner/enzymer eller DNA blir skadet på en måte som cellen ikke klarer reparere. Ved DNA-skade aktiveres ulike proteiner som er involvert i stopp av celledeling, reparasjon av skaden og/eller apoptose.

Apoptose er blitt et svært aktivt forskningsområde. Den store interessen startet på 1970-tallet. I perioden fram til 2001 ble det publisert nærmere 80 000 artikler om temaet (Lockshin et al., 2001), og siden den gang er det publisert mer enn 43 000 (pubmed april 2005).

Apoptose deles inn i utvendige og innvendige apoptotiske-signalveier. Den utvendige, aktiveres av endogene signal som bindes til dødsreseptorer på celleoverflaten. Den innvendige signalveien kan initieres ved ulike typer ekstracellulært og/eller intracellulært stress (Okada et al., 2004).

Proteiner og gener involvert

p53 - Et gen som koder for et tumorsupressor-protein. p53-proteinet aktiveres som en følge av enkelt- eller dobbeltrådbrudd i DNA. Proteinet er svært viktig for å hindre tumordannelse ved at det fører til aktivering, hemming eller

transkripsjon av proteiner involvert i reparasjon av DNA, cellesyklusarrest og apoptose. Det fører til oppregulering av blant annet signalproteinet bax og redusert mengde av proteinene bcl-2 og $bclx_L$, som alle er svært sentrale i apoptosen (Oren 2003; Schuler et al., 2001; Slee et al., 2004).

Apoptosom -Et protein kompleks som består av cytokrom c, APAF-1 og caspase 9. Apoptosomet aktiverer caspase 3.

Caspaser - Caspaser er en familie cysteinproteaser. Disse proteinene er svært viktig for både aktivering og gjennomføring av apoptosen. Navnet caspaser er en forkortelse for cystein aspartat-spesifikkeproteaser. Den katalytiske aktiviteten er avhengig av et cysteinresiduum som er felles for alle caspasene. Proteasene kutter andre proteiner/enzymer etter aminosyren aspartat og fører til aktivering eller inaktivering av substratet (Salvesen et al., 1997). Caspasene deles inn i initiator- og effektorcaspaser. Initiatorcaspaser (caspase 8 og 9) starter en kaskade som fører til aktivering av effektorcaspaser. Effektorcaspaser (caspase 3) induserer apoptosen direkte ved og blant annet føre til aktivering av nukleaser som gir fragmentering av DNA (Bratton et al., 2001).

Bcl-proteiner - Strukturelt beslektede proteiner som enten er proapoptotiske eller antiapoptotiske. De proapoptotiske fører til frisettelse av cytokrom c til cytosol og aktivering av apoptosen. De antiapoptotiske hemmer denne frisettelse av cytokrom c. (Bratton et al., 2001).

4.4.2.1. Utvendig apoptotisk-signalvei (Fig. 11)

Den utvendige apoptotiske-signalvei aktiveres av endogene eller endogenliknende ligander som bindes til reseptorer på celleoverflaten (Okada et al., 2004). En av disse reseptorene er Fas som også kalles CD95. Fas tilhører en reseptorgensuperfamilie ved navnet tumornekrose faktor (TNF). Proteinene fra denne familien har cysteinrike ekstracellulære domener. Fas er spesielt viktig i hovedsakelig tre typer fysiologisk apoptose som fjerning av aktiverte T celler etter en immunrespons, dreping av celler som er virusinfiserte eller kreftceller via cytotoksiske T celler og NK-celler (natural killer) og i dreping av inflammatoriske celler. Den apoptotiske signalveien aktiveres ved at det bindes en ligand til reseptoren (Fas-ligand). Dermed vil reseptoren føre til aktivering av caspase 8 som oligomeriserer og aktiveres ved at

den kløyver seg selv (Ashkenazi et al., 1998). Caspase 8 kan påvirke den apoptotiske-signalveien på flere måter. Den kan enten direkte aktivere caspase 3 eller kløyve bid, som er et proapoptotisk bcl-protein. Aktivering av bid vil føre til apoptose via mitokondriene som beskrevet under den innvendige apoptotisk-signalveien.

4.4.2.2. Innvendig apoptotisk-signalvei.

Den innvendige apoptotiske-signalveien aktiveres av ekstra- eller intracellulært stress. Signalene som initieres som en følge av denne type stress konvergeres hovedsakelig via mitokondriene (Fig. 11). Apoptose tilknyttet mitokondriene kan initieres via dødsreseptorene som er beskrevet over, eller som en følge av for eksempel DNA-skade (Okada et al., 2004). Aktivering av caspase 8 gir en kløyving av bid. Kløyvet bid medfører en translokering av bax til mitokondriene. Bax er et proapoptotisk bcl-protein og kan sammen med bid bindes til mitokondriemembranen og føre til frisetting av cytokrom c. Dette gjøres enten ved å danne en pore i membranen eller ved å aktivere en tilstedeværende pore slik at cytokrom c skilles ut. Cytokrom c danner et proteinkompleks med APAF-1 og initiatorcaspase 9, dette komplekset gir en kløving av caspase 3 som igjen vil kløyve ulike ”dødssubstrat” (som fører til aktivering av nukleaser) som resulterer i apoptose. Utskillelsen av cytokrom c kan hemmes av bcl-2 og $bclx_L$ som begge er antiapoptotiske bcl-proteiner. Disse hemmer cytokrom c-utskillelsen direkte (porene i mitokondriemembranen) eller indirekte (hemming av APAF-1). p53 påvirker apoptosen ved blant annet oppregulering av Fas og bax (Bratton et al., 2001), eller direkte ved å redusere nivået av bcl-2 og $bclx_L$ (Slee et al., 2004).

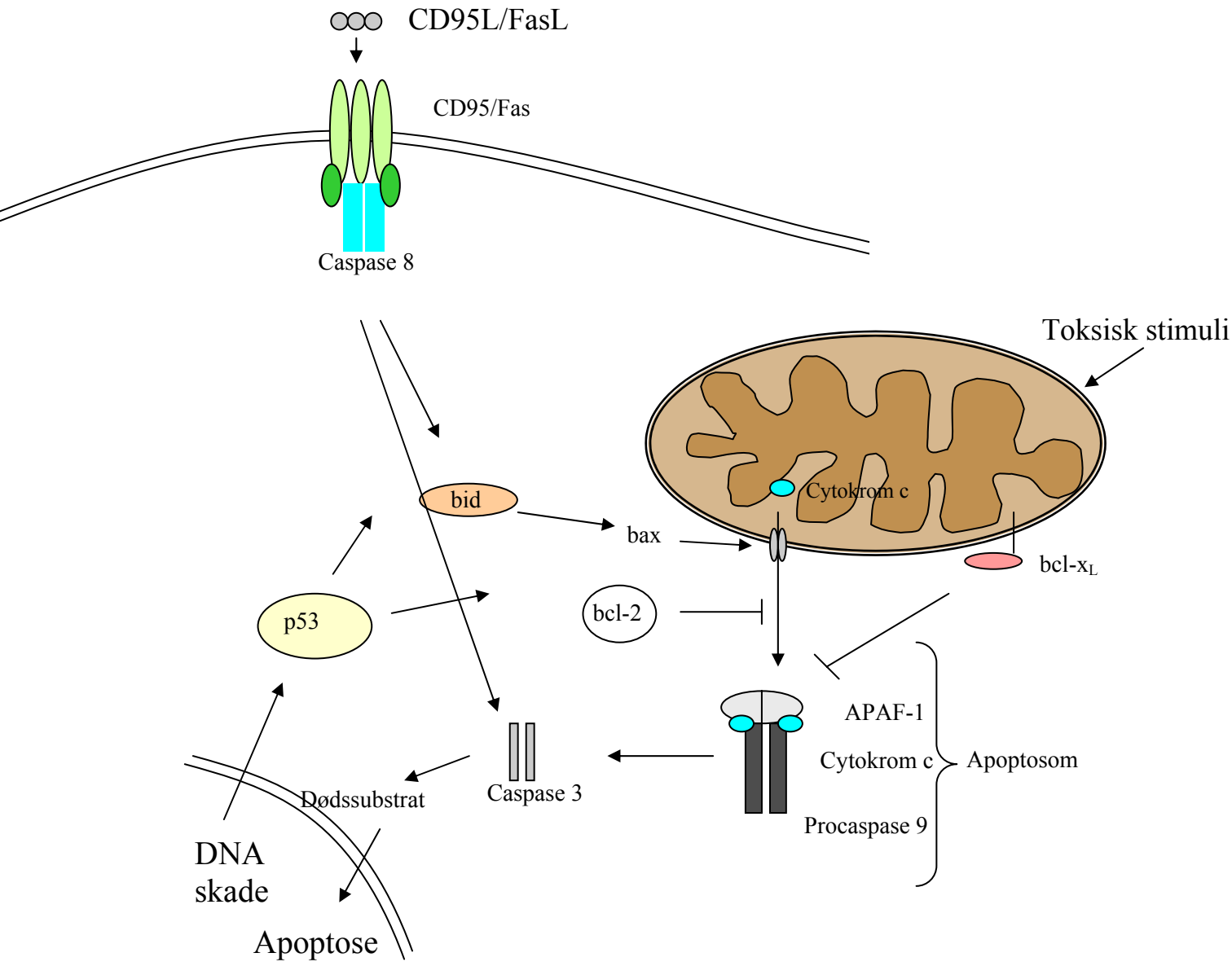


Fig. 11. Utvendig og innvendig apoptotisk-signalvei (forenklet oversikt)

MÅL

Kartlegge toksisiteten til de ulike nitro-PAH (1-NP, 1,3-DNP, 1,8-DNP) og DEPE i hepatoma-cellelinjen Hepa1c1c7. Cellelinjen er innarbeidet på avdelingen og har vist seg som et godt modellsystem for å teste PAH.

- Undersøke hva slags type celledød som er dominerende for de ulike stoffene.

- Undersøke om forbindelsene induserer cyp1-enzymer, og om cyp1-enzymene metaboliserer stoffene til reaktive forbindelser.

- Undersøke om nitro-PAH og DEPE fosforylerer p53-protein, og om det er endringer i cellesyklus.

- Undersøke om nitro-PAH og DEPE induserer overlevelses og dødsignaler.

- Sammenligne effektene av nitro-PAH med effektene etter eksponering av DEPE.

KONKLUSJON

- Hepa1c1c7 celler eksponert for nitro-PAH og DEPE førte til både apoptotisk og nekrotisk celledød, den relative potensen var: 1,3-DNP>1-NP>>DEPE>>1,8-DNP. Celler eksponert for 1-NP førte til økt dannelse av granuler eller cytoplasmiske vesikler, noe som indikerte at en annen type celledød enn apoptose og nekrose også kunne være involvert. 1,3-DNP var den mest potente forbindelsen til å initiere apoptose. 1,8-DNP gav ingen markert økning av apoptose og nekrose. De høyeste konsentrasjonene som ble testet av DEPE induserte både apoptose og nekrose, men på et senere tidspunkt enn 1-NP og 1,3-DNP.

- Alle forbindelsene induserte cyp1a1 og cyp1b1. Toksisiteten til 1-NP ble ikke hemmet ved bruk av CYP-hemmeren α-NF. Dette tyder på at andre enzymer kan være involvert i aktiveringen av 1-NP. På den andre siden, ble toksisiteten til 1,3-DNP nesten fullstendig

hemmet ved bruk av α-NF. Dette tyder på at toksisiteten til 1,3-DNP avhenger av dens metabolisme gjennom cyp1a1 og cyp1b1.

- Alle forbindelsene gav økt fosforylering av p53-protein. 1-NP, 1,3-DNP og DEPE førte til translokasjon av p53 til kjernen. 1,8-DNP gav ingen markert økning i celledød, men var den mest potente forbindelsen til å fosforylere p53-protein og førte til akkumulering av cellene i S-fase. Dette kan tyde på at 1,8-DNP førte til DNA-skader selv om forbindelsen ikke førte til celledød. Mangelen av celledød skyldes at 1,8-DNP ikke førte til en translokasjon av p53-protein til kjernen. Det at 1,8-DNP ser ut til å føre til DNA-skader, men at den apoptotiske prosessen er hemmet kan bidra til å forklare forbindelsens mutagene og kreftfremmende egenskaper.

- 1-NP, 1,3-DNP og DEPE førte til økt nivå av proapoptotiske- og antiapoptotiske-/overlevelses- signaler. Celler eksponert for 1-NP induserte proapoptotiske-signaler som fosforylering av p53 og dens translokasjon til kjernen, kløyving av caspase 8 og caspase 3, nedregulering av $bclx_L$ og aktivering av p38 og JNK MAPK. 1-NP førte også til økt nivå av overlevelsessignaler som fosforylering av akt, inaktivering og nedregulering av bad og fosforylering av ERK MAPK. 1,3-DNP induserte signaler i den apoptotiske responsen som fosforylering av p53 og dens translokasjon til kjernen, kløyving av caspase 8, bid og caspase 3, nedregulering av $bclx_L$ og fosforylering av p38 og JNK MAPK. Forbindelsen førte også til økt nivå av overlevelsessignaler som fosforylering av akt og inaktivering og nedregulering av bad. DEPE induserte signaler i den apoptotiske responsen som translokasjon av p53 til kjernen og fosforylering av p38 og JNK MAPK. Forbindelsen førte også til en økning i overlevelsessignalet fosforylert akt.

- Funnene i dette studiet er i samsvar med at 1-NP, 1,3-DNP og 1,8-DNP bidrar til effektene en observerer ved DEPE eksponering. Av de ulike nitro-PAH ser spesielt bidraget fra 1-NP ut til å være vesentlig.

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Effects of diesel exhaust particle extracts and some of the major nitrated polycyclic aromatic hydrocarbon components on apoptotic and cell signalling in Hepa1c1c7 cells.

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ABBREVIATIONS

α-NF, α-naphtaflavone; AhR, aryl hydrocarbon receptor; B[*a*]P, benzo[*a*]pyrene; CP-PAH, cyclopenta-polycyclic aromatic hydrocarbons; CYP, cytochrome P450; cyt c, cytochrome c; DEP, diesel exhaust particles; DEPE, diesel exhaust particle extracts; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulphoxide; DNP, dinitropyrene; 1,3-DNP, 1,3-dinitropyrene; 1,8-DNP, 1,8-dinitropyrene; ERK, extracellular signal-related kinase; FCS, foetal calf serum; ICAD, inhibitor of caspase activated deoxynuclease; JNK, c-jun N-terminal kinase; MAPK, mitogen activated kinases; NQOI, NADPH:quinone oxidoreductase; 1-NP, 1-nitropyrene; nitro-PAH, nitrosubstituted- polycyclic aromatic hydrocarbons; NOS, nitrogen oxide synthase; NR, nitroreductases; PAH, polycyclic aromatic hydrocarbons; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; siRNA, small interference RNA; tbid, truncated bid; cdk, cyclin dependent kinases; XO, xanthine oxidase

ABSTRACT

In the present study we show that the Hepa1c1c7 cells exposed to nitrosubstituted-polycyclic aromatic hydrocarbons (nitro-PAH) and diesel exhaust particle extracts (DEPE) induced apoptosis to a various extend and in the following order: 1,3-dinitropyrene (1,3-DNP) > 1-nitropyrene (1-NP) >> DEPE >> 1,8-dinitropyrene (1,8-DNP) as measured by flow cytometry and microscopic analysis. The compounds all induced cyp1a1 and cyp1b1 and seemed to affect different intracellular signalling pathways. Microscopic analysis showed that 1-NP resulted in cells with increased formation of cytoplasmic vesicles, suggesting that cell death pathways other than apoptosis and necrosis could also be involved. The CYP-inhibitor, α-naphtaflavone (α-NF), almost completely blocked 1,3-DNP induced cell death. This suggests that the toxicity that results from 1,3-DNP are due to its metabolism through cyp1a1 and 1b1. In contrast, 1-NP caused an increase in cell death when α-NF was added. 1,3-DNP resulted in activation of pro-apoptotic proteins, including increased phosphorylation of p53 and its translocation to the nucleus, cleavage of bid, caspase 8 and caspase 3, down-regulation of $bclx_L$ and phosphorylation of p38 and JNK MAPK. 1,3-DNP also resulted in an increased level of survival signals such as activation of akt and inactivation and down-regulation of bad. 1,8-DNP did not seem to induce apoptosis to a large extend, but was the most potent compound in regard to phosphorylation of p53 and resulted in accumulation of cells in S-phase. Interestingly, in cells exposed to 1,8-DNP, p53 was phosphorylated, but did not enter the nucleus. The lack of apoptosis despite of DNA damage could contribute to 1,8-DNP mutagenic and carcinogenic properties. DEPE changed the gene expression and increased the level of apoptosis as observed by increased phosphorylation of p53 and its translocation to the nucleus, cleavage of caspase 3 and phosphorylation of p38 and JNK MAPK. DEPE also resulted in an increase of the survival signal akt. These findings indicate that the nitro-PAH contributes to the effects of DEPE observed in the Hepa1c1c7 cells, furthermore when considering the potency and amount of the different nitro-PAH, the contribution of 1-NP seems to be substantial.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are environmental pollutants formed during incomplete combustion of organic material. They are present in many mixtures such as cigarette smoke, coal fly ash, grilled food and diesel exhaust (IARC 1989; Purohit et al., 2000; Rosenkranz et al., 1980; Rosenkranz et al., 1983). Nitro-PAH are an important subgroup of the PAH, found on diesel exhaust particles (DEP) that has been suggested to be related to the development of lung cancer (Garshick et al., 2004; Pope, III et al., 2002; Vineis et al., 2004). Studies indicate that several of the nitro-PAH are highly mutagenic in bacterial systems (Rosenkranz et al., 1980; Rosenkranz et al., 1983) and are found to cause mutations and tumors in animal models (El Bayoumy 1992; Guengerich 2000).

Nitro-PAH may be metabolized by ring oxidation, nitroreduction as well as conjugation reactions. These reactions may lead to detoxified as well as reactive metabolites. Ring hydroxylations may result in reactive epoxides and are catalysed by cytochrome P450 enzymes like CYP1A1, 1A2 or 1B1 (Yamazaki et al., 2000). These CYP-enzymes are induced by aryl hydrocarbon receptor (AhR) which is known to be activated by many aromatic compounds (Chen et al., 2003; Nebert et al., 2000; Nebert et al., 2004). Some of these CYP-enzymes have also been suggested to be able to *N*-hydroxylate the nitro-PAH. This may lead to formation of nitroso, *N*-hydroxylamine and the corresponding amine. A further acetylation or sulphatation of the *N*-hydroxylamine can result in the formation of reactive nitrenium ions that may form DNA adducts (Arlt et al., 2005; Hatanaka et al., 2001). The nitroreduction of the nitro-PAH to the corresponding amine is a six electrons reduction reaction. In the presence of oxygen, a redox cycle can regenerate the nitro compound and produce superoxide anion ($O_2^{\bullet -}$) whose dismutation yields hydrogen peroxide. These reactions can be catalyzed by nitroreductases (NR) like nitrogen oxide synthase (NOS), DT-diaphorase, xanthine oxidase (XO), aldehyde oxidase and NADPH:quinone oxidoreductase (NQOI) (Ask et al., 2003; Ask et al., 2004; Rafil et al., 1991; Silvers et al., 1997). Both ring oxidation and nitroreduction may both lead to production of reactive metabolites that can covalently bind to macromolecules like DNA and cause DNA damage.

DNA damage is considered to be an important part of the mutagenic and carcinogenic effects of PAH. It is, however, no simple correlation between the DNA damage, mutations and cancer. DNA damage may be repaired, cause mutations or result in cell death like apoptosis if the DNA damage is to massive (Campisi 2005; Norbury et al., 2004). The induced cell death may, however, also be an important part in the tumor development, resulting in increased proliferation in surrounding cells with possible fixation of DNA damage, as well as cause a selection of cells that are more resistant to chemical induced cell death. Similarly, PAH induced cell death may be an important part of the cancer development. PAH induced cell death measured as necrosis as well as apoptosis have been studied in several experimental systems, including Hepa1c1c7 hepatoma cells (Lei et al., 1998; Solhaug et al., 2004b; Solhaug et al., 2004a), F258 rat liver epithelial cells (Huc et al., 2004), Daudi human B cells (Salas et al., 1998), human ectoviral cells (Rorke et al., 1998) and A20.1 murine B cells (Burchiel et al., 1993).

Apoptosis is a controlled form of cell death characterized by nuclear and cell fragmentation, cell shrinkage and no inflammation (Lockshin et al., 2004). In mammalian cells, apoptosis may be mediated by a family of cystein proteases known as caspases. There are two main pathways in which the activation of caspases are triggered – the extrinsic and the intrinsic apoptotic pathways. The extrinsic pathway is activated through engagement of death receptors on the cells surface. This leads to the recruitment and cleavage of pro-caspase 8, which in turn promotes the further cascade of pro-caspase activation. The intrinsic pathway starts within the cells, and is triggered by various intra- and extracellular stresses. Signals that are transduced in response to these stresses converge mainly on the mitochondria, leading to cytochrom c (cyt c) release and activation of caspase 9. Caspase 8 and 9 activates by oligomerization and they cleave the precursor forms of effector caspases, such as caspase 3. Activated caspase 3 in turn cleaves a specific set of cellular substrates resulting in the well known constellation of biochemical and morphological changes that are associated with the apoptotic phenotype (Okada et al., 2004). Members of the bcl-2 family regulate the activation of the mitochondrial pathway. Bax, bid and bad are pro-apoptotic bcl-2 proteins resulting in release of cyt c from the mitochondria, whereas bcl-2 and $bclx_L$ are anti-apoptotic bcl-2 proteins, inhibiting this process (Boatright et al., 2003; Bratton et al., 2001; Gross et al., 1999).

PAH like benzo[*a*]pyrene (B[*a*]P) are known to cause an accumulation of the tumor suppressor protein p53 (Bjelogrlic et al., 1994b), which have an important role in induction of temporary and permanent cell cycle arrest as well as apoptosis (Campisi 2005; Oren 2003). p53 can be activated as a result of cellular stress like hypoxia, oncogene activation, telomere erosion and DNA damage (Bernstein et al., 2002; Vousden 2000). Activated p53 may act as a transcription factor, thus inducing transcription of proteins involved in cell death. It may also directly bind to proteins like bax, leading its translocation to the mitochondria, or binding to bcl-2 and $bclx_L$ and inhibit their anti-apoptotic properties (Oren 2003; Slee et al., 2004).

B[*a*]P (Solhaug et al., 2005) and DEP extracts (DEPE) (Bonvallot et al., 2001; Hiura et al., 1999) has been found to activate several intracellular signalling pathways, including the mitogen activated protein kinase (MAPK) family. MAPK consisting of the classical mitogen kinase extracellular signal-regulated kinase (ERK) and the stress activated kinases p38 and c-jun N-terminal kinase (JNK). The precise role of these kinases, in the apoptotic process is still not fully understood.

In a previous study Solhaug and co-workers (Solhaug et al., 2004b) have found that B[*a*]P and several cyclopenta-PAH (CP-PAH) induce pro- as well as anti-apoptotic signals that may be important for their mutagenic properties. In the present study we will extend these findings by examine and characterizing the apoptotic effects of the nitro-PAH, 1-nitropyrene (1-NP), 1,3-dinitropyrene (1,3-DNP) and 1,8-dinitropyrene (1,8-DNP) in Hepa1c1c7 hepatoma cells. Furthermore, we also compare the responses obtained with that observed following exposure to diesel exhaust particle extracts (DEPE).

Materials and methods

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), 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 was purchased from Merck and Co, Inc (New Jersey, USA). Standard reference material 1975 containing diesel exhaust particle extract (DEPE) was purchased from 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 were obtained 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, foetal calf serum (FCS) 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, 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). CYP1B1 from alpha diagnostics (San Antonio, USA) and 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) and FITC-conjugated goat anti-rabbit IgG from Dako (Glostrup, Denmark) was applied.

Cell culture

The mouse hepatoma Hepa1c1c7 cell line was purchased from European Collection of Cell Culture (ECACC), the cell line expresses Ah-receptor and is able to induce cyp1-enzymes. Maintenance of the cells was done according to ECACC's guidelines and they were grown in alpha MEM medium with 2 mM L-glutamine, without ribonucleotides and deoxyribonucleotides. Heat-inactivated foetal calf serum (FCS, 10%) and 0.1 mg/mL of the fungicide gentamycin were added to the medium. The cells were incubated in 5% CO_2 at 37ºC, where they were kept in logarithmic growth 1-9x10^6 cells/75 cm^2 flasks and split twice a week. When splitting the cells, they were washed with phosphate-buffered saline (without Ca^{2+} and Mg^{2+}; PBS) and added 0.0025 % trypsin, which was removed shortly afterwards. The cells were then incubated for 1 minute in 37ºC. The trypsin was inactivated by adding culture medium with FCS, and $^1/_{10}$ - $^1/_{20}$ of the cells was transferred into new flasks for further growth. All the experiments were preformed before passage 40.

Exposure

The cells were seeded in dishes or trays at a concentration of 100 000 per cm^2 the day before exposure. Fresh medium was added before exposure. When inhibitors were used the cells were pre-incubated with the inhibitor for one hour before adding test substances. All the compounds used (1-NP, 1,3-DNP, 1,8-DNP, B[*a*]P and DEPE) were dissolved in DMSO. The amount of DMSO added to the cells was ≤ 0.5 %, except when the cells were exposed to 70 µg/mL DEPE the DMSO amount increased to 0.7 %. After exposure they were analysed by flow cytometry, microscopy, DNA fragmentation, immunocytochemistry, western blotting or cell survival assay.

Fluorescence microscopy

Plasma membrane damage, changes in nuclear morphology associated with apoptosis and necrosis was determined after trypsination and staining of the cells with Hoechst 33342 (5 µg/mL) and PI (10 µg/mL) in the dark for 30 min. Hoechst stains DNA and makes the nucleus blue fluorescent. PI is a red fluorescent dye that stains cells that have a disrupted membrane (necrotic cells). After staining the cell suspension, cells were centrifuged and resuspended in FCS and smears were prepared on microscopic slides. Cell morphology was

determined using a Nikon Eclipse E 400 microscope (magnification x 1000). At least 300 cells were counted per slide.

Flow cytometry

In flow cytometry, the cell suspension is passed through a highly focused beam of UV light. Flow cytometry measures both the deviation of light (light scatter) as a function of size and optical properties of the cells, as well as the absorption/emission of the fluorescence from cell components stained with fluorescent dyes (Otsuki et al., 2003).

After treatment cells were trypsinated and prepared for flow cytometry. The DNA of the cells were stained by incubating approximately $0.5x10^6$ cells with Hoechst 33258 (1.0 µg/mL) and Triton X-100 (0.1%) in the dark for 15 min. Hoechst 33258 stains DNA and Triton X-100 make the cells permeable. The blue fluorescence was measured using an Argus 100 Flow cytometer (Skatron, Lier, Norway). The 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). The percentages of cells in the 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.

Due to chromatin condensation and nuclear fragmentation during the process of apoptosis, the apoptotic cells and bodies arising from G_1 emit fluorescent signals that are lower than those of G_1 cells. The fluorescent signals, which are detected and recorded, can be much smaller than G_1 cells and thus represent only a fraction of the original amount of DNA in a whole nucleus. Therefore, if a particular treatment causes a great increase in the percentage of apoptotic cells/bodies, it must be remembered that only one G_1 nucleus can fragment into many smaller condensed chromatin units. As apoptosis progresses, the size of the fluorescent signal become progressively smaller and at some point will be gated out together with the background fluorescence.

DNA fragmentation assay

One of the characteristic features of apoptosis is internucelosomal DNA fragmentation following endonuclease digestion. Multiple DNA fragments 180-200 bp in length can be visualized as a DNA ladder on an agarose gel stained with a nucleic acid stain under UV light. The method, however, needs a high number of cells or high apoptotic rates for visualization (Otsuki et al., 2003).

DNA fragmentation assay was performed according to the method of Gorczyca and co-workers (Gorczyca et al., 1993), with minor modifications (Bjelogrlic et al., 1994a). The cells were concentrated by centrifugation and washed in PBS (without Ca^{2+} and Mg^{2+}). Approximately $2.5x10^6$ 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 37ºC for 30 min. Proteinase K (1 mg/mL final concentration) was added and the samples were incubated for an additional 30 min. The samples were then added 50 µL loading buffer (1mL containing: 0.01 ml 1 M Tris, pH 7.5; 0.04 mL 0.5 M EDTA, pH7.5; 0.5 mL glycerol (85%); 0.8 mg bromophenol blue; 0.45 mL H_2O) and incubated at 65ºC for 10 min just prior to application to the gel. Horizontal 1.5% agarose gels were run for at least 3 hrs at room temperature at 2 V/cm. The DNA bands (laddering) were visualized under UV light in gels run with SYBR®Green I nucleic acid gel stain.

Cell lysis and western blotting

In western blotting equal amounts of cellular proteins are separated by SDS-PAGE (sodium dodecyl sulphate polyachrylamide gel electrophoresis), and transferred electrophoretically from the gel to a nitrocellulose (used in our study) support membrane. Primary monoclonal or polyclonal antibodies are added to interact with a specific antigenic epitope, which is presented by the target protein attached to the support membrane. Furthermore, a secondary antibody, conjugated with HRP (horse raddish peroxidase) is added and binds to the primary antibody-antigen complex. Luminescent substrates are then used to visualize the bound components.

The cells were exposed as described earlier in 60 mm dishes. To extract the proteins, the 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 mg/mL leupeptin, 1 mM PMSF, 10 mg/mL aprotinin and 10 mg/mL pepstatin A. After cell lysis, the solutions were sonicated and centrifuged. The supernatant contained the proteins and were collected. Furthermore, protein concentration was measured using a Bio-Rad DC protein assay kit. The samples were adjusted to an equal protein concentration with lysis buffer and diluted with 5x SDS-PAGE sample buffer (0.312 M Tris-HCL, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.05% bromophenol blue) so that the sample buffer was diluted 5x. Finally a total of 10% glycerol was added, and the samples were boiled for 5 min.

A sample of 12.5 µg protein in each well was subjected to 12% or 15% SDS-PAGE. The proteins were electrotransferred to nitrocellulose membranes and stained with ponceau S. Blots that contained equal amount of protein loading were probed with a particular primary antibody in 1% fat-free dry milk or 5% bovine serum albumin, according to the recommendations of the manufacturer. The incubations with primary antibodies were overnight at 4ºC or 2 hrs at room temperature. The blots were then incubated with horseradish peroxidase-conjugated antibody for 1 hr at room temperature. The western blots were developed using the ECL chemiluminescence system according to the manufacturer's instructions (Amersham Pharmacia, Little Chalfont, UK). The results from one representative experiment are shown.

Immunocytochemistry

Immunocytochemistry is a technique as western, based on specific antibody-antigen binding for detection. Immunocytochemistry detects the antigen in the cells, so that it is possible to visualize were in the cells the protein in question is located.

The cells were exposed as described earlier, fixed in cold absolute ethanol for 10 min and permeabilized in phosphate-buffered saline (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 hr in anti-mouse IgG, the cells were incubated for 30 min with primary rabbit antibody diluted in MOM-diluent. The cells were then incubated with biotin-conjugated secondary antibody for 10 min followed by fluorescein-avidin DCS for 5

min. Between each step, the cells were washed in PBS. The cells were finally mounted in Vectashield mounting medium (Vector Laboratories).

Cellular survival assay

To examine how the proliferation of the exposed cells was affected, a cellular survival assay was assessed. The cells were exposed as described earlier. Following 24 hrs exposure, they were trypsinated, counted and seeded out in a lower density (800 cells per cm^2). After 72 hrs the cells were fixed with methanol for 2-3 min, stained with May-Grunwald for 15 min and Giemsa for 10 min. The May-Grunwald and Giemsa colour was diluted in Soerensen buffer (31 mM Na_2HPO_4 and 28 mM KH_2PO_4, pH 7.0), May-Grunwald in an equal volume and Giemsa in a $^1/_9$ relation. After the cells were stained they were washed in tap water and left for 3-4 min in Soerensen buffer. The cells were then dried and covered with a cover slip fastened with polyvinyl alcohol.

Statistical method

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

Results

Induction of apoptosis

Hepa1c1c7 hepatoma cells were treated with the different nitro-PAH and DEPE for 24 hrs and added as a positive control. As can be seen in Fig. 1 and 2, all the compounds except 1,8-DNP, induced apoptosis and necrosis as measured by fluorescence microscopy.

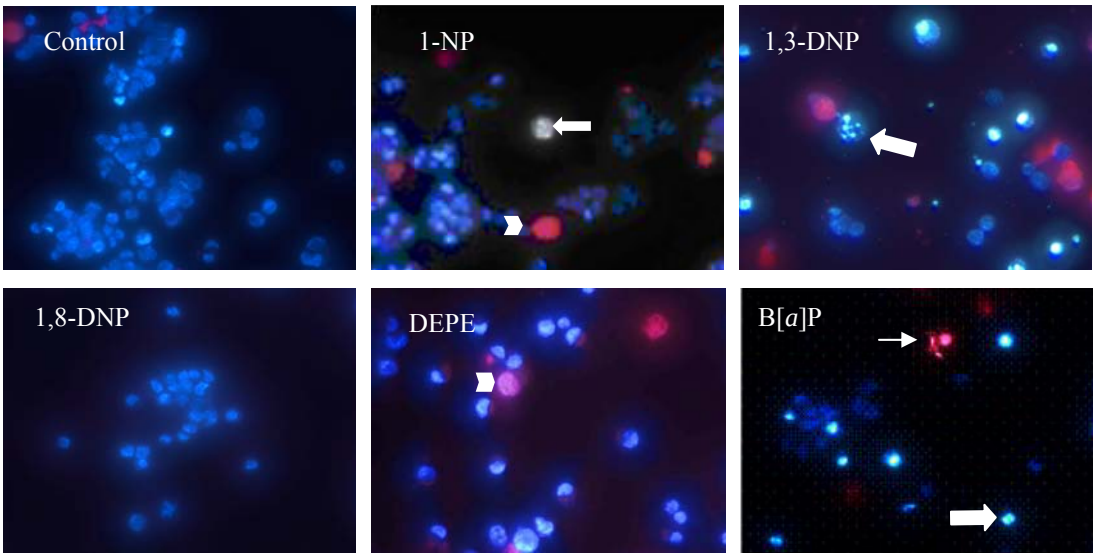

Fig. 1. Effects of nitro-PAH on cell morphology. Hepa1c1c7 cells were treated with or without B[a]P, 1-NP, 1,3-DNP, 1,8-DNP and DEPE for 24 hrs. The concentrations used were 30 µM for the nitro-PAH and B[*a*]P and 70 µg/mL for DEPE. Arrows indicate apoptotic cells, arrow-head indicate necrotic cells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

1,3-DNP was most potent giving a significant change at 3 µM. The highest increase in apoptosis was 31% seen at 30 µM 1,3-DNP also resulting in 14% necrotic cells. 1-NP induced both apoptosis and necrosis starting at 10 µM and giving maximal responses, 17% and 23 % respectively, at 60 µM. In contrast, 1,8-DNP did not induce cell death at any of the concentrations tested. Furthermore, neither 30 - 70 µg/mL DEPE caused any marked increase in apoptosis. However, an increased amount of necrotic cells were measured at concentrations of DEPE above 30 µg/mL and in cells exposed to 70 µg/mL 11% necrosis was observed.

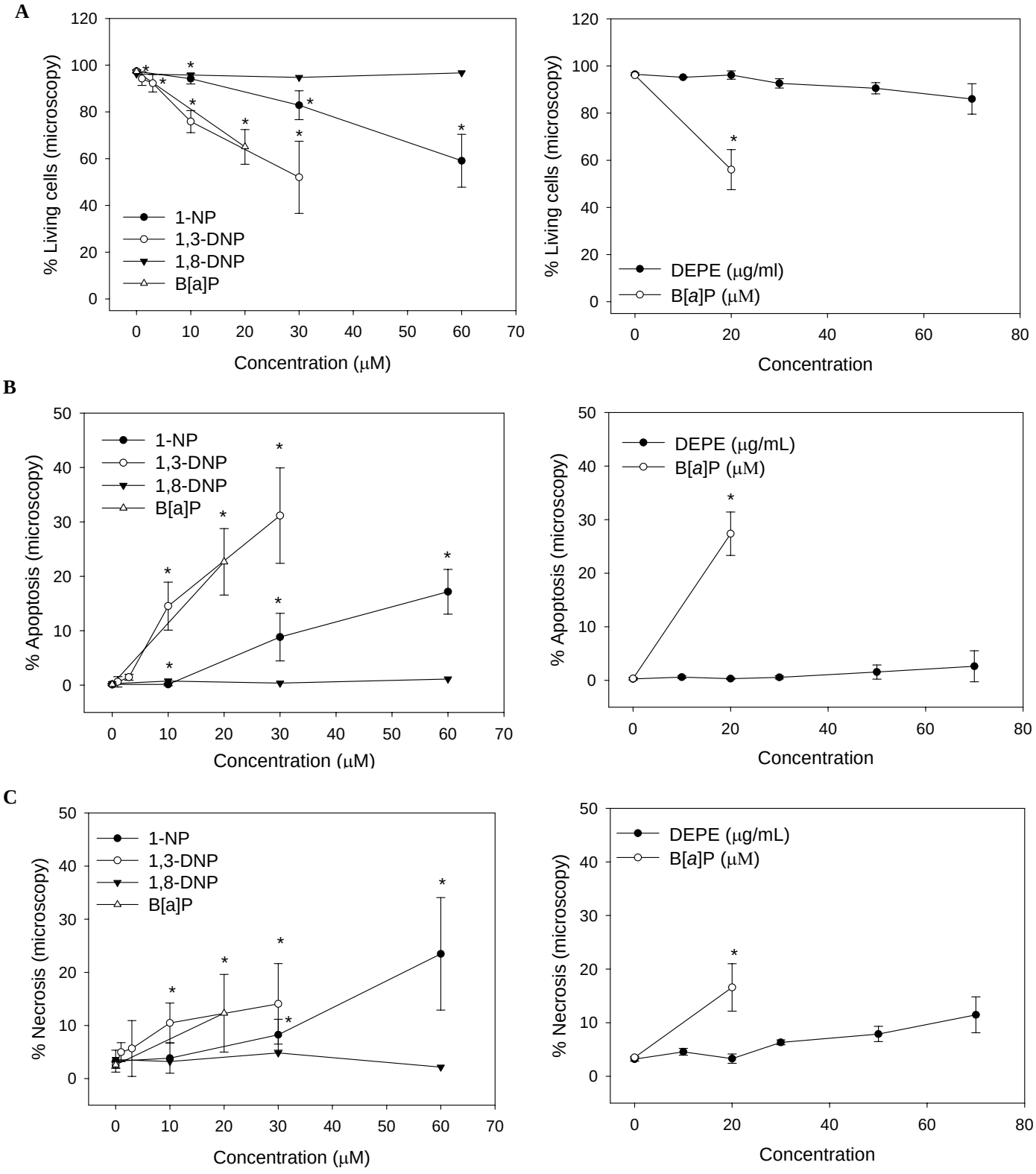

Fig. 2. Concentration dependent effects of nitro-PAH and DEPE on Hepa1c1c7 cells after 24 hrs exposure. The results are from fluorescence microscopy and presented as % living cells (**A),** apoptotic cells (**B)** and necrotic cells **(C)** . The data are means ± SD for ≥ 3 separate experiments. Data from 1,8-DNP are means from two separate experiments. The lacks of effects of 1,8-DNP have been observed in additional four experiments by microscopic examination.

Furthermore, flow cytometric analysis was used to measure apoptosis. The data in Fig. 3 show that 1,3-DNP was the most potent compound followed by 1-NP. No increase in apoptosis was seen after 1,8-DNP, whereas exposure to DEPE resulted in a minor increase. The results from these studies correlated well with that from microscopic analyses. Flow cytometry was considered to be a faster and more convenient technique and thus used in the following experiments. Results obtained were only confirmed by microscopic examination.

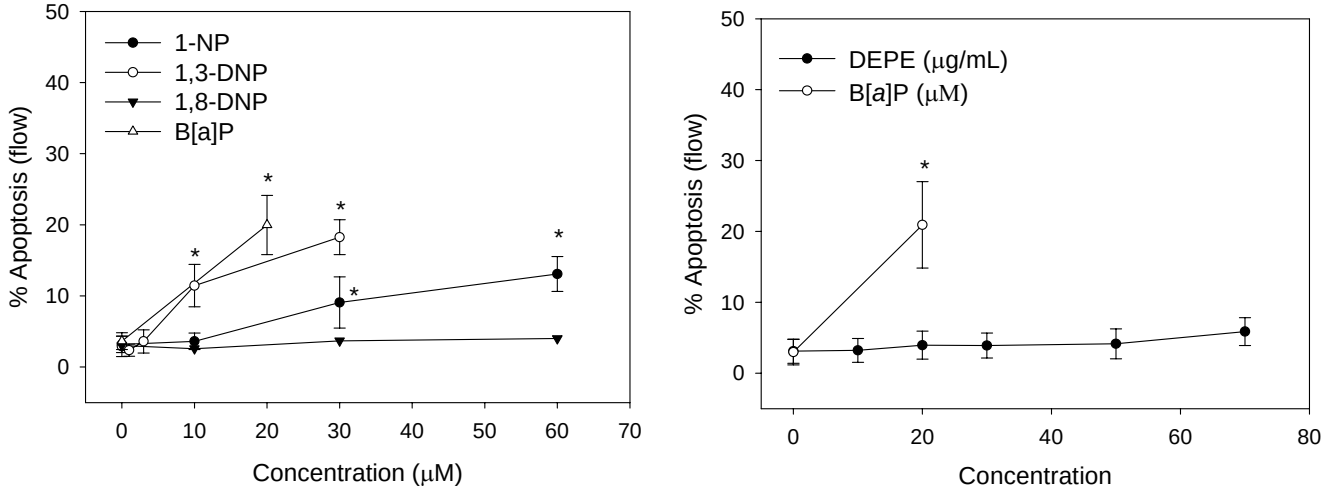

Fig. 3. Concentration dependent apoptotic effect of nitro-PAH and DEPE on Hepa1c1c7 cells. Flow cytometric analysis following staining with Hoechst 33258 was measured after 24 hrs exposure of various concentrations of the test compounds. The data are means ± SD for separate experiments (n ≥ 3). Data from 1,8-DNP are means from two separate experiments.

Flow cytometry analysis was also used to examine any possible change in cell cycle after exposure to test substances. Following 24 hrs exposure, 1-NP, 1,3-DNP and 1,8-DNP were found to increase the amount of cells in S-phase (Fig. 4), whereas no changes were observed with DEPE (data not shown). 1,8-DNP gave a concentration depended S-phase accumulation starting at 3 μM (data not shown), whereas the highest increase were seen at 10 μM (Fig. 4) and higher (data not shown) giving approximately 50 % cells in S-phase. With B[a]P, a marked increase in S-phase could be observed at 3 μM (Solhaug et al., 2005).

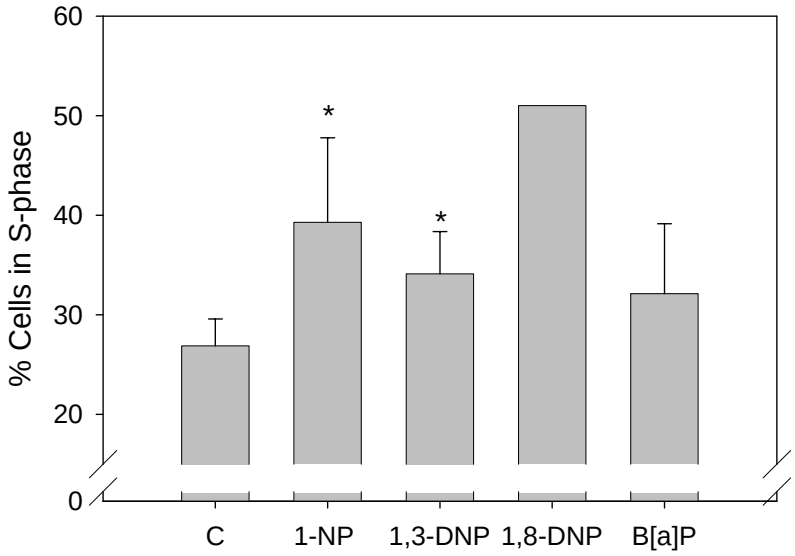

Fig. 4. Accumulation of cells in S-phase when exposed to nitro-PAH, measured by flow cytometry. The cells were analysed after 24 hrs exposed to 60 μM 1-NP, 30 μM 1,3-DNP, 10 μM 1,8-DNP and 20 μM B[a]P. The concentrations shown for 1-NP and 1,3-DNP were the lowest and only concentration to give a significant 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. The data are means ± SD for separate experiments (n ≥ 3). Data from 1,8-DNP are means from two separate experiments

Furthermore, the time course using flow cytometry analysis tested at a concentration of 30 μM revealed that apoptosis was significantly induced after 16 hrs exposure for 1-NP and 1,3-DNP, whereas at 24 hrs a more marked increase were observed. 1,8-DNP gave a slight increase in apoptosis after 40 hrs. At a concentration of 70 μg/mL, DEPE induced 6 % apoptosis after 24 hrs, increasing to 18 % following 48 hrs incubation (Fig. 5).

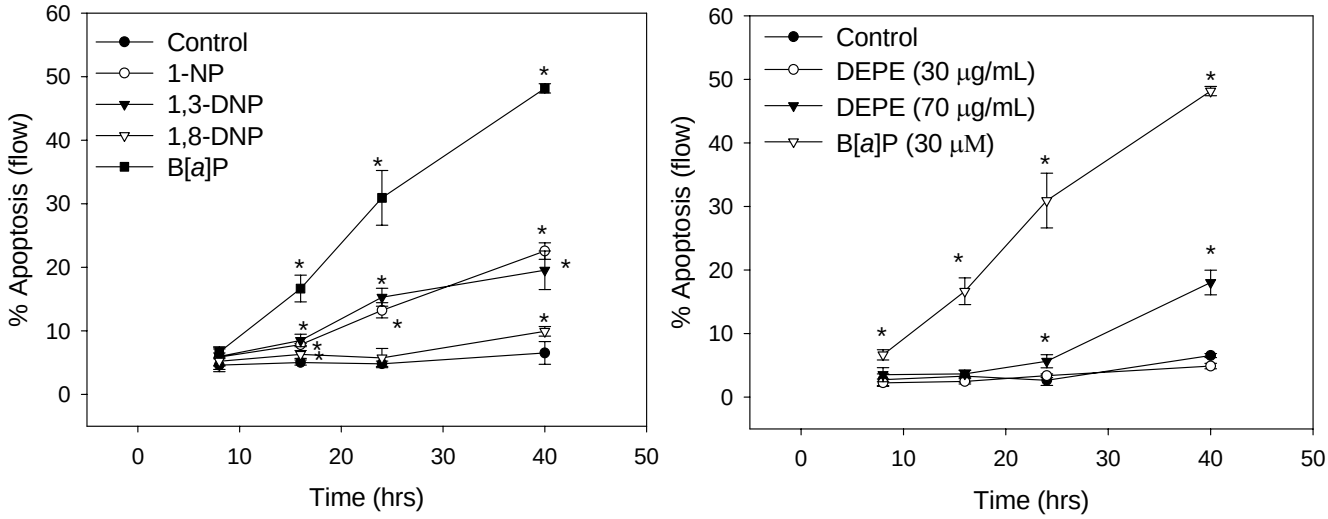

Fig. 5. Time dependent apoptotic effects of nitro-PAH (30 μM) in Hepa1c1c7 cells. Cells were analysed by flow cytometry after 8, 16, 24, and 40 hrs exposures of the test compounds. The data are means ± SD of three parallels in one representative experiment.

Cleaving of caspases and DNA-fragmentation.

Caspase 3 is an effector caspase that is activated by the initiator caspases, caspase 8 and caspase 9 (Bratton et al., 2001). Western analysis of cell lysates following 24 hrs exposure showed increased amount of cleaved caspase 3 after exposure to all the nitro-PAH except for 1,8-DNP. 1,3-DNP was most potent starting at 10 μM and an increase would be seen after 16 hrs at a concentration of 30 μM. Whereas 1-NP resulted in an increase at the highest concentration tested (30 μM) at 24 hrs. DEPE also caused a minor activation at the two highest concentrations (50 and 70 μg/ml; Fig. 6A and B). Further more the level of pro-caspase 8 was reduced when exposed to the highest concentration tested (30 μM) for 1-NP and 1,3-DNP. No change was observed for 1,8-DNP (Fig.6C).

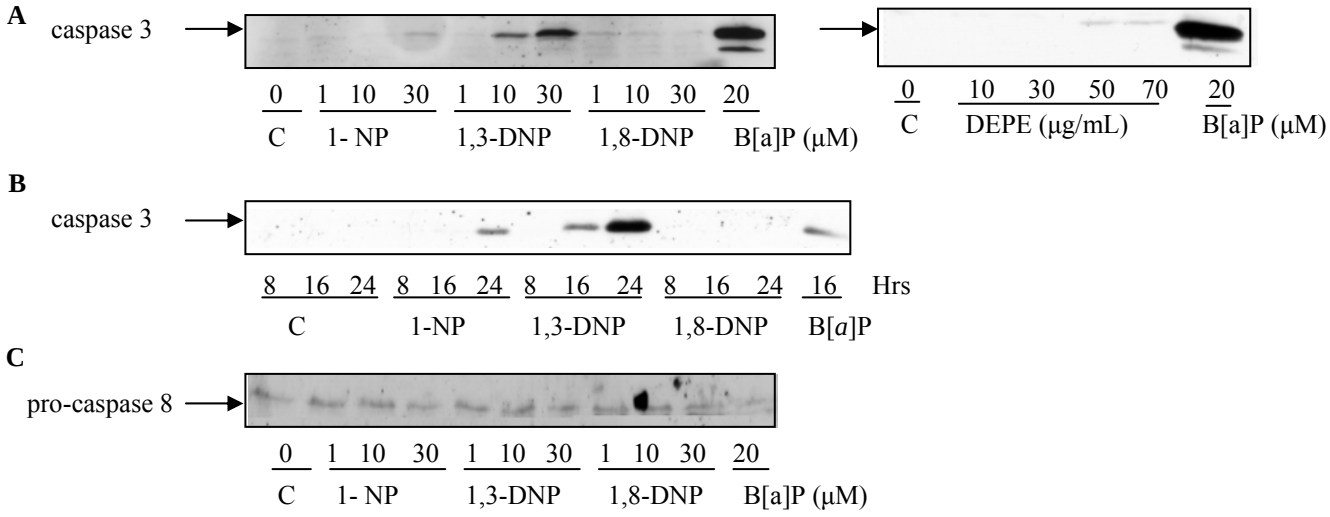

Fig. 6. Effects of nitroPAH and DEPE on the active form of caspase 3 and nitro-PAH on procaspase 8. **A**: cleaved caspase 3 analysed by western after 24 hrs exposure of various concentrations of the test compounds. **B**: Time depended effects on nitro-PAH, analysed by western after 8, 16 and 24 hrs exposure. The concentration used were 30 μM for all the compounds tested. **C**: Pro-caspase 8 analysed by western after 24 hrs exposure of various concentrations of the test compounds.

The cleaved caspases activates DNA endonucleases resulting in fragmentation of DNA. The gel presented in Fig. 7 show that 1,3-DNP induce fragmentation of DNA following exposure to 10 and 30 μM, while no marked fragmentation was observed for the other nitro-PAH and DEPE.

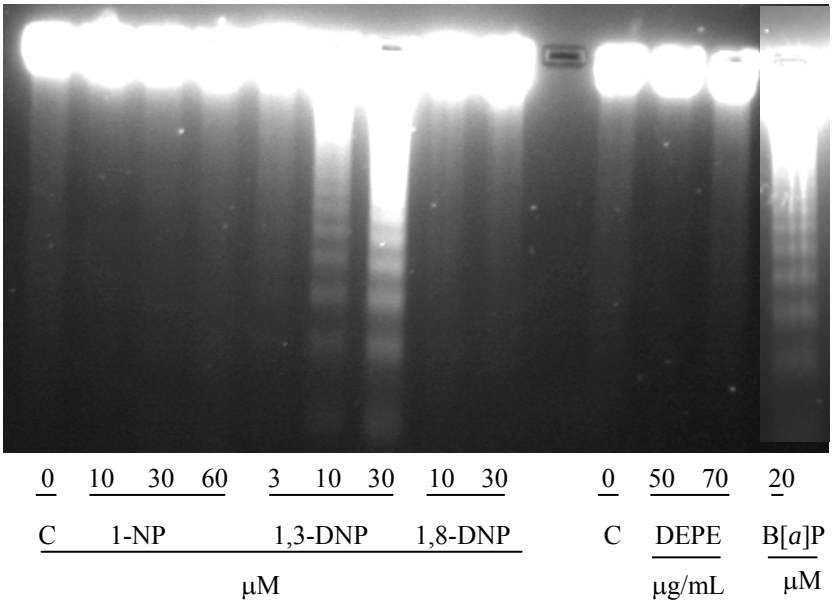

Fig. 7. The characteristic apoptotic fragmentation pattern showed by DNA fragmentation assay. Hepa1c1c7 cells were exposed of various concentrations of the test compounds for 24 hrs before analysed.

CYP1 induction and metabolism

CYP1-enzymes are central in the activation of PAH to reactive molecules that may bind covalently to macromolecules like DNA. To examine the relative potency of the nitro-PAH compounds and DEPE to increase the amount of the different cyp1-enzymes, cyp1a1, cyp1a2 and cyp1b1 respectively, we exposed the cells for 8 or 24 hrs and analysed them by western blotting. The results showed that all the compounds increased the level of cyp1a1 compared to the control following 8 hrs exposures (Fig. 8A). 1,3-DNP, 1,8-DNP and DEPE enhanced cyp1a1 level at the lowest concentration tested (1 μM for 1.3-DNP/1.8-DNP and 1 μg/mL for DEPE) whereas 1-NP induced cyp1a1 at a higher concentration (10 μM). Furthermore, all the compounds also induced cyp1b1 after 24 hrs exposures, 1,8-DNP yielding the strongest response. 1-NP, 1,3-DNP and 1,8-DNP all induced cyp1b1 at 1 μM. DEPE increased the level of cyp1b1 at 1, 3 and 10 μg/mL, but resulted in a decreased response when exposed to higher concentrations (30 μg/mL and above; Fig. 8B). No induction of cyp1a2 following exposure to nitro-PAH nor DEPE were observed (data not shown). To further analyse the possible involvement of cyp1a1 in the bioactivation of the compounds, we used the CYP-inhibitor α-NF and assessed the induced toxicity. The compounds tested were 1,3-DNP and 1-NP, since the most marked effects were seen after exposures to these compounds. Results show that the

toxicity of 1,3-DNP was almost completely blocked by α-NF at 10 and 30 μM. Interestingly, α-NF rather increased the level of apoptosis in the cells exposed to 1-NP (Fig. 8C).

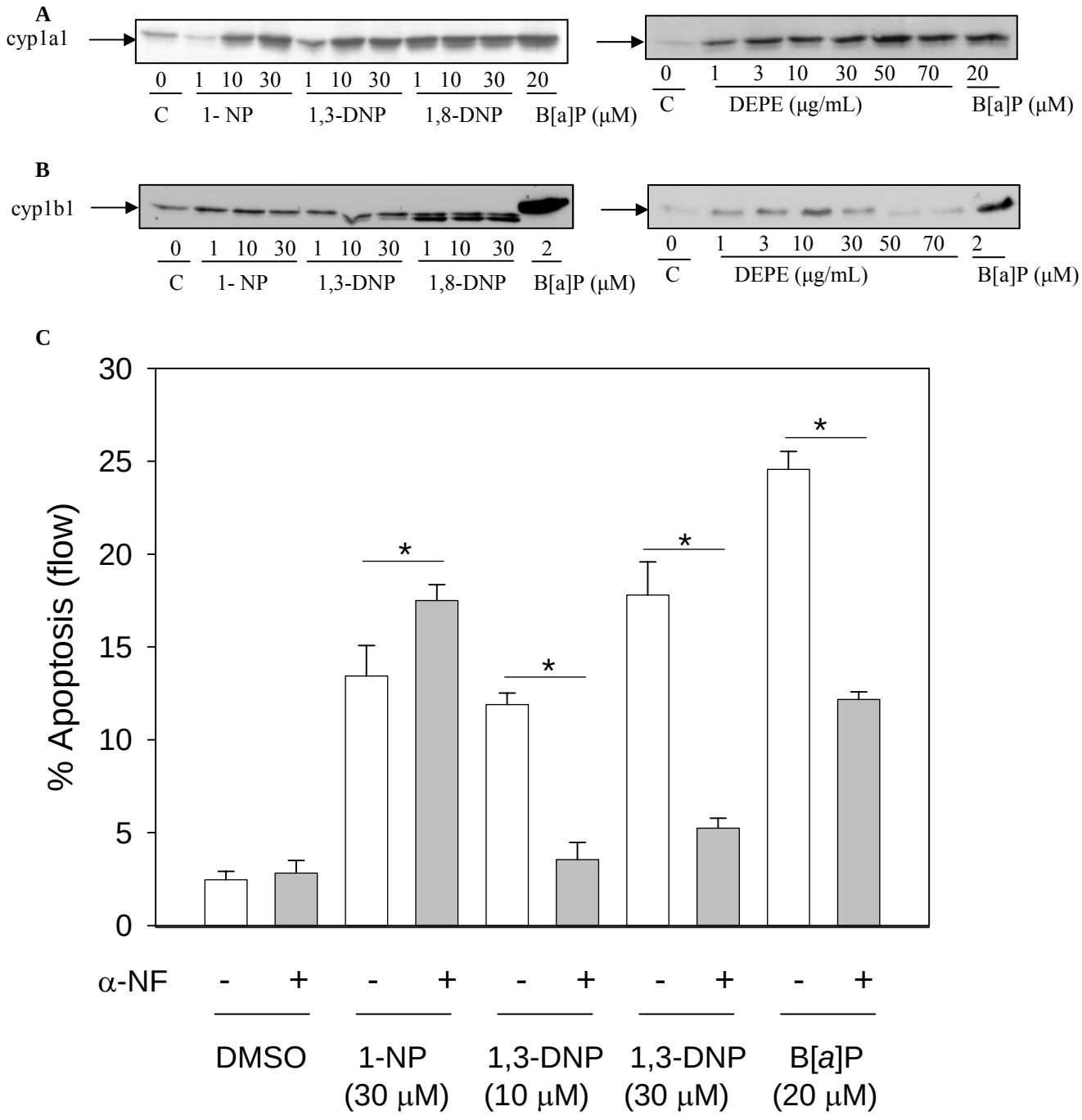

Fig. 8. **A**: Effects of nitro-PAH and DEPE on cyp1a1. Analysed by western after 8 hrs exposure of various concentrations of the test compounds. **B**: Effects of nitro-PAH and DEPE on cyp1b1. Analysed by western after 24 hrs exposure of various concentrations of the test compounds. **C**: Effects of α-NF on 1-NP and 1,3-DNP induced apoptosis. Flow cytometry showing 1-NP and 1,3-DNP with and without α-NF. Hepa1c1c7 cells were exposed for 24 hrs. The data are means ± SD of one representative experiment of two are shown.

p53 phosphorylation

p53 is a transcription factor that is important in the regulation of cell cycle and apoptosis (Oren 2003). Western analysis of cell lysates after 24 hrs exposure showed that all the compounds gave an increased amount of phosphorylated p53 (p-p53) at serin 15. 1,3-DNP and 1,8-DNP phosphorylated p53 at 1 µM (which was the lowest concentration tested) with 1,8-DNP yielding the largest increase. 1-NP resulted in an increased level of p-p53 at the highest concentration tested (30 µM) whereas DEPE caused a slight increase in p53 phosphorylation at 50 µg/mL (Fig. 9A). Immunocytochemistry showed that p53 translocated to the nucleus following exposure to all the compounds except 1,8-DNP (Fig. 9B). However, cells exposed to 1,8-DNP had an increased fluorescence that correlated well with the increased phosphorylation of p53 observed with western analysis. The concentration used were 30 µM for the nitro-PAH and 50 µg/mL for DEPE.

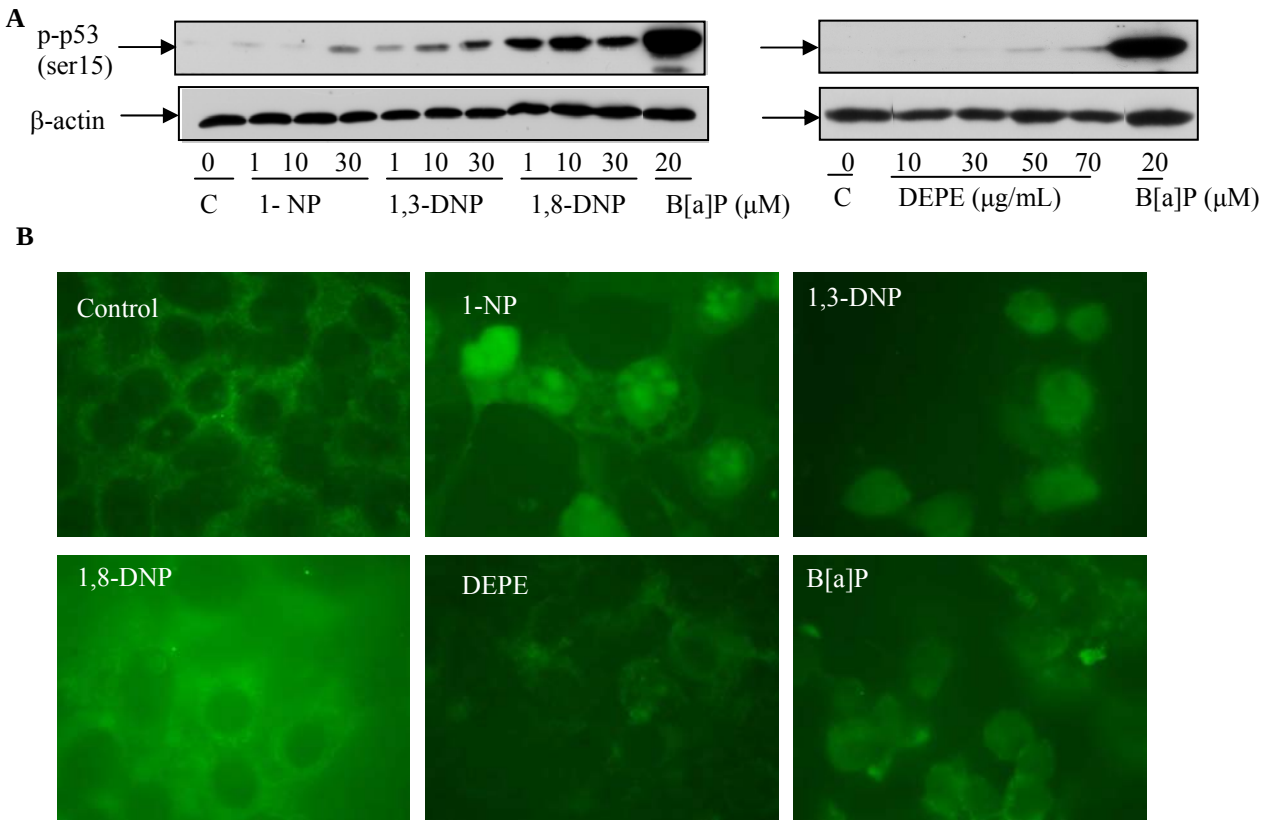

Fig. 9. Effects of nitro-PAH and DEPE on p53 expression. **A**: Analysed by western after 24 hrs exposure of various concentrations of the test compounds. **B**: Analysed by immunocytochemistry after 24 hrs exposure. The concentrations used were 30 µM for the nitro-PAH. 50 µg/mL of DEPE. 20 µM for B[*a*]P

Akt phosphorylation

Akt is a protein kinase known to promote growth factor-mediated cell survival and block apoptosis (Song et al., 2005). Results were analysed by western blotting following 24 hrs exposures. 1-NP gave an increased phosphorylation (activation) of Akt at 30 μM 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 also seen at 10 μM when the cells were exposed to 1,8-DNP. DEPE at the concentrations 3 - 50 μg/mL gave a higher amount of phosphorylated Akt (Fig. 10).

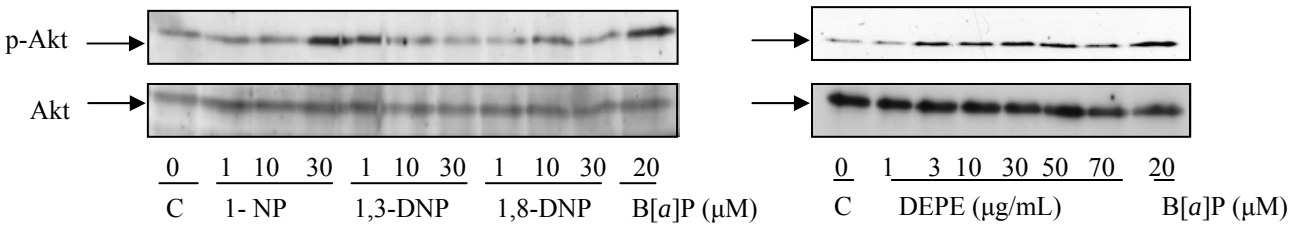

Fig. 10. Effects of nitro-PAH and DEPE on phosphrylated Akt and total Akt. Analysed by western after 24 hrs exposure of various concentrations of the test compounds.

Bcl-proteins

Bcl-proteins can either be pro-apoptotic or anti-apoptotic (Gross et al., 1999). Anti-apoptotic $bclx_L$ was markedly decreased following exposure to 10 μM for 1-NP and 1 μM for 1,3-DNP. DEPE gave a slight decrease in $bclx_L$ level at the highest concentration tested (70 μg/ml), whereas no changes for 1,8-DNP were observed (Fig. 11). Pro-apoptotic bid showed a reduced level following 30 μM 1,3-DNP. Interestingly, 1-NP, 1,8-DNP and DEPE seemed to rather increase the 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. 11). Furthermore, the anti-apoptotic signal of phosphorylated bad was also increased following exposure to 10 and 30 μM 1-NP and 10 μM 1,3-DNP. However, at 30 μM, 1,3-DNP decreased phosphorylation of bad (Fig. 11). There was no change in the level of pro-apoptotic bax and anti-apoptotic bcl-2 from nitro-PAH after 24 hrs exposure. DEPE gave a decrease in bcl-2 at 70 μg/mL, but no change in bax was observed (Fig. 11).

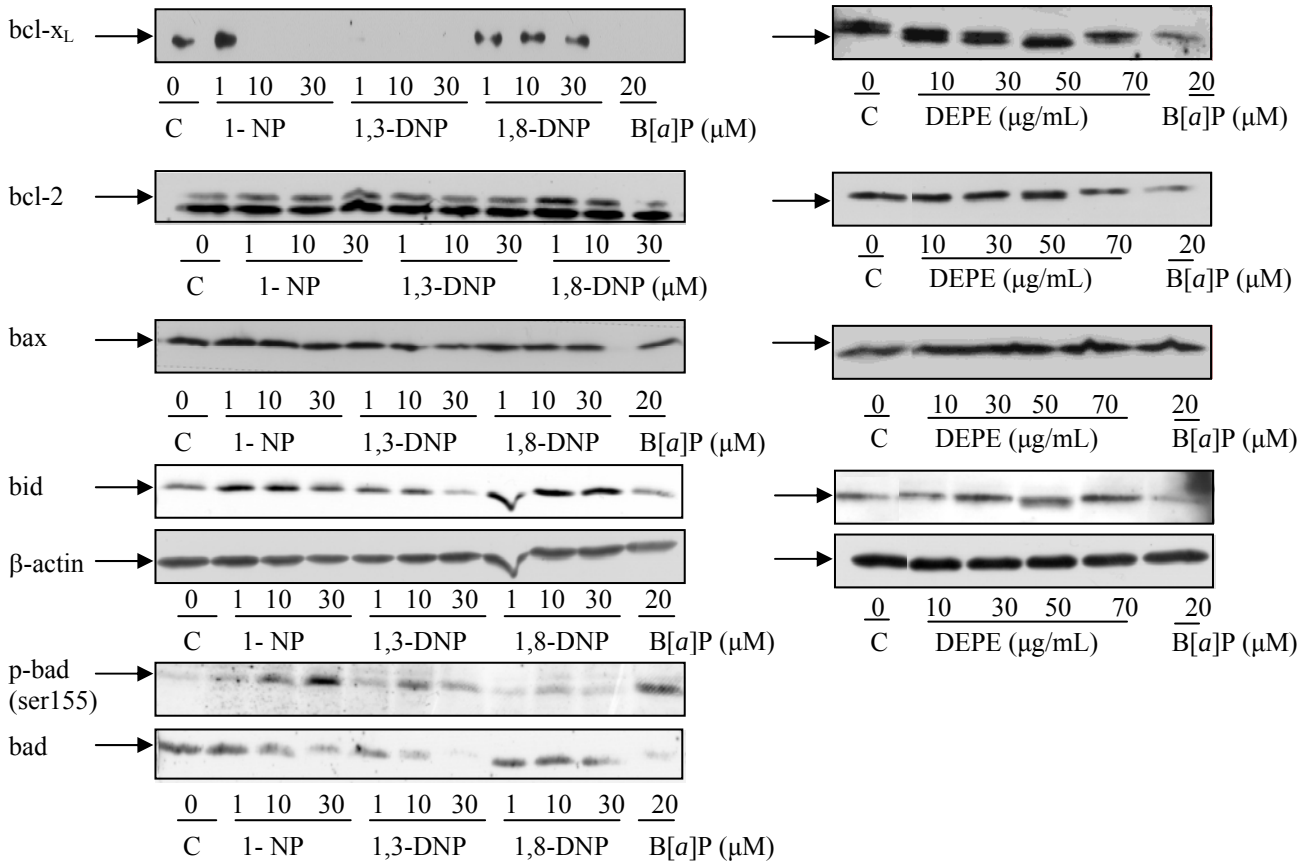

Fig. 11. Effects of nitro-PAH and DEPE on bcl-proteins. Analysed by western after 24 hrs exposure of various concentrations of the test compounds. Anti-apoptotic bcl-x_L and bcl-2, pro-apoptotic bax and bid, phosphorylated bad at ser155 and total bad.

MAPK phosphorylation

MAPK are often found to be involved in the apoptotic signalling pathway. Exposure to 1-NP gave a higher level of phosphorylated ERK1/2 following 24 hrs treatments (all concentrations). 1,3-DNP and 1,8-DNP did not seem to cause any marked change in the level of p-ERK1/2 while DEPE decreased the phosphorylation (Fig. 12). 1-NP, 1,3-DNP and DEPE resulted in phosphorylation of p38 following 8 hrs exposure. With 1-NP and 1,3-DNP, an increased concentration depended phosphorylation of p38 were seen starting at 10 μM, whereas DEPE at a concentration of 3 μg/mL. 1,8-DNP did not have a marked increase at any of the concentrations tested (Fig. 12). 1-NP, 1,3-DNP and DEPE enhanced the level of phosphorylated JNK after 24 hrs exposure. 1-NP resulted in a concentration depended increase starting at 1 μM, whereas 1,3-DNP and DEPE activated p-JNK at 10 μM and 10 μg/mL and higher, respectively. No activation of JNK was observed for 1,8-DNP (Fig. 12).

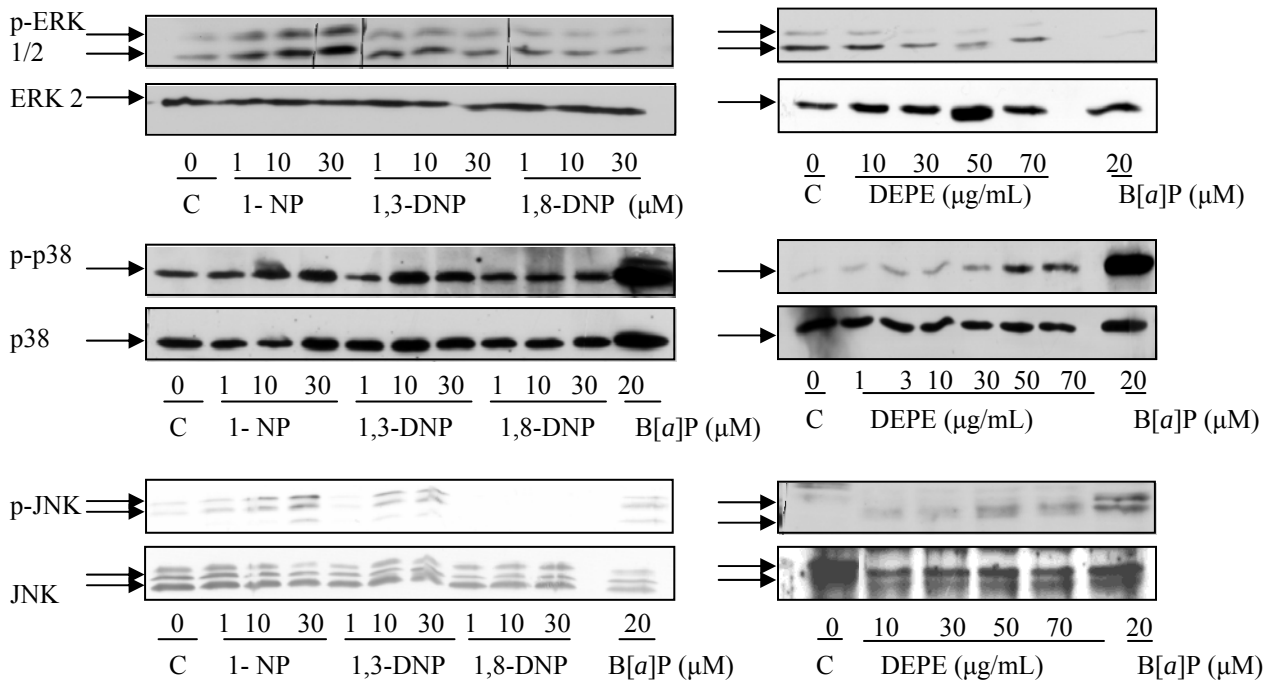

Fig. 12. Effects of nitro-PAH and DEPE on MAPK. Analysed by western. p-ERK1/2 and ERK2 after 24 hrs exposure. p-p38 and p38 after 8 hrs exposure. p-JNK and JNK after 24 hrs exposure.

Cell survival

Microscopic analysis revealed that exposure to 1-NP resulted in changes in cell morphology not seen with the other compounds. The cells incubated with 1-NP showed an increase of both in number and size of cytoplasmic vesicles/granules. This change was seen very distinct after staining for p53 using fluorescence microscopy (Fig. 9B). In order to further characterize the toxic effects of 1-NP, we examined its effects on cell proliferation. We first analysed amount of cells in the different phases of the cell cycle after exposure to 1-NP for 24 hrs. The results indicated an increased level of cells in S-phase (Fig. 4). To further examine if the accumulation of cells in S-phase implicated increased proliferation or delayed S-phase, we looked at the cell survival 72 hrs after a period of 24 hrs exposures. As can be seen in Fig. 13, microscopic analysis of the cells showed similar distinct cytoplasmic vesicles/granules as seen using immunocytochemistry (Fig. 9B). Some changes in cell morphology were seen after exposure to 10 μM 1-NP, but most of the granules were seen after treatment with 30 μM.

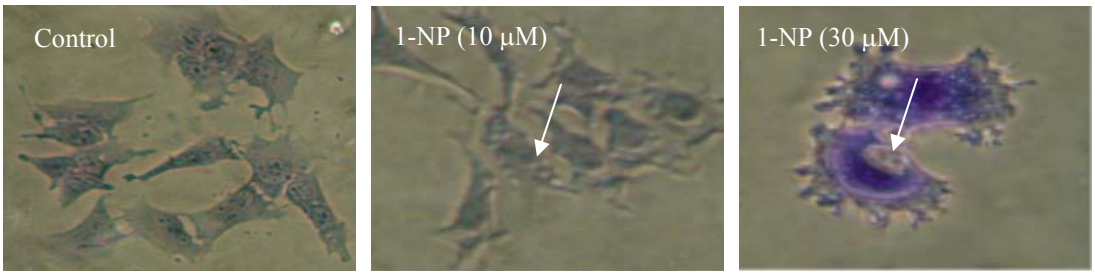

Fig. 13. Effects of 1-NP on cell morphology. The cells were treated with 10 and 30 μM 1-NP for 24 hrs, then seeded out in a lower density and further incubated for 72 hrs where after they were stained with Gimsa and May-Grunewald. Arrows indicate vacuole-like structures in the exposed cells

As can be seen in Fig. 14, 1-NP both reduced the number of colonies and the number of cells in the colonies. Exposure to 10 μM 1-NP reduced the total number of viable cells to 46 % of the control whereas 30 μM 1-NP reduced the number of viable cells to 27 %. Furthermore, 10 μM 1-NP reduced the number of cell colonies to 69 % whereas 30 μM 1-NP reduced the number to 63 % (Fig. 14).

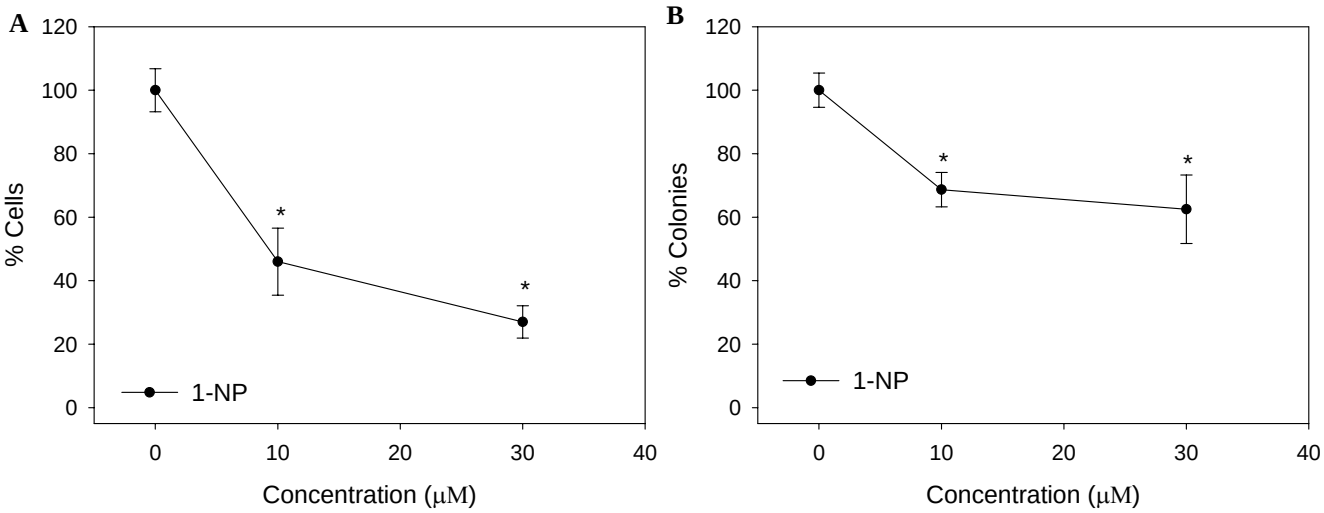

Fig. 14. Effects of 1-NP on number of cells and colonies. The cells were treated with 10 and 30 μM 1-NP for 24 hrs, then seeded out in a lower density and further incubated for 72 hrs. After incubation they were analysed by microscopic analysis **A** show % viable cells and **B** show % cell colonies. The data are means ± SD from three parallels in one representative experiment of two.

Discussion

Recent studies show that PAH like CP-PAH and B[*a*]P induce apoptosis in Hepa1c1c7 hepatoma cells (Solhaug et al., 2004a). In the present study, we find that DEPE and the nitro-PAH, 1-NP, 1,3-DNP and 1,8-DNP induced apoptosis to a various extent in the Hepa1c1c7 cells (Fig. 2 and 3). Interestingly, the compounds also seemed to affect the intracellular signalling pathways as well as cell morphology somewhat differently. 1-NP and 1,3-DNP induced both apoptosis as well as necrosis judged by fluorescence microscopy analysis and flow cytometry, whereas 1,8-DNP did not seem to induce apoptosis nor necrosis in any of the concentrations tested until a later time point (Fig. 2, 3 and 5). With regard to DEPE, no significant increases in cell death were detected by microscopic analysis as well as flow cytometry until 48 hrs after exposure (Fig. 2, 3 and 5). The necrosis that were observed in parallel with apoptosis after exposure of cells to 1-NP and 1,3-DNP (Fig. 2B and C) are often seen following exposure to toxic chemicals. This might be due to an inhibition of the apoptotic process due to low energy levels in the cells or oxidation of SH-groups on the caspases resulting in an inactivation (Nicotera et al., 1999; Solhaug et al., 2004b). The various compounds induced cell death in the following order, 1,3-DNP>1-NP>>DEPE>>1,8-DNP. Earlier studies indicate that the DNP isomers in general are more mutagenic than 1-NP, and that the potency of different DNP varies much (Rosenkranz et al., 1980). 1-NP, 1,3-DNP and 1,8-DNP have been reported to induce tumors in rats (Imaida et al., 1995; Ohgaki et al., 1984). 1,8-DNP are generally considered as a more potent genotoxic and carcinogenic compound than 1,3-DNP (Imaida et al., 1991; Takayama et al., 1983). Thus, it is interesting to note that we find that 1,8-DNP did not induce much cell death in the Hepa1c1c7 cells, when compared to other compounds.

The family of caspases is important for activation of the apoptotic pathway. Caspase 3 is an effector caspase that is cleaved by initiator caspase 8 and 9 and the activation cleaves a number of structural and regulatory proteins e.g. ICAD (inhibitor of caspase activated deoxynuclease), PARP, bcl-2, $bclx_L$, lamins and cytokeratins. Cleavage of these proteins finally results in the characteristic morphology of apoptotic cells, including DNA and cell fragmentation (Earnshaw et al., 1999). In this study we show that exposure of Hepa1c1c7 cells to 1-NP, 1,3-DNP and DEPE resulted in cleaved caspase 3 (Fig. 6A and B), whereas

only 1,3-DNP increased fragmentation of the DNA as judged by DNA gel electrophoresis (Fig. 7). Thus, DNA laddering seems to be a less sensitive method to measure apoptosis than microscopic analysis, flow cytometry and western analysis of caspase 3.

CYP1A1 and 1B1 is considered to be the most efficient enzymes to metabolise B[*a*]P to its reactive metabolite (Gelboin 1980; Shimada et al., 2001). Solhaug and co-workers (Solhaug et al., 2004b) showed that in addition to B[*a*]P, several CP-PAH induced cyp1a1 in Hepa1c1c7 cells, and that the induction correlated well with the toxicity of the compounds. Furthermore, the CYP-inhibitor α-NF reduced the toxicity of B[*a*]P and CP-PAH in the Hepa1c1c7 cells. Reactive compounds that cause DNA damage are known to activate p53 and result in temporary cell cycle arrest, senescence and/or apoptosis. Binding of PAH to the AhR have been reported to affect cell cycle progression and cause apoptosis by other means than metabolising the PAH to reactive compounds. The receptor can, when activated by compounds like PAH, directly interact with proteins and cause cell cycle arrest (Nebert et al., 2000; Puga et al., 2002). Recent findings have also indicated a role for the AhR in the apoptotic process. Bax, a pro-apoptotic bcl-2 protein has been suggested to be induced by the AhR binding to 7,12-dimethylbenz[*a*]anthracene (DMBA), thereby resulting in apoptosis (Matikainen et al., 2001). In the present study, we find that all the nitro-PAH and DEPE induced cyp1a1 and 1b1 (Fig. 8A and B) suggesting a role for AhR in the apoptotic process. However, their ability to induce cyp1a1 and cyp1b1 did not directly reflect their toxicity. Cyp1a1 and cyp1b1 was induced by 1,3-DNP (Fig. 8A) and α-NF almost completely reduced its toxicity (Fig. 8C). This suggests that the toxicity that results from 1,3-DNP are due to its metabolism through cyp1-enzymes, and that its induction of these enzymes are an important part of the process. In accordance with this, both CYP-enzymes (CYP1A2 and 1B1) and NR has been proposed to metabolise nitro-PAH to *N*-Hydroxy-metabolites (Hatanaka et al., 2001), which may be further metabolized by phase II enzymes to the reactive nitrenium ion (Arlt et al., 2005; Glatt 2000; Hein et al., 1997). In contrast to 1,3-DNP, 1-NP caused an increase in cell death when α-NF was added (Fig. 8C). Inactivation of 1-NP by CYP1A1 has been reported in *S. typhimurium* strains with recombinant human P450 family 1 enzymes (Yamazaki et al., 2000). The fact that α-NF rather increased the toxicity of 1-NP, however, does not necessarily exclude cyp1a1 and 1b1 as a metabolic pathway since the enzymes are involved in both activation as well as detoxification pathways.

Mutations in the tumor suppressor *p53* gene is known to be one of the most frequently observed alterations in cancers (Hecht 1999). Activation of p53 modulates cellular functions such as gene transcription, DNA synthesis, DNA repair, cell cycle arrest, senescence and apoptosis (Slee et al., 2004). Studies indicate that the p53 protein may act not only as a transcription factor, but also more directly induce apoptosis through the mitochondria pathway (Marchenko et al., 2000; Moll et al., 2001; Schuler et al., 2000). In the present study all the nitro-PAH and DEPE induced phosphorylated p53 at serin 15 as judged by western blotting (Fig. 9A). Interestingly, the most potent of the compounds was 1,8-DNP, even though it did not seem to induce apoptosis to a large extent. The results from immunocytochemistry correlated well with and extended the understanding of the western findings (Fig. 9B). Whereas, 1-NP, 1,3-DNP and DEPE resulted in a translocation of p53 to the nucleus, no such translocation was observed with 1,8-DNP. This indicates that 1,8-DNP causes some conformational changes in the phosphorylated p53 protein thereby reducing its ability to cause apoptosis. It is interesting to note that in human breast cancer MCF-7 cells, Cd(II) resulted in suppression of the p53 mediated cell cycle arrest due to Cd(II) induced changes in p53 (Hartwig et al., 2002).

The present study showed that 1,8-DNP was by far the most potent compound with regard to cause an accumulation of cells in S-phase (Fig. 4) which together with the increase in phosphorylated p53 suggests that 1,8-DNP was more potent than 1,3-DNP in causing DNA damage. This is in accordance with the findings that 1,8-DNP is a more potent mutagen and carcinogen than 1,3-DNP (Imaida et al., 1991; Takayama et al., 1983). The finding that 1,8-DNP induced DNA damage, but inhibit the p53-dependent induced apoptosis could be of great importance when explaining the mutagenic and carcinogenic properties of 1,8-DNP. Furthermore, this might be related to other studies which indicate that some PAH may induce p53 without G_1-arrest, resulting in accumulation of cells in S-phase and cells with an increased probability of having mutations (Khan et al., 1998). Dipple and co-workers (Dipple et al., 1999) referred to this as stealth properties and suggest that it may contribute to the carcinogenic properties of these PAH.

Another interesting finding was that the cells exposed to 1-NP had different cell morphology than cells exposed to the other nitro-PAH and DEPE. The morphology of the cells exhibited

an increased granulation or formation of cytoplasmic vesicles (Fig. 13), which may indicate that the cells also partly died through other mechanisms than apoptosis and necrosis, such as permanent cell cycle arrest (senescence) or autophagic cell death. Senescence is activated by telomere shortening, but may also be a result of tumorigenic stresses such as DNA damage. The morphological characterization of senescent cells shows flattened cytoplasm and increased granularity (Okada et al., 2004). Autophagy is described as a controlled form of cell death that is different from apoptosis, and it is characterized morphological by an increased number of double- or multiple-membrane cytoplasmic vesicles (Gozuacik et al., 2004). In the present study, cellular survival assay showed that 1-NP decreased the proliferation rate as well as reduced the number of cell colonies (Fig. 14). This could suggest together with increased granulation/vesicles that senescence could be another type of cell death involved. To further elucidate cell death induced by 1-NP it could be interesting to assess the inhibitor of autophagy, 3-methyl adenine (3MA) and to closer study the vesicles by electron microscopy.

The bcl-2 family members are central in the apoptotic process, and are mainly converged to the mitochondria. In the present study we look into the pro-apoptotic members bax, bid and bad, and the anti-apoptotic members bcl-2 and $bclx_L$. The pro-apoptotic members are often localized in the cytosol or cytoskeleton. Death signals cause conformational changes of the proteins, which enable them to target and integrate into membranes such as the mitochondria, resulting in release of cyt c to the cytosol and triggering of apoptosis. In the presence of survival signals pro-apoptotic bad is phosphorylated, resulting in its sequestration, whereas in the absence of survival signals bad is free to interact and inhibit bcl-2 and $bclx_L$, thereby inducing apoptosis (Gross et al., 1999). In this study the nitro-PAH and DEPE did not change the levels of bax and bcl-2 (Fig. 11). Similar findings has also been reported with CP-PAH and B[*a*]P, however, further studies revealed that B[*a*]P caused a translocation of bax to the mitochondria (Solhaug et al., 2004b; Solhaug et al., 2005). Thus, the fact that we did not see any changes in the level of bax, does not exclude the possible involvement of bax in nitro-PAH induced apoptosis.

Recruitment of death receptors like Fas are known to activate caspase 8, leading to the initiation of extrinsic and intrinsic apoptotic pathways (Boatright et al., 2003). DNA damage

can result in the activation of caspase 8 by p53 dependent transcription of death receptors (Slee et al., 2004). Recently, activation of caspase 8 by anti-cancer drugs through changes in membrane fluidity causing death receptor clustering has been suggested (Dimanche-Boitrel et al., 2005; Micheau et al., 1999). Others report that some PAH may activate caspase 8 through a protein kinase which is independent of death receptors (Page et al., 2002). It has also been suggested that caspase 8 can be cleaved directly by reactive oxygen species (ROS) (Zhuang et al., 2000). Thus, PAH induced cleavage of caspase 8 is not necessarily directly linked to the activation of death receptors. Caspase 8, which is an initiator caspase can cause cleavage of pro-caspase 3 or bid. Truncated bid (tbid) is translocated to the mitochondria where it is believed to lead to cyt c release (Korsmeyer et al., 2000). In the present study 1-NP and 1,3-DNP resulted in decreased levels of pro-caspase 8 (Fig. 6C). 1,3-DNP also resulted in reduced levels of bid (Fig. 11). These findings suggests a role of death receptors at least in 1,3-DNP-induced apoptosis, but needs further investigations.

Another interesting finding was that 1-NP and 1,3-DNP resulted in decreased levels of bad and increased levels of phosphorylated bad at serin 155 (Fig. 11). Bad is phosphorylated at this site by protein kinase A (PKA), leading to cell survival (Lizcano et al., 2000). Bad may also be phosporylated by the PI-3 kinase Akt/PKB at serin 136, giving the same survival outcome. Akt is a central kinase in the signal transduction pathway triggered by growth factors or insulin. Its activation is particularly important in regulating cell survival (Song et al., 2005). In this study, 1-NP, 1,3-DNP and DEPE had increased activation of Akt (Fig. 10). Thus, it will be important to further elucidate if and how Akt suppress cell death when exposed to these compounds, and if activation of Akt results in phosphorylation of bad at serin 136.

Activation of the MAPKs have in previous studies been associated to B[a]P (Li et al., 2004; Solhaug et al., 2005) and DEPE (Bonvallot et al., 2001; Hiura et al., 1999) exposure. In the present study, only 1-NP caused an activation of ERK (Fig. 12). ERK is usually associated with cell survival and growth, however some reports indicate that ERK may induce pro-apoptotic signals (Tang et al., 2002; Wang et al., 2000). It would be interesting to further elucidate the effects of ERK in 1-NP exposed Hepa1c1c7 cells. In the present study all the compounds except 1,8-DNP induced phosphorylation of JNK (Fig. 12), which could suggest

that JNK phosphorylation depended on a nuclear translocalisation of p53. There are several postulated mechanisms in which the JNK pathway may be activated. Some groups indicate that the pathway is triggered by death receptors like Fas (Mielke et al., 2000), others have showed that the JNK pathway is dependent on the activation of effector pro-caspases (Dent et al., 2003). Interestingly, both Fas and effector pro-caspases are down stream targets of the p53 protein which could support the suggestion that 1,8-DNP JNK phosphorylation is dependent on p53 activity. However, p53 has mostly been associated as a down stream target of JNK (Herr et al., 2001; She et al., 2002). Phosphorylation of p38 and JNK activity (Fig. 12) correlate somewhat with the toxicity of the compounds and it could thus be interesting to further investigate the importance of JNK induced apoptosis in the nitro-PAH exposed Hepa1c1c7 cells. However, since the inhibitors of ERK and p38 all seem to influence the metabolism of B[*a*]P (Solhaug et al., 2005), and may therefore also affect the activation of the nitro-PAH, siRNA techniques could be used in addition to the inhibitors. The use of siRNA on the different MAPK (ERK, p38 and JNK) have been reported in several experimental systems, including, human melanoma cells (Hong et al., 2005) and human osteoblast cells (Kuo et al., 2005). Another possibility are to make/use cell lines in which these activities have been knocked down.

DEP is a major component of PM_{10} pollutants. In Los Angeles basin, DEP comprise of 40 % of the total PM_{10} levels, and the daily rate of exposure may be as high as 300 µg DEP (Diaz-Sanchez et al., 1994; Hiura et al., 1999). DEP consists of a vast number of PAH as well as nitro-PAH. In the standard reference material (SRM) 2975 for DEP (NIST 2000b) there is about 700 times more 1-NP than the well known carcinogenic compound B[*a*]P. DNP are generally considered to be more potent than 1-NP, but the SRM 1975 for DEPE (NIST 2000a) contains more than 10 times the amount of 1-NP then the DNP. In the present study the nitro-PAH seemed to be potent inducers of DNA damage, the DNP more potent than 1-NP in regard to p53 phosphorylation. DEPE increased the level of cleaved caspase 3 (Fig. 6A) and phosphorylated p53 (Fig. 9A), but in a lower degree than the nitro-PAH on the mass basis. This was to be expected since the concentrations of the nitro-PAH were higher when exposed as single components than in DEPE. DEPE gave a marked increase in cyp1a1 and cyp1b1 level (Fig. 8A and B), which reflects the high levels of PAH it contains. The nitro-PAH seem to contribute to the effects of DEPE observed in the Hepa1c1c7 cells, furthermore when

considering the amount of 1-NP compared to the DNP, the effects of 1-NP will possibly be higher or the same as the DNP.

In conclusion, these findings indicate that the nitro-PAH contributes to the effects of DEPE observed in the Hepa1c1c7 cells, furthermore when considering the potency and amount of the different nitro-PAH, the contribution of 1-NP seems to be substantial.

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