Differential mechanisms of cell death induced by nitro-polycyclic aromatic hydrocarbons

Dissertation for the degree of philosophiae doctor (ph.d.)

by

Nana Y. O. Asare

Division of Environmental Medicine
Norwegian Institute of Public Health

Faculty of Medicine
University of Oslo

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 768

ISBN 978-82-8072-783-1

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2009.

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To the One whose supernatural life, death and resurrection has been under investigation for ages and still is

Immanuel

ACKNOWLEDGEMENTS

The present work was carried out at the Department of Air Pollution and Noise (MILS), Division of Environmental Medicine, Norwegian Institute of Public Health, from the year 2003 to 2008. I appreciate the financial support provided by the Research Council of Norway for 3 years and additional financial support and good working conditions made available at the Norwegian Institute of Public Health.

I am grateful to my main supervisor, Dr. Jørn A. Holme for excellent supervision and guidance throughout the course of this work. My sincere gratitude goes to my co-supervisor, Dr. Marit Låg and head of department, Dr. Per E. Schwarze for their interest and support whenever needed. I thank Prof. Henrik Huitfeldt for being my contact supervisor at the University of Oslo despite his busy schedule.

My appreciation goes to all collaborators, especially those at the University of Rennes, France for their invaluable contributions and excellent working relations. Now, to all 'MILsere', thank you for your contributions in diverse ways and providing a good working environment. Special thanks to my office-mates, Annike I. Totlandsdal and Mistuko Komada for just being there. Not forgetting Dr. Rune Becher for his excellent support with microscopy and concern in general. Skilled technical assistance received from Leni Ekeren, Hans J. Dahlman and Tonje W. Elvestad is also very much appreciated.

Finally, I am deeply indebted to my wonderful family, Ed Sr. & Jr., Emil and Evangelin for their patience and just being them! Not to mention the support and encouragement from my parents, brothers and sisters, members of International Victory Fellowship (IVF), and all loved ones!

ABSTRACT

Cell death characterization is becoming an integral part in toxicological studies. Mechanistic studies of nitro-PAHs (polycyclic aromatic hydrocarbons) of interest might help elucidate which chemical characteristics are most important in eliciting toxic effects. The nitroarene fraction of MW 247 in diesel exhaust which comprises Nitropyrene (NP) and Nitrofluoranthene (NF) isomers is of particular interest due to the fact that about 10% of direct mutagenicity is associated with this fraction.

In the present study, we observed that involvement of cyp1a1 as well as intracellular accumulation of lipids is common to 1-NP and 3-NF cytotoxicity. We found that 1-NP and 3-NF, cause single strand DNA breaks as well as oxidative DNA damage in Hepa1c1c7 cells and the damages are recognized by ATR-CHK1 pathway and p53, which appear to initiate diverse signalling pathways of physiological relevance reflected in different morphological features. 1-NP- and 3-NF-exposed Hepa1c1c7 cells exhibited marked changes in cellular morphology, decreased proliferation and different forms of cell death. Upon exposure to 1-NP or 3-NF for 24 h, both typical apoptotic and necrotic cells were observed. However, with 3-NF in particular a large number of the cells exhibited a characteristic partial nuclear chromatin condensation. The caspase inhibitor Z-VAD-FMK inhibited only the apoptotic cell death. Nec-1 (an inhibitor of RIP-1-dependent necroptotic cell death) exhibited no inhibitory effects on either cell death or vacuolization in 1-NP-exposed cells, but reduced 3-NF-induced cell death. Additionally, cycloheximide completely attenuated 1-NP- and 3-NF-induced cell death. Flow cytometric analyses indicated that caspase-3 was activated differentially in the cell populations after exposure to 1-NP or 3-NF and TUNEL assays showed comparable patterns, with one population described as classic apoptotic cells. Mitochondrial release and translocation of LEI-DNase II, AIF and EndoG to the nucleus appear to be involved in the cell death processes, particularly that of 3-NF. Furthermore, Nec-1 appears to block the mitochondrial release of AIF and EndoG and thereby prevents membrane damage and eventually cell death, suggesting involvement of RIP-1 in 3-NF- induced necroptotic cell death. In contrast, the corresponding amine forms (1-aminopyrene and 3-aminofluoranthene) elicited only minor apoptotic and necrotic cell death, and cells with characteristics typical of either paraptosis or necroptosis were absent.

All the MAPKs; ERK1/2, p38 and JNK, appear to be involved in the death process since marked activations were observed upon 1-NP or 3-NF exposure, and their inhibitors partly reduced the induced cell death. Electron microscopic examination revealed that the characteristic vacuolization exclusive in 1-NP-exposed cells was due to swelling of mitochondria, autophagosomes and endoplasmic reticulum (ER). Myelinosomes, increases in number as well as size of lysosomes were observed in 3-NF-exposed cells. Interestingly, reactive oxygen species (ROS) and ionic imbalance, typical of paraptosis, appear to be prominent mechanistic factors in 1-NP-induced cell death, but not 3-NF. Taken together, 1-NP and 3-NF elicit apoptotic and non-apoptotic forms of programmed cell death (PCD), which are physiologically relevant processes in normal and pathological conditions. Closer insight into interactions between the different death pathways may be of importance in risk assessment and could additionally lead to novel drug discovery targets.

LIST OF PAPERS

Listed below are the publications of this thesis and will be referred to as Paper I, II and III accordingly in the text.

Paper I

1-Nitropyrene induces apoptosis and apparently a non-apoptotic programmed cell death (paraptosis) in Hepa1c1c7 cells. N. Asare, N.E. Landvik, D. Lagadic-Gossmann, M. Rissel, X. Tekpli, K. Ask, M. Låg, J. A. Holme. Toxicology and Applied Pharmacology 230 (2008) 175–186

Paper II

3-Nitrofluoranthene but not 3-Aminofluoranthene elicits apoptosis as well as programmed necrosis in Hepa1c1c7 cells. N. Asare, M. Låg, D. Lagadic-Gossmann, M. Rissel, P. Schwarze, J. A. Holme. (under consideration in Toxicology)

Paper III

Signalling pathways involved in 1-Nitropyrene- (1-NP) and 3-Nitrofluoranthene (3-NF)-induced cell death in Hepa1c1c7 cells. N. Asare, X. Tekpli M. Rissel, A. Solhaug, N. Landvik, V. Lecureur, G. Brunborg, M. Låg, D. Lagadic-Gossmann, J. A. Holme. (manuscript)

ABBREVIATIONS

3-AF, 3-aminofluoranthene
1-AP, 1-aminopyrene
AIF, apoptosis inducing factor
ATM, ataxia telangiectasia mutated
ATR, ATM- and Rad3-related
B[*a*]P, benzo[*a*]pyrene
BKC, big potassium channels
BNIP3, BCL2/adenovirus E1B 19kDa interacting protein 3
Cdk, cyclin-dependent kinases
cyp, cytochrome P450
EndoG, endonuclease G
ER, endoplasmic reticulum
ERK, extracellular signal-related kinase
fpg, formamidopyrimidine-DNA glycosylase
gamma-H2AX, phosphorylated H2AX
IBT, iberiotoxin
JNK, c-jun N-terminal kinase
LEI-DNase, Leucocyte elastase inhibitor-DNase
LD, lipid droplet
MAPKs, mitogen activated protein kinases
MC, mitotic catastrophe
MMP, mitochondrial membrane permeabilization
Nec-1, Necrostatin-1
3-NF, 3-nitrofluoranthene
Nitro-PAHs, nitro-polycyclic aromatic hydrocarbons
1-NP, 1-Nitropyrene
NOS, nitric oxide synthase
PAHs, polycyclic aromatic hydrocarbons
PARP, poly (ADP-ribose) polymerase
PCD, programmed cell death
PI, propidium iodide
RIP, receptor interacting protein
RNS, reactive nitrogen species
ROS, reactive oxygen species
TUNEL, TdT-mediated dUTP-biotin nick end labelling
Z-VAD-FMK, N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)fluoromethyl ketone.

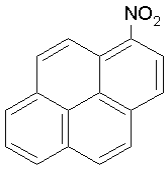
INTRODUCTION

POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

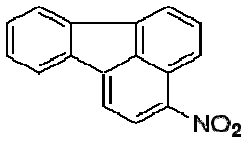
PAHs represent a major class of chemical carcinogens present in the environment in relation to production, refining and application of coal, mineral oil and oil shale [1-3]. Such sources as coal tar, vehicular exhaust, petroleum residues, coke oven emissions, and tobacco smoke condensate comprises a mixture of PAHs and heterocyclic aromatic hydrocarbons containing one or more nitrogen, sulphur, or oxygen atoms [1-3]. Human exposure to a variety of these complex mixtures have been associated with increased cancer incidence and other pathological conditions [1-3].

NITRO-PAHs

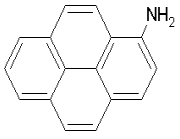
Exposure to nitro-PAHs is an environmental health concern. Nitro-PAHs can be formed as direct or indirect products of incomplete combustion of organic materials and have been found to be ubiquitous in ambient air [2,4]. Indoor air exposure from kerosene heaters and cooking oils has also been reported [2]. In diesel exhaust, the nitroarene fraction of MW 247 which comprises of nitropyrene (NP) and nitrofluoranthene (NF) isomers (in particular 1-NP, 3- and 8-NF) is of interest because about 10% of direct mutgenicity is associated with this fraction [2,5-7].



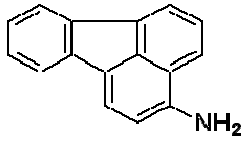
1-Nitropyrene (1-NP)



3-Nitrofluoranthene (3-NF)



1-Aminopyrene (1-AP)



3-Aminofluoranthene (3-AF)

Figure 1: Chemical structures of 1-NP, 1-AP, 3-NF and 3-AF.

METABOLIC ACTIVATION OF NITRO-PAHs

Both reductive and oxidative metabolism of these nitro-PAHs has been implicated in their ability to cause mutagenic activity and possibly carcinogenicity [2,5-12]. 1-NP undergoes extensive metabolism on the pyrene moiety as well as the nitro function of the molecule (Figure 2). 1-NP can be metabolized via cytochrome P450-mediated ring C-oxidation to epoxides, which may undergo subsequent rearrangement to nitropyrenols and conjugation or hydration to dihydrodiols [2,13]. Nitroreduction in one- or two-electron steps to form in sequence 1-nitroso-pyrene, *N*-hydroxy-1-AP and 1-AP, with or without subsequent acetylation also occurs [2,4,14-16]. A combination of ring oxidation and nitroreduction followed by acetylation also exists [2]. This complex biotransformation is reflected in the large variety of metabolites in plasma and tissue homogenates as well as adducts formed with blood proteins and DNA [2,8,17-19].

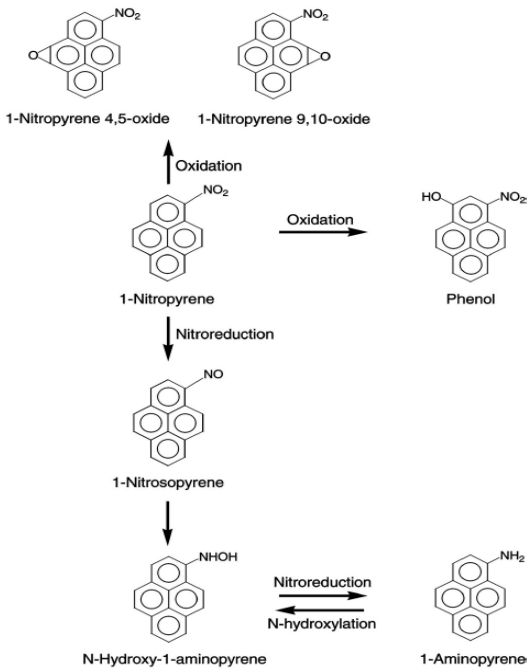


Figure 2: Metabolic pathway of 1-NP (Source- Kim HJ et al, Mol Cells. 2005 19 (1): 114-23)

Metabolism of 3-NF may follow similar patterns. Rat hepatic enzymes are known to catalyze both oxidative and reductive NF metabolism *in vitro*. Under aerobic conditions hydroxylation of the aromatic ring is the main pathway, whereas only reduction of the nitro group occurs under anaerobic conditions [20]. However, it appears that the isomeric position of the nitro group and the coplanar conformation with respect to the plane of aromatic rings has some influence on the biological activity of NFs [20]. The major adduct formed from 3-NF *in vitro* is *N*-(deoxyguanosin-8-yl)-3-AF, in the presence of xanthine oxidase and DNA [21].

CELL GROWTH AND DEATH

Intracellular as well as extracellular signalling regulate cell growth and death, and the balance between cell division and death is crucial for development and maintenance of multi-cellular organisms [22,23]. Disorders in these processes can lead to fatal pathological consequences. DNA damage checkpoint is one of the monitoring systems that maintain genomic integrity. Checkpoint systems including, ataxia telangiectasia mutated (ATM)-CHK2 and ATM- and Rad3-related (ATR)-CHK1 signalling pathways are in place to sense DNA damage and execute cell cycle arrest by inhibition of the activity of cell cycle regulators. [24]. If cells with DNA damage are able to evade the various checkpoint mechanisms and enter mitosis, cell death during mitosis may be the final resort to avoid mutation [25]. This is essential for the maintenance of genomic stability and prevention of tumour development.

Recently, it has become increasingly evident that the classic dichotomous classification of cell death into active apoptosis versus passive necrosis is a simplification of more complex processes that guard organisms against potentially harmful or unwanted cells [22,23,26-28]. The demise of cells by programmed cell death (PCD) is marked by well-defined morphological changes which until now had been synonymous to apoptosis. Apoptosis is an active process often characterized by cell shrinkage, nuclear and cytoplasmic condensation, DNA fragmentation, and formation of apoptotic bodies, generally phagocytosed by other cells [23,29-31]. One of the defining features of apoptosis is the sequential activation of multiple caspases, cysteine proteases that are involved in apoptotic cell death by a proteolytic cascade [30,32]. On the other hand, classic necrosis is passive and displays gross morphological and ultrastructural features that contrast apoptosis such as extensive cellular and organelle swelling, and an early disruption of cellular plasma membrane [29,33].

Since the precise molecular mechanisms of the different cell deaths have not been fully characterized, others have suggested alternative basis for their classification based on nuclear morphology of dying cells in an attempt to include the other indeterminate forms of PCDs [28,34]. Based on this general guideline, PCD can be differentiated into classic apoptosis, apotosis-like PCD with less compact, lumpy chromatin masses and necrosis-like PCDs exhibiting either complete absence of chromatin condensation or chromatin clustering leading to formation of loose speckles [28,34]. Notably, there have also been attempts to classify PCD according to cellular compartments involved in the process since nuclear morphology does not take into account the death signalling pathways involved [28].

Autophagy is a catabolic process involving the degradation of a cell's own components by the lysosomal machinery and autophagic-mediated cell death, which is often considered to be caspase-independent, is characterized mainly by the accumulation and subsequent degradation of various-sized cytoplasmic vacuoles by the cell's lysosomal system [23,27]. Other forms of PCDs, termed non-apoptotic/caspase-independent/necrosis-like PCDs have been described [35-37]. One such PCD dubbed paraptosis is characterized by cytoplasmic vacuolation, along with mitochondrial and ER swelling, resistance to apoptotic inhibitors and the involvement of mitogen-activated protein kinase (MAPKs) [23,36,38,39]. Necroptosis is another PCD that shares the combined biochemical and ultrastructural features of apoptosis and necrosis, with the selective inhibition of receptor interacting protein 1 (RIP-1) by Nec-1 [35,37,40]. Although autophagy is activated and reactive oxygen species (ROS) levels may be elevated, they play no critical roles in necroptosis [35,41]. Despite the broad spectrum of necrosis-initiating conditions, a growing body of evidence suggest that the execution of necrotic cell death may be carried out by a finite common set of mechanisms [33,34,40].

The balance in lipid metabolism appears to be critical in determining the fate of a cell. Lipid accumulation leading to a PCD termed lipoptosis has been reported [42,43]. Anoikis is the apoptosis of cells that have lost contact with extracellular matrix or interact through an inappropriate integrin-matrix combination [44-47]. Mitotic catastrophe (MC) has been considered another death pathway triggered by mitotic failure caused by defective cell cycle checkpoints [22,48-50]. More recent evidence suggests that MC may not be a separate mode of cell death but rather a preceding process which can end up in either apoptosis or necrosis, and the final outcome is dependent on the molecular profile of the cell [48,51]. Cellular senescence, a phenomenon whereby normal differentiated cells lose the ability to divide may

also be considered as a type of death [29,52]. It is widely believed that cellular senescence evolved as a way to prevent the initiation and spread of cancer because somatic cells that have divided several times will have accumulated DNA mutations, and would thus have increased probability of becoming cancerous if cell division continued [28,29].

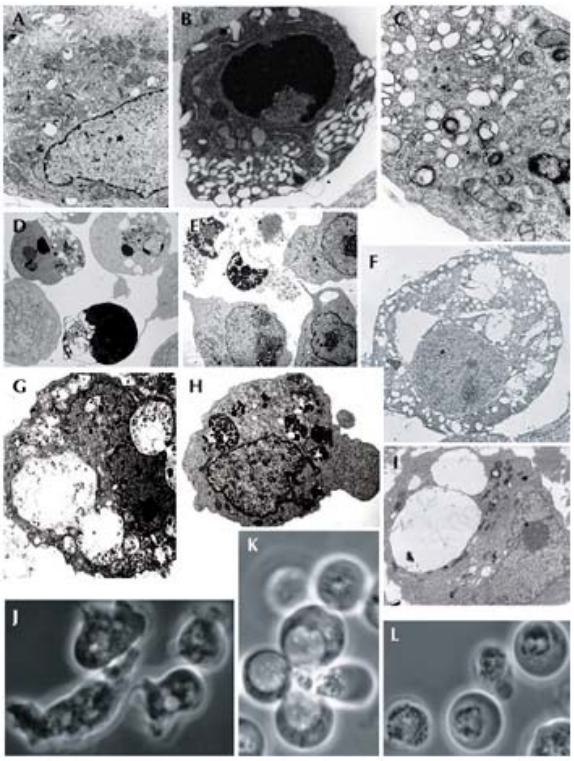


Figure 3: Micrographs of some types of cell death
(Source–http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1973949, Pierre Golstein and Guido Kroemer, 2007) **(A–I)** Electron microscopy; **(J–L)** phase contrast. **(A)** Normal HeLa cells; **(B)** treated with staurosporin to induce apoptosis; or **(C)** treated with thapsigargin to induce autophagy (Galluzzi *et al*, 2007). **(D)** Apoptotic and **(E)** necroptotic Jurkat cells (Degterev *et al*, 2005). **(F)** Paraptotic 293T cells (Sperandio *et al*, 2000; copyright (2000) National Academy of Sciences, USA). **(G)** Necrotic and **(H)** autophagic iBMK cells (Degenhardt *et al*, 2006; Mathew *et al*, 2007). **(I)** Ras-expressing U251 glioblastoma cells showing macropinosomes (J.H. Overmeyer, A. Kaul, E.E. Johnson & W.A. Maltese, unpublished data). **(J)** *Dictyostelium* cells, vegetative, **(K)** undergoing vacuolar autophagic cell death, and **(L)** undergoing necrotic cell death (Laporte *et al*, 2007).

SOME ORGANELLES INVOLVED IN CELL DEATH

Upon activation of a death stimulus, cells have access to different death programs executed either by caspases or independent of caspases. The mitochondria, lysosomes and ER may play prominent roles in certain PCDs but can be involved in the different pathways [22,28,53]. Signals from the different cellular organelles have been found to be linked, acting upstream as well as downstream of each other [22,28,53].

The mitochondria appear to be essential for a broad spectrum of death pathways and its membrane permeabilization (MMP) is reported to define the point of no return in most PCD models [28,53]. A large number of pathways are upstream of MMP, however, they are strictly controlled by the Bcl-2 family members with only few exceptions [28,54]. Bax and Bak being the 'multi-domain' pro-apoptotic members are considered to be crucial pore-forming molecules that trigger MMP and the subsequent release of death-inducing molecules such as cytochrome *c*, apoptosis inducing factor (AIF), endonuclease G (EndoG) and leucocyte elastase inhibitor-DNase (LEI-DNase) from the mitochondrial intermembrane space [28,54]. ROS generation independent of the Bcl-2 family members is also known to trigger the loss of mitochondrial trans-membrane potential resulting in a PCD with features of autophagic degeneration [55] or necrotic cell death.

In the classic apoptosis-necrosis paradigm, lysosomes were considered to be solely involved in necrotic and autophagic cell death through the release of unspecific enzymes [22,28]. Although lysosomal leakage is induced by undefined mechanisms, effector molecules released include ROS, cathepsins and H^+ [22,28,53]. Cathepsins released from lysosomes function in the apoptotic pathway by causing MMP via cleavage of Bid or activation of PLA2 and the subsequent increase in arachidonic acid [22,28,53]. Cathepsins may also cause caspase-independent chromatin condensation directly. Acidification of the cytosol is known to stabilize BNIP3, triggering autophagy or activate LEI-DNase II which can cause partial chromatinolysis in a caspase-independent manner [22,28,53,55]. The ER, another prominent sensor of cellular stress may initiate PCD by two main distinct mechanisms, the unfolded protein response and release of Ca^{2+} [22,28]. These two events eventually lead to MMP and may either activate the classical apoptosis pathway or other non-apoptotic death pathways [22,28].

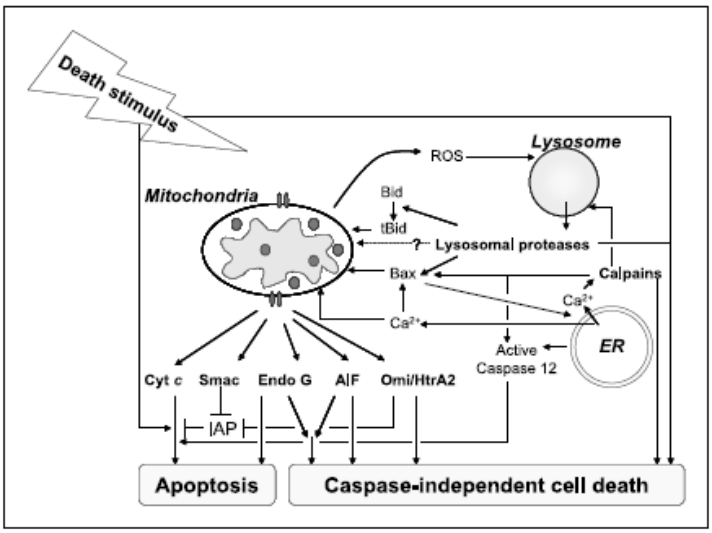


Figure 4: Diagramatic representation of cross-talk between organelles in cell death (Source-[22])

PHYSIOLOGIC ROLE AND HEALTH IMPLICATIONS OF CELL DEATH

Ironic as it may be, death happens to be an essential part of living. Cells divide repetitively from conception onwards and their endless proliferation is compensated for by death. Cellular suicide, to the right extent, in the right cells, at the right times and places is therefore crucial to the development of organisms. Defects in the program or process can be disastrous leading to cancer, autoimmune disorders such as AIDS, viral infections, ischemic injury, myocardial infarction, sepsis, multiple organ dysfunction syndrome, fibrosis, artherosclerosis, birth defects and neurodegenerative diseases such as Alzheimer's, stroke, Parkinson's [22,28,29].

Generally, multiplicity or redundancy of cell death pathways may represent an adaptive advantage, considering the imperative need to remove damaged or abnormal cells in order to preserve integrity of organisms [56]. It has been shown that both apoptotic and necrotic death markers are concomitantly present in the same cell after cerebral ischemia, indicating the

simultaneous activation of different death programs [57]. This paradigm is also reflected in cases where cell death triggers that induce caspase-dependent apoptosis shifts to caspase-independent death outcomes in cells with inhibition of apoptosis such as neurons, tumors or virally-infected cells [56]. There is accumulating evidence *in vitro* as well as *in vivo* that AIF can act as a safeguard death executioner in cancer cells with faulty caspase activation [58-60]. Interestingly, some proteins that block paraptosis may have complementary effects on apoptosis in development and cancer protection [61]. Consistent with this notion, AIF, EndoG, other endonucleases and proteases, as well as MAPKs are common mediators in the various death processes and as such some convergence of the pathways downstream may exist [39,61-63]. The dominant cell death phenotype has therefore been postulated to be determined by the relative speed of the available death programs. Thus, although characteristics of several death pathways can be displayed, only the fastest and most efficient is usually evident [64].

EXPERIMENTAL CONSIDERATIONS

THE EXPERIMENTAL SYSTEM

Preliminary studies of the nitro-PAHs in different cell types (primary rat lung epithelial T2 and lung epithelial A549 cells), proved Hepa1c1c7 cells to be a more promising choice for the mechanistic studies aimed at in this work. 1-NP in particular, exhibited a characteristic vacuolization process only in the mouse hepatoma cell line which apparently demanded further investigations. More importantly, previous reports show that Hepa1c1c7 cells are capable of cyp1a1 induction, making them suitable for mechanistic studies on PAHs [65-67]. It would, however, have been very insightful to have a comparative study on the signalling and mechanistic differences in the different cell types, but as time would not permit that, such studies are considered in the future plans of this project.

Although the mechanistic investigations in this study were performed at concentrations that may seem high when compared to environmentally-relevant concentrations, with highly lipophilic compounds such as 1-NP and 3-NF, a delayed absorption in the lung is known to result in a potentially higher dose to certain lung cells [68]. Furthermore, other routes of

exposure of PAHs such as dietry [69] and skin [70] have been found to profoundly contribute to their uptake in organisms.

CHARACTERIZATION OF CELL DEATH

Various techniques are available for the characterization of the different forms of cell death. In the present study, fluorescent microscopy employing Hoechst/PI staining has proven to be one of the most reliable techniques, though it is time-consuming, tedious and may be subjective. Flow cytometry has the advantage of being time-effective but when measured only at sub-G1, this method was unable to recognize the typical partial nuclear condensation evident in both apoptotic and non-apoptotic PCDs elicited in this system. However, employing apoptotic markers such as cleaved caspase-3 and TdT-mediated dUTP-biotin nick end labelling (TUNEL)-stained cells in combination with flow cytometric analyses as used in this study proves to be more efficient in determining the differences in different cell populations that are otherwise masked. Notably, techniques like western blotting also provide insightful and highly reliable information, such as cleavage of caspase-3 and its substrate, PARP, examined in this study. A combination of immunocytochemical and western blotting analyses, as used for activation of caspase-8 and its subsequent cleavage of important substrates like Bid and RIP-1, works very well. It is therefore important to use different techniques in toxicity assessments before any firm judgements are made.

Cellular morphological changes alone are not enough to draw conclusions on cell death pathways. Cellular organelles such as mitochondria, lysosomes and ER can be involved in various pathways and may play prominent roles in certain PCDs. Prominent organelles involved in the death process may provide information for the basis of classification. Biochemical interactions in support of such findings are of utmost importance. Use of inhibitors that block certain processes such as protein synthesis (cycloheximide/actinomycin) [71], protease activation (pan-caspase inhibitors like Z-VAD-FMK or Z-FA-FMK, specific on cathepsin B) [72-74], BKC opening (Iberiotoxin) [75,76] and MAPKs activation [77] may give a clue. Notably, Nec-1 is claimed to selectively inhibit necroptosis via RIP-1 and is as such used as an operational definition of necroptosis [35,37,40]. In general, care should be taken in concentrations used and interpretation of results, since most inhibitors may have

unspecific effects. Ideally, other techniques such as antisense, siRNA and gene-knockdown/knockouts may be used in addition, even though they may also have limitations.

AIM OF STUDY

Although it is well known that 1-NP and 3-NF are metabolically activated to mutagenic or carcinogenic compounds, less is known about their effect on cellular signalling and toxicity. The main objective of this work was thus to elucidate the toxic effects and mechanisms of 1-NP, 3-NF and their corresponding amine metabolites in Hepa1c1c7 cells. Below are the specific goals to achieve our aim.

- Examine the potency, characterize and compare the cell death elicited by 1-NP, 3-NF and their amine forms (Papers I, II and III)
- Investigate the role of metabolic activation of the nitro-PAHs and cell signalling pathways in the induced cell death(s) (Paper I, II and III)
- Examine perturbations in cellular organelles elicited upon exposure to the compounds and their role in the induced cell death (Paper I, II and III)
- Further characterize the induced cell death and examine the involvement of key factors in the cell death processes such as DNA damage, ROS, and ionic homeostasis (Paper III)

SUMMARY OF FINDINGS

Paper I

1-NP-exposed Hepa1c1c7 cells exhibited marked changes in cellular morphology, decreased proliferation and different forms of cell death. A dramatic increase in cytoplasmic vacuolization was observed already after 6 h of exposure and the cells started to round up at 12 h. The rate of cell proliferation was markedly reduced at 24 h and apoptotic as well as propidium iodide (PI)-positive cells appeared. Electron microscopic examination revealed that the vacuolization was partly due to mitochondria swelling. The pan-caspase inhibitor Z-VAD-FMK inhibited only the apoptotic cell death and Nec-1 (an inhibitor of necroptosis) exhibited no inhibitory effects on either cell death or vacuolization. In contrast, cycloheximide markedly reduced both the number of apoptotic and PI-positive cells as well as the

cytoplasmic vacuolization, suggesting that 1-NP induced a non-apoptotic PCD. All the MAPKs; ERK1/2, p38 and JNK, which are important intracellular signalling mediators appear to be involved in the death process, since marked activations were observed upon 1-NP exposure, and their inhibitors partly reduced the induced cell death. The ERK1/2 inhibitor PD 98057 completely blocked the induced vacuolization, whereas the other MAPKs inhibitors (UO126, SP600125 and SB202190) only had minor effects on this process. These findings suggest that 1-NP may cause apoptosis and paraptosis. In contrast, the corresponding amine (1-AP) elicited only minor apoptotic and necrotic cell death, and cells with characteristics typical of paraptosis were absent.

Paper II

In this study, we show that the environmental pollutant, 3-nitrofluoranthene (3-NF) but not its amine form, 3-aminofluoranthene (3-AF), induces apoptosis as well as regulated necrosis with necroptotic features in Hepa1c1c7 cells. Upon exposure to 3-NF, both typical apoptotic and necrotic cells were observed. A large number of the cells exhibited a characteristic partial nuclear chromatin condensation. Cycloheximide completely attenuated 3-NF-induced cell death. Activation of caspase-8, -9, and -3 were observed. Moreover, Z-VAD-FMK decreased the apoptotic cells, whereas the number of propidium iodide (PI)-positive cells with partial chromatin condensation was reduced by Nec-1, an inhibitor of receptor interacting protein (RIP-1). Cyp1a1 but not nitric oxide synthase (NOS) appear to be involved in activation of 3-NF to reactive metabolites. Increase in the number as well as size of lysosomes, myelinosomes, and activation of autophagy were also observed. 3-NF induced phosphorylation of ERK1/2, JNK and p38 MAPKs. Interestingly, while inhibitors of ERK1/2 (PD 98057, UO126) and JNK (SP600125) reduced apoptotic as well as necrotic cell death, the p38 inhibitor, SB202190, reduced only the necrotic cell death. Taken together, 3-NF elicits both apoptosis and a caspase-independent programmed cell death (PCD) with autophagic characteristics. Conversely, with 3-AF, no apparent cytotoxic effects besides a reduction in cell proliferation was observed.

Paper III

Here, we observed that 1-NP and 3-NF elicited apoptotic as well as non-apoptotic programmed cell deaths (PCDs) with paraptotic and necroptotic characteristics, respectively, in Hepa1c1c7 cells. Here, we observed intracellular accumulation of lipids in both 1-NP and

3-NF treated cells. 1-NP and 3-NF induced single-stranded as well as oxidative DNA-damages which appear to correspond well with the induced cytotoxicity. The DNA damage activated CHK1, but not ATM-CHK2 pathway in both 1-NP- and 3-NF-induced cell deaths. Flow cytometric analyses showed caspase-3 was activated in some cell populations but not in other cell populations after exposure to 1-NP or 3-NF. This distribution pattern is reflected in TUNEL assays which exhibited two populations, with one population described as classic apoptotic cells. 1-NP treated cells exhibited a more diffuse cell population pattern. Translocation of mitochondrial DNA-cleaving enzymes, LEI-DNase II, AIF and EndoG to the nucleus suggest that these DNases are involved in the cell death processes, especially for 3-NF. Nec-1, an inhibitor of RIP-1-dependent necroptotic cell death, reduced the induced mitochondrial release of AIF and EndoG by 3-NF, but not by 1-NP. Oxidative damage, big potassium channels (BKC) and intracellular accumulation of Ca^{2+}, typical of paraptosis, appear to be prominent mechanistic mediators involved in 1-NP-induced cell death, but not 3-NF.

GENERAL DISCUSSION

The ubiquitous nature of PAHs poses an environmental and occupational health concern to their exposure. Traffic is an important source of PAHs in urban areas and traffic-related pollutants seems to be implicated in the observed associations between air pollution and adverse health effects [2]. PAHs and nitro-PAHs are metabolized by a variety of xenobiotic-metabolizing enzymes [3,38,65,66,78,79]. In diesel exhaust particles (DEP), in particular, PAHs and nitro-PAHs form part of the organic component responsible for DEP induction of cytochrome P450 family enzymes, among others, which are critical for their metabolism in the lung as well as the liver [80,81]. Consistent with these findings, we observed marked inductions of cyp1a1 for all PAHs tested in this study and previous ones (Paper I and II, [65,66]). The Cyp inhibitor, α-naphthoflavone, markedly blocked 1-NP- and 3-NF-induced cell death (Paper I and II), which is indicative of the fact that their activation to metabolites toxic to cells involves cyp1a1. Neither NOS nor NQO1 appeared to be involved in this activation process in our cell model (Paper I and II).

Reactive intermediates resulting from the metabolic activation of PAHs have the ability to bind DNA and other macromolecules resulting in adduct formation [1,2,4,7,81-86]. In

general, oxidation of PAHs by Cyp enzymes is known to be the common initial step in the activation process that results in the production of polar biochemically reactive metabolites that are capable of interacting with cellular macromolecules such as DNA and proteins [3,87]. Electrophilic nitrenium ions generated from nitroreduction of nitro-PAHs, including 1-NP and 3-NF, are also capable of interacting with DNA [21,88]. Both 1-NP and 3-NF have been reported to be genotoxic and carcinogenic in different systems [1,2,89]. In the present study, the intensity of DNA damage caused by the individual compounds correlated well with the observed cytotoxicity, suggesting that DNA damage is an important part of triggering factors in the observed cell death (Paper III). The nitro-PAHs were more cytotoxic and exhibited more genotoxicity, by way of single-stranded DNA breaks as well as oxidative DNA damage as opposed to their amine forms (unpublished data). 3-NF gave the highest score in single-stranded DNA damage and is the most cytotoxic. Formation of 8-oxo-7,8-dihydro-2'-guanosine (8-oxo-dG) is reported to be a relevant indicator of oxidative base damage that may lead to DNA dysregulation [90]. Interestingly, significant levels of oxidative DNA damage were observed only in 1-NP-exposed cells as judged by 8-oxo-dG formation (Paper III). 3-NF did not elicit a significant increase in oxidative DNA damage. 1-NP was also found to cause lipid peroxidation (Paper III). A possible role of ROS is further supported by the fact that thiourea partially blocked 1-NP-induced cell death, but had no significant effect on 3-NF-induced cell death (Paper III). These findings are in line with previous reports implicating ROS in paraptotic cell death [76]. Although, ROS levels may be elevated in necroptotic signalling, it has been found to play no functional role in the death process [41].

In response to genotoxic stress, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) become activated and sequentially activate CHK2 and CHK1, respectively [29]. Several checkpoints are thus activated to delay cell cycle progression and coordinate repair [25,29,48,49]. Cells in different cell cycle phases happen to use diverse mechanisms to arrest their progression. Mitotic catastrophe (MC) is used to describe dysregulated or failed mitosis usually as a response of mammalian cells to mitotic DNA damage [25,29,48,49]. In G1, DNA damage activates p53, which sequentially activates a Cdk inhibitor, p21Cip1 [25,48]. Thus, p53 mutant tumours that are resistant to genotoxic damage are known to exhibit mitotic death as a delayed response [48]. In the present study, flow cytometry analyses showed marked increase in phosphorylated forms of CHK1 and H2AX but not ATM and CHK2 in 1-NP and 3-NF-exposed cells (Paper III). Thus, with regard to these compounds ATR signalling is prominent in their death pathway, leading to stalled replication forks as

opposed to double strand (ds) breaks in DNA which are more closely related to ATM signalling. However, gamma-H2AX showed a dramatic increase, which could be due to secondary dsDNA break formation, or most probably dsDNA breaks resulting from the activity of AIF, EndoG and LEI-DNaseII. Additionally, cell proliferation was markedly inhibited after exposure of Hepa1c1c7 cells to both 1-NP and 3-NF as judged by TB exclusion cell counts and cloning efficiency of surviving cells (Paper I and II). Flow cytometric analyses revealed only a small increase of cells in S-phase. Interestingly, time-laps observations of cells under exposure revealed impaired cell divisions associated with mitotic catastrophe after 1-NP exposure (unpublished data). We frequently observed that cells started the mitotic process but were unable to complete it normally and eventually died off.

A wide variety of stimuli such as DNA damage, oxidative or nitrosative stress and ligand binding to cell surface death receptors can initiate apoptosis [33,91]. One of the defining features of apoptotic cell death pathway mechanism is the formation of multiprotein complexes that provide the scaffolding for the activation of initiator caspases like caspase-8 and -9 by autoproteolytic cleavage [33]. The respective complexes are the death-induced signalling complex and apoptosome [33]. Eventually, activation of effector caspase-3 and the subsequent cleavage of PARP lead to association with caspase-activated DNase which executes the typical internucleosomal DNA fragmentation upon activation [92]. This canonical pathway of caspase activation via mitochondria, attained by way of cytochrome c release appears to be common with both 1-NP and 3-NF-induced apoptotic pathways (Paper I and II). Notably, flow cytometric analyses revealed partial to no activation of caspase-3 in some population of cells, suggesting an early arrested apoptotic process which shifts to necrotic cell death as judged by the phenotype observed especially in 3-NF-exposed cells (Paper III). The activation of initiator caspase-8 and its subsequent cleavage of Bid in particular, were more clearly evident in 3-NF cellular signalling (Paper II). Other non-mitochondrial caspase-dependent PCDs have also been discovered [93]. The death receptor-mediated pathway is the most studied in this group [94,95].

A caspase-independent mitochondrial pathway mediated by AIF release from the intra-membrane mitochondria space and subsequent translocation to the nucleus has been characterized lately [92]. AIF is known to trigger DNA fragmentation into large fragments corresponding to peripheral chromatin condensation typical of apoptosis-like PCD [92]. LEI-DNase II and EndoG have been found to function similarly in paraptotic and other PCD

pathways [92,96]. Flow cytometry analyses of TUNEL-stained cells, allowed as to distinguish two cell populations with differential DNA fragmentation, suggesting partial DNA fragmentation and the involvement of AIF, EndoG and LEI-DNaseII in the induced cell death (Paper III). A potential role of these endonucleases was further supported by immunocytochemical analyses showing a translocation of LEI-DNaseII, AIF and EndoG to the nucleus in 3-NF-exposed cells in particular (Paper III). In addition, we found that the translocation with AIF and EndoG is partly blocked by Nec-1, suggesting involvement of RIP-1 in 3-NF-induced necroptotic-like PCD. Thus the population of cells exhibiting more DNA damage may be classified as true apoptotic cells, whereas the other population could include necroptotic cells with regard to 3-NF. This correlates well with the distribution pattern observed with cleaved caspase-3 (Paper III), and is in accordance with previous reports in which necroptosis has been suggested to be a cellular back-up system where apoptosis is inhibited [35,40]. A cell's commitment to die has been repeatedly suggested to switch from apoptosis to other PCD types whereby caspase activation cannot be obtained [97-99]. However, both pathways may coexist in some paradigms [Paper I and II, [92,100]. Overall, some commonality in initiating signals which lead to different biochemical pathways, effecting different ultrastructural features appear to exist in 1-NP and 3-NF-exposed Hepa1c1c7 cells [Paper I, II and III]. Such paraptotic or necroptotic features were neither seen upon exposure to the corresponding amine forms, nor the prototype PAH, B[*a*]P.

The idea that apoptosis can be initiated by caspase-independent processes had been previously challenged by the difficulty in outlining complete pathways in which the molecular actors are defined [53,101]. Lysosomes may function as death signal integrators in several apoptotic pathways [33,34,53,102,103]. Lysosomal cathepsins are known to translocate from the lysosomal lumen to the cytosol in response to p53 activation, oxidative stress, TNF and the lipid second messenger, sphingosine [33,53,103,104]. The lysosomal membrane permeabilization and cathepsin release is reported to be a crucial step of the death cascade [33,53,103,104]. The early phase of commitment to apoptosis is suggested to be a caspase-independent step, followed by a caspase-mediated degradation step [28,102]. Of note, lysosomal leakage inevitably leads to intracellular acidification which has many consequences that may contribute to PCDs. This cytosolic acidification has been shown to induce post-translational modification of the anti-apoptotic serine protease inhibitor, LEI, resulting in its conversion to LEI-DNase II that mediates nuclear changes typical of apoptosis-like PCDs [28]. Additionally, lysosomal leakage leading to cytosolic acidification has been shown to

trigger JNK-mediated caspase- and Bcl-2-independent necrosis-like PCD in bladder cancer cells [105]. Other pathways associated with ER stress and dependence receptor-induced apoptosis have been reported [63,106]. Evidence suggests intracellular lipid accumulation leading to apoptosis [42,43]. We observed lipid droplet accumulation in both 1-NP and 3-NF-exposed cells which may be implicated in the induced cell deaths (Paper III). This possibility will be elucidated in another study. Another interesting variant is the apoptosis of cells that lose contact with the extra-cellular matrix or interact through an inappropriate integrin-matrix combination [44-46]. Preliminary studies suggest that the nitroaromatic drug, nilutamide may cause apoptosis via this mechanism in Hepa1c1c7 cells (personal communication).

Toxic insults such as DNA damage, ROS/RNS elevation or binding of inflammatory cytokines to their receptors on the cell surface may activate both death and survival signalling pathways [Paper I and II, [65,66,100]. Interplay between survival and death promoting complexes ensues until one finally dominates and determines the cell's fate. RIP1 is known to be a crucial adaptor kinase mediating these stress-induced signalling pathways and a cell's commitment to death or survival [40,100,107]. RIP1 is the first member of a family of seven, all of which contain a kinase domain (KD) [40,100,107]. Depending on the cellular context, increased RIP1 expression may lead to activation of MAPKs, NF-κB, apoptosis or necrosis [40,100,107]. An active KD allows RIP1 to autophosphorylate. Besides, RIP1 also interacts with a pool of adaptor proteins through its intermediate domain which leads to the recruitment of other kinases such as MEKK1, MEKK3 and RIP3 [108]. The activation of ERK, which was very marked in our study (Paper II), as well as necrotic cell death is known to depend on the kinase activity which is not essential for the activation of the other MAPKs or NF-κB [100]. Notably, RIP-1 is known to be a critical substrate of caspase-8 as with Bid and other downstream caspases such as caspase-3 [109]. Both 3-NF and 1-NP exposures appear to result in RIP-1 cleavage (Paper III). The efficiency of RIP-1 cleavage is suggested to be a critical factor in determining the execution of cell death, apoptosis in particular, by different death receptors [109]. Several death receptor-induced necrotic cell deaths including necroptosis implicate RIP1 [40,100]. The existence of a programmed necrotic pathway reported by Holler et al [110] showed convincing evidence that death domain receptor-induced necrosis of T lymphocytes required functional RIP-1. Recently, the necrostatins, Nec-1 in particular was identified as an allosteric inhibitor of the RIP-1 kinase step in necroptotic pathway [40]. Thus, the involvement of RIP-1 in 3-NF-induced necroptotic-like PCD is suggested since Nec-1 inhibits the necroptotic features and cell death (Paper II and III). RIP1

integrates several different upstream signals which culminate in a limited number of cellular responses and abolishing its kinase function may allow interference with necrotic signalling pathways in certain disease states.

The MAPKs are serine/threonine protein kinases which play pivotal roles in a variety of cell functions in many cell types [111,112]. This family of proteins shares many structural similarities and includes three major sub-families; the extracellular signal-regulated kinases (ERKs), the stress related c-Jun NH_2-terminal kinases (JNKs) and p38 kinases [111,112]. Various biochemical and molecular processes including reactive toxic metabolites lead to activation of cell signalling mechanisms involving the MAPKs [39,113,114]. However, specificity of response is achieved by the influence on gene expression and regulation of downstream kinases or transcription factors [111,112]. The cellular localization of these transducers brings them in contact with appropriate molecular targets that alter the expression of genes that are involved in apoptosis, necroptosis, necrosis and paraptosis [39,40,100,111,112]. Taken together, the mediation of the MAPKs, particularly, ERK and JNK pathways in the different cell death processes suggest interplay in the death pathways (Papers I and II). This notion is consistent with previous studies where the different cell deaths have been suggested to be physiologically complementary processes [40,91,98,99,115]. The final outcome of MAPKs activation is very difficult to predict and is determined by parallel interactions with several other signaling pathways. ERK is known to be involved in survival signalling [77]. However, recent studies have shown that ERK may promote cell death as well [77,111]. Thus, some duality in the function of ERK is evident. The PI3K-Akt survival pathway is also activated by various intracellular and extracellular signals [116]. Akt kinase modulates transducers that function in apoptotic pathways upon its activation, leading to the ability of cells to resist death signals [29,116]. Akt signalling is known to induce the expression of anti-apoptotic Bcl-xl and inhibit pro-apopototic activity by promoting nuclear translocation of MDM2, thereby negatively regulating p53-mediated apoptosis [29]. These cell survival pathways are therefore crucial in tumorigenesis because they can suppress or cause alterations in PCDs [29,116]. Dysregulation of various signalling pathways associated with induction of cell proliferation, modulation of cellular differentiation and apoptosis has been proposed to contribute to carcinogenicity of PAHs [117].

Ionic homeostasis is a major function of plasma membranes as well as intracellelular organelle membranes [118]. Specific balance in the major inorganic cations and anions, Ca,

K, Na, H, Cl, phosphate and bicarbonate is fundamental to all cells [118]. Cell-specific differences in the expression of membrane transport proteins and regulatory factors, which permits variations in the absolute rates of transmembrane flux of these ions have been reported [118]. One interesting study demonstrated that prolonged activation of big potassium channels (BKCs) in response to the respiratory burst induced by monocytes initiate paraptosis in selected glioma cells [76]. The characteristic swelling and cytoplasmic vacuolization as well as cell death were abolished by iberiotoxin, a specific BKC inhibitor [76]. Confocal fluorescence microscopy in the same study revealed colocalization of BKCs with the two targeted organelles, mitochondria and ER, implicated in parpatosis [76]. Although mitochondria and ER are affected in 1-NP-exposed Hepa1c1c7 cells in the present study, IBT was unable to visibly block the cytoplasmic vacuolization process but partially reduced cell death (Paper III). Calcium homeostasis is also widely appreciated as a crucial determining step with the onset of various types of cell death [119]. Mitochondria and ER again, are the key organelles involved in calcium handling [28,119]. Calcium overload happens to be a central feature in necrotic cell death [119]. Interestingly, cellular Ca^{2+} accumulation appears to be involved in 1-NP-induced cell death, but not 3-NF, in the Hepa1c1c7 cells**.**

Autophagy plays a central role in the maintenance of cellular homeostasis [119]. Previous reports have implicated autophagy as the mechanism by which several anti-cancer drugs induce their cytotoxic effects [29,119]. However, upon nutrient deprivation, autophagy operates as a survival pathway particularly in organisms [119]. This duality of roles in the autophagic process has led to unresolved discussions as to whether it should be considered as a cell survival mechanism or a form of cell death [119,120]. Although necroptotic death exhibits autophagic characteristics, it is considered as a downstream event and not an integral part of the cell death pathway [35,40]. In line with these reports we observed autophagosomes, lysosomal leakage as well as increased expression in the autophagic marker, LC3b but 3-methyladenine, an inhibitor of autophagy, did not have any effect on the induced cell deaths (Papers II and III). Moreover, neither cathepsin B inhibitor (benzyloxycarbonyl-phenyl-alanyl-fluoromethylketone, Z-FA-FMK), Pepstatin A (inhibitor of cathepsin D) nor the calpain inhibitor (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal, ALLN) had any effect on either 3-NF or 1-NP-induced cell death (Paper III). Consistent with the notion that though several features may be exhibited, only the fastest and most efficient is usually evident [64]. A link between autophagosomal and lysosomal systems have been implicated in PCDs [64].

Interestingly, lysosomal leakage leading to intracellular acidification has also been reported to stabilize the autophagy-inducing Bcl-2 family member, BNIP-3 [121].

Amphiphilic compounds have been reported to consistently cause cellular accumulation of myelinosomes [122-124]. These compounds are known to affect lipid metabolism by direct interference with the action of phospholipases in the ER, lysosomes and plasma membrane [122-124], or intercalation into the phospholipid portion of cytoplasmic membranes, stabilizing these lipids and eventually prevent their catabolism [122-124]. This leads to the accumulation of lipids with altered physicochemical properties which are recognized by the cell as abnormal and concentrated in myelinosomes or autophagic vesicles [122-124]. This mode of action might be one of the mechanisms by which 3-NF causes cytotoxicity in Hepa1c1c7 cells (Papers II and III). A direct link between myelinosomes, ER, and nuclear envelope has also been established [124]. An association between lipid and myelin accumulation have also been observed to have relevance in certain pathological states such as acute atherosis in relation to preeclampsia and a broad spectrum of pregnancy disorders [125]. Advances in our understanding of the molecular machinery that regulates the functional interaction of lipid droplets with other organelles and the cytoskeleton may have physiological relevance in diseases such as the Niemann-Pick type C, artherosclerosis and thrombosis.

The balance between cell growth, mitosis and cell death maintains cellular homeostasis in organisms [126]. Defects in genes regulating either mitosis or mitotic death may therefore contribute to tumorigenesis. Cell death is an intrinsic part of development, tissue homeostasis and immune regulation in organisms [126,127]. PCD is the predominant form of physiologic cell death through which organisms eliminate unwanted or damaged cells [23,29]. A wide range of physiological and pathological stimuli can initiate PCD by eliciting DNA or plasma membrane damage, receptor mechanisms and biochemical agents [23,33,91,128]. The mode of cell death, be it apoptotic, necrotic or indeterminate depends on the initiating stimuli and available cellular energy. Certain mutations allow a cell to resist death stimuli and thereby provide it with a selective growth advantage. Enormous mechanistic investigations unravelling dysregulated PCDs, apoptosis in particular, suggest that defects in the control of cell death or survival is implicated in pathogenesis of a wide range of diseases [29,61]. Deficient PCDs is associated with cancer, autoimmune disorders and viral infections among others, whereas excessive PCD is linked to ischemic injury, AIDS, myocardial infarction,

stroke, multiple organ dysfunction syndrome, and most neurodegenerative diseases [22,23,28,29,61].

Different morphological features reflecting different biochemical pathways of physiological relevance are accumulating. Dysregulation of these signalling pathways associated with either cell growth or death has been proposed to contribute to carcinogenicity of PAHs [117,129]. A better understanding of mechanisms of the various PCDs and clarity in the interplay between apoptotic and non-apoptotic PCDs may be informative in risk assessment evaluations of toxic compounds and promises to be of great importance to attempts being made to treat diseases associated with PCDs.

CONCLUSIONS

The work presented here has provided novel information about different cell death pathways induced by nitro-PAHs. A summary of conclusions drawn from these findings are listed below.

- The nitro-PAHs appear to be more toxic than their corresponding amines and also cause more single-stranded as well as oxidative DNA damage, which is largely recognized by ATR-CHK1 genotoxic signalling pathway.
- Both 1-NP and 3-NF induced apoptotic as well as non-apoptotic PCDs with paraptotic and necroptotic characteristics, respectively.
- The activation of 1-NP and 3-NF to metabolites toxic to Hepa1c1c7 cells involves cyp1a1 but neither NOS nor NQO1.
- ERK, JNK and p38 MAPKs appear to play active roles in the different cell death pathways elicited, consistent with the notion of interplay in these cell deaths.
- Whereas mitochondrial and ER damage may be prominent in 1-NP-induced cell death, AIF, EndoG and LEI-DNase II and Nec-1-dependent signalling appear to be prominent mediators in 3-NF-induced cell death.
- ROS, activation of BKCs as well as Ca^{2+} dysregulation are important factors that contribute to the cell death process induced by 1-NP, but not 3-NF.

FUTURE STUDIES

- Futher clarify mechanisms of the different PCDs elicited by 1-NP and 3-NF employing such techniques as siRNA, antisense and gene-knockdowns
- Investigate interplays between the apoptotic and non-apoptotic PCDs by microarray or proteomic labelling techniques
- Comparative analyses of these nitroarenes in different cell systems, such as primary lung cells, hepatocytes, other cell lines and possibly in vivo studies would be insightful

Reference List

[1] IARC. IARC - Summaries and Evaluations. 33, 201. 1984.
Ref Type: Report

[2] WHO, Selected nitro- and nitro-oxy-polycyclic aromatic hydrocarbons, 229 ed. World Health Organization, Geneva, 2003.

[3] W. Xue D. Warshawsky, Toxicol.Appl.Pharmacol. 206 (2005) 73-93.

[4] Chan, P. C. Toxicity studies of 1-Nitropyrene. National Toxicology Program 34, 1-62. 1996. NIH (USA).
Ref Type: Generic

[5] K. El Bayoumy, S. S. Hecht, T. Sackl, G. D. Stoner, Carcinogenesis 5 (1984) 1449-1452.

[6] T. L. Gibson, Mutat.Res. 122 (1983) 115-121.

[7] H. S. Rosenkranz, Mutat.Res. 101 (1982) 1-10.

[8] K. El Bayoumy, B. Johnson, S. Partian, P. Upadhyaya, S. S. Hecht, Carcinogenesis 15 (1994) 119-123.

[9] T. Kinouchi Y. Ohnishi, Microbiol.Immunol. 30 (1986) 979-992.

[10] A. P. Li J. S. Dutcher, Mutat.Res. 119 (1983) 387-392.

[11] K. El Bayoumy, Y. H. Chae, J. G. Rosa, L. K. Williams, D. Desai, S. Amin, E. Fiala, Cancer Lett. 151 (2000) 7-13.

[12] E. Dybing, J. E. Dahl, F. A. Beland, S. S. Thorgeirsson, Cell Biol.Toxicol. 2 (1986) 341-355.

[13] H. J. Kim, T. H. Kim, S. Y. Lee, D. H. Lee, S. I. Kim, G. P. Pfeifer, S. K. Kim, C. S. Lee, Mol.Cells 19 (2005) 114-123.

[14] Y. H. Chae, T. Thomas, F. P. Guengerich, P. P. Fu, K. El Bayoumy, Cancer Res. 59 (1999) 1473-1480.

[15] R. P. Mason J. L. Holtzman, Biochemistry 14 (1975) 1626-1632.

[16] I. T. Reeve M. G. Miller, Chem.Res.Toxicol. 15 (2002) 352-360.

[17] Y. M. van Bekkum, P. T. Scheepers, P. H. van den Broek, D. D. Velders, J. Noordhoek, R. P. Bos, J.Chromatogr.B Biomed.Sci.Appl. 701 (1997) 19-28.

[18] Y. M. van Bekkum, P. H. van den Broek, P. T. Scheepers, R. P. Bos, Chem.Res.Toxicol. 11 (1998) 1382-1390.

[19] Y. M. van Bekkum, P. H. van den Broek, P. T. Scheepers, J. Noordhoek, R. P. Bos, Chem.Biol.Interact. 117 (1999) 15-33.

[20] M. A. Belisario, R. Pecce, M. R. Della, A. R. Arena, A. Cecinato, P. Ciccioli, N. Staiano, Carcinogenesis 11 (1990) 213-218.

[21] A. M. Dietrich, C. R. Guenat, K. B. Tomer, L. M. Ball, Carcinogenesis 9 (1988) 2113-2119.

[22] L. E. Broker, F. A. Kruyt, G. Giaccone, Clin.Cancer Res. 11 (2005) 3155-3162.

[23] W. Bursch, A. Ellinger, C. Gerner, U. Frohwein, R. Schulte-Hermann, Ann.N.Y Acad.Sci. 926 (2000) 1-12.

[24] M. Shimada M. Nakanishi, J Mol.Histol. 37 (2006) 253-260.

[25] X. Huang, T. Tran, L. Zhang, R. Hatcher, P. Zhang, Proc.Natl.Acad.Sci.U S A 102 (2005) 1065-1070.

[26] W. Bursch, B. Grasl-Kraupp, A. Ellinger, L. Torok, H. Kienzl, L. Mullauer, R. Schulte-Hermann, Biochem.Cell Biol. 72 (1994) 669-675.

[27] W. Bursch, K. Hochegger, L. Torok, B. Marian, A. Ellinger, R. S. Hermann, J Cell Sci. 113 (Pt 7) (2000) 1189-1198.

[28] M. Jaattela, Oncogene. 23 (2004) 2746-2756.

[29] H. Okada T. W. Mak, Nat.Rev.Cancer 4 (2004) 592-603.

[30] T. Hirsch, P. Marchetti, S. A. Susin, B. Dallaporta, N. Zamzami, I. Marzo, M. Geuskens, G. Kroemer, Oncogene. 15 (1997) 1573-1581.

[31] J. F. Kerr, A. H. Wyllie, A. R. Currie, Br J Cancer 26 (1972) 239-257.

[32] P. Nicotera, M. Leist, L. Manzo, Trends Pharmacol.Sci. 20 (1999) 46-51.

[33] P. S. Tang, M. Mura, R. Seth, M. Liu, Am J Physiol.Lung Cell Mol.Physiol. 294 (2008) L632-L641.

[34] M. Leist M. Jaattela, Nat.Rev Mol.Cell Biol. 2 (2001) 589-598.

[35] A. Degterev, Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G. D. Cuny, T. J. Mitchison, M. A. Moskowitz, J. Yuan, Nat.Chem.Biol. 1 (2005) 112-119.

[36] S. Sperandio, B. de, I, D. E. Bredesen, Proc.Natl.Acad.Sci.U S.A 97 (2000) 14376-14381.

[37] X. Xu, C. C. Chua, J. Kong, R. M. Kostrzewa, U. Kumaraguru, R. C. Hamdy, B. H. Chua, J Neurochem. 103 (2007) 2004-2014.

[38] N. Asare, N. E. Landvik, D. Lagadic-Gossmann, M. Rissel, X. Tekpli, K. Ask, M. Lag, J. A. Holme, Toxicol.Appl.Pharmacol. 230 (2008) 175-186.

[39] S. Sperandio, K. Poksay, B. de, I, M. J. Lafuente, B. Liu, J. Nasir, D. E. Bredesen, Cell Death.Differ. 11 (2004) 1066-1075.

[40] A. Degterev, J. Hitomi, M. Germscheid, I. L. Ch'en, O. Korkina, X. Teng, D. Abbott, G. D. Cuny, C. Yuan, G. Wagner, S. M. Hedrick, S. A. Gerber, A. Lugovskoy, J. Yuan, Nat.Chem.Biol. 4 (2008) 313-321.

[41] W. Han, L. Li, S. Qiu, Q. Lu, Q. Pan, Y. Gu, J. Luo, X. Hu, Mol.Cancer Ther 6 (2007) 1641-1649.

[42] S. Brandlein, N. Rauschert, L. Rasche, A. Dreykluft, F. Hensel, E. Conzelmann, H. K. Muller-Hermelink, H. P. Vollmers, Mol.Cancer Ther 6 (2007) 326-333.

[43] T. Pohle, S. Brandlein, N. Ruoff, H. K. Muller-Hermelink, H. P. Vollmers, Cancer Res. 64 (2004) 3900-3906.

[44] S. Marastoni, G. Ligresti, E. Lorenzon, A. Colombatti, M. Mongiat, Connect.Tissue Res. 49 (2008) 203-206.

[45] M. A. Pallero, C. A. Elzie, J. Chen, D. F. Mosher, J. E. Murphy-Ullrich, FASEB J (2008) .

[46] N. M. Munoz, J. Y. Baek, W. M. Grady, Growth.Factors. (2008) 1.

[47] R. Lock J. Debnath, Curr Opin.Cell Biol. (2008) .

[48] J. Erenpreisa M. S. Cragg, Cancer Cell Int 1 (2001) 1.

[49] M. Castedo, J. L. Perfettini, T. Roumier, K. Andreau, R. Medema, G. Kroemer, Oncogene. 23 (2004) 2825-2837.

[50] M. Castedo, J. L. Perfettini, T. Roumier, A. Valent, H. Raslova, K. Yakushijin, D. Horne, J. Feunteun, G. Lenoir, R. Medema, W. Vainchenker, G. Kroemer, Oncogene. 23 (2004) 4362-4370.

[51] H. Vakifahmetoglu, M. Olsson, B. Zhivotovsky, Cell Death.Differ. 15 (2008) 1153-1162.

[52] L. HAYFLICK P. S. MOORHEAD, Exp.Cell Res. 25 (1961) 585-621.

[53] M. Jaattela, C. Cande, G. Kroemer, Cell Death.Differ. 11 (2004) 135-136.

[54] M. G. Annis, J. A. Yethon, B. Leber, D. W. Andrews, Biochim.Biophys.Acta 1644 (2004) 115-123.

[55] V. C. Vande, J. Cizeau, D. Dubik, J. Alimonti, T. Brown, S. Israels, R. Hakem, A. H. Greenberg, Mol.Cell Biol. 20 (2000) 5454-5468.

[56] H. Boujrad, O. Gubkina, N. Robert, S. Krantic, S. A. Susin, Cell Cycle. 6 (2007) 2612-2619.

[57] I. Unal-Cevik, M. Kilinc, A. Can, Y. Gursoy-Ozdemir, T. Dalkara, Stroke 35 (2004) 2189-2194.

[58] M. Alonso, C. Tamasdan, D. C. Miller, E. W. Newcomb, Mol.Cancer Ther 2 (2003) 139-150.

[59] B. Joseph, P. Marchetti, P. Formstecher, G. Kroemer, R. Lewensohn, B. Zhivotovsky, Oncogene. 21 (2002) 65-77.

[60] D. J. Liao R. B. Dickson, Lab.Invest. 83 (2003) 1437-1449.

[61] D. E. Bredesen, Curr Mol.Med 8 (2008) 173-186.

[62] S. Castro-Obregon, R. V. Rao, G. del Rio, S. F. Chen, K. S. Poksay, S. Rabizadeh, S. Vesce, X. K. Zhang, R. A. Swanson, D. E. Bredesen, J Biol.Chem. 279 (2004) 17543-17553.

[63] R. V. Rao, K. S. Poksay, S. Castro-Obregon, B. Schilling, R. H. Row, G. del Rio, B. W. Gibson, H. M. Ellerby, D. E. Bredesen, J Biol.Chem. 279 (2004) 177-187.

[64] W. Bursch, Cell Death.Differ. 8 (2001) 569-581.

[65] N. E. Landvik, M. Gorria, V. M. Arlt, N. Asare, A. Solhaug, D. Lagadic-Gossmann, J. A. Holme, Toxicology 231 (2007) 159-174.

[66] A. Solhaug, M. Refsnes, M. Lag, P. E. Schwarze, T. Husoy, J. A. Holme, Carcinogenesis 25 (2004) 809-819.

[67] C. B. Ko, S. J. Kim, C. Park, B. R. Kim, C. H. Shin, S. Choi, S. Y. Chung, J. H. Noh, J. H. Jeun, N. S. Kim, R. Park, Toxicology 199 (2004) 35-46.

[68] P. Gerde, B. A. Muggenburg, G. G. Scott, J. L. Lewis, K. H. Pyon, A. R. Dahl, Carcinogenesis 19 (1998) 493-500.

[69] T. J. Buckley P. J. Lioy, Br J Ind.Med 49 (1992) 113-124.

[70] J. G. Van Rooij, E. M. Van Lieshout, M. M. Bodelier-Bade, F. J. Jongeneelen, Scand J Work Environ.Health 19 (1993) 200-207.

[71] R. Gardner, S. Cronin, B. Leader, J. Rine, R. Hampton, Mol.Biol.Cell 9 (1998) 2611-2626.

[72] A. C. Johansson, L. Norberg-Spaak, K. Roberg, Acta Otolaryngol. 126 (2006) 70-81.

[73] C. P. Lawrence, A. Kadioglu, A. L. Yang, W. R. Coward, S. C. Chow, J Immunol 177 (2006) 3827-3836.

[74] K. Matsuura, M. Wakasugi, K. Yamashita, T. Matsunaga, J Biol.Chem. (2008) .

[75] K. Y. Kim H. G. Cheon, J Biol.Chem. 281 (2006) 13503-13512.

[76] N. T. Hoa, J. G. Zhang, C. L. Delgado, M. P. Myers, L. L. Callahan, G. Vandeusen, P. M. Schiltz, H. T. Wepsic, M. R. Jadus, Lab.Invest. 87 (2007) 115-129.

[77] S. J. Harper N. Wilkie, Expert Opin.Ther Targets. 7 (2003) 187-200.

[78] K. Ask, S. Dijols, C. Giroud, L. Casse, Y. M. Frapart, M. A. Sari, K. S. Kim, D. J. Stuehr, D. Mansuy, P. Camus, J. L. Boucher, Chem.Res.Toxicol. 16 (2003) 1547-1554.

[79] T. Shimada, Drug Metab.Pharmacokinet. 21 (2006) 257-276.

[80] J. Y. Ma J. K. Ma, J Environ.Sci.Health C Environ.Carcinog.Ecotoxicol.Rev 20 (2002) 117-147.

[81] K. Saito, T. Kamataki, R. Kato, Cancer Res. 44 (1984) 3169-3173.

[82] V. M. Arlt, Mutagenesis 20 (2005) 399-410.

[83] F. A. Beland, Res.Rep.Health Eff Inst. (1991) 1-22.

[84] Y. H. Chae, P. Upadhyaya, B. Y. Ji, P. P. Fu, K. El Bayoumy, Mutat.Res. 376 (1997) 21-28.

[85] H. S. Rosenkranz P. C. Howard, Dev.Toxicol.Environ.Sci. 13 (1986) 141-168.

[86] K. Saito, S. Mita, T. Kamataki, R. Kato, Cancer Lett. 24 (1984) 121-127.

[87] H. C. Pitot Y. P. Dragan, Prog.Clin.Biol.Res. 391 (1995) 21-38.

[88] F. A. Beland M. M. Marques, IARC Sci.Publ. (1994) 229-244.

[89] H. Yamazaki, N. Hatanaka, R. Kizu, K. Hayakawa, N. Shimada, F. P. Guengerich, M. Nakajima, T. Yokoi, Mutat.Res. 472 (2000) 129-138.

[90] S. Shibutani, M. Takeshita, A. P. Grollman, Nature 349 (1991) 431-434.

[91] N. Bidere A. Senik, Apoptosis 6 (2001) 371-375.

[92] J. Fombonne, L. Padron, A. Enjalbert, S. Krantic, A. Torriglia, Apoptosis 11 (2006) 367-375.

[93] J. Fombonne, S. Reix, R. Rasolonjanahary, E. Danty, S. Thirion, G. Laforge-Anglade, O. Bosler, P. Mehlen, A. Enjalbert, S. Krantic, Mol.Biol.Cell 15 (2004) 4938-4948.

[94] T. Kondo, T. Yokokura, S. Nagata, Proc.Natl.Acad.Sci.U S A 94 (1997) 11951-11956.

[95] J. R. Ortaldo, R. T. Winkler-Pickett, S. Nagata, C. F. Ware, J Leukoc.Biol. 61 (1997) 209-215.

[96] C. A. Belmokhtar, A. Torriglia, M. F. Counis, Y. Courtois, A. Jacquemin-Sablon, E. Segal-Bendirdjian, Exp.Cell Res. 254 (2000) 99-109.

[97] R. A. Lockshin Z. Zakeri, Nat.Rev Mol.Cell Biol. 2 (2001) 545-550.

[98] R. A. Lockshin Z. Zakeri, Curr Opin.Cell Biol. 14 (2002) 727-733.

[99] R. A. Lockshin Z. Zakeri, Oncogene. 23 (2004) 2766-2773.

[100] N. Festjens, T. Vanden Berghe, S. Cornelis, P. Vandenabeele, Cell Death.Differ. 14 (2007) 400-410.

[101] X. Tang R. Edenharder, Food Chem.Toxicol. 35 (1997) 373-378.

[102] M. Gorria, X. Tekpli, M. Rissel, O. Sergent, L. Huc, N. Landvik, O. Fardel, M. T. Dimanche-Boitrel, J. A. Holme, D. Lagadic-Gossmann, Toxicol.Appl.Pharmacol. 228 (2008) 212-224.

[103] M. Leist M. Jaattela, Cell Death.Differ. 8 (2001) 324-326.

[104] N. Bidere, H. K. Lorenzo, S. Carmona, M. Laforge, F. Harper, C. Dumont, A. Senik, J Biol.Chem. 278 (2003) 31401-31411.

[105] B. W. Zanke, C. Lee, S. Arab, I. F. Tannock, Cancer Res. 58 (1998) 2801-2808.

[106] R. V. Rao, E. Hermel, S. Castro-Obregon, G. del Rio, L. M. Ellerby, H. M. Ellerby, D. E. Bredesen, J Biol.Chem. 276 (2001) 33869-33874.

[107] M. A. O'Donnell, D. Legarda-Addison, P. Skountzos, W. C. Yeh, A. T. Ting, Curr Biol. 17 (2007) 418-424.

[108] E. Meylan J. Tschopp, Trends Biochem.Sci. 30 (2005) 151-159.

[109] Y. Lin, A. Devin, Y. Rodriguez, Z. G. Liu, Genes Dev. 13 (1999) 2514-2526.

[110] N. Holler, R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J. L. Bodmer, P. Schneider, B. Seed, J. Tschopp, Nat.Immunol 1 (2000) 489-495.

[111] S. Subramaniam, U. Zirrgiebel, H. O. Bohlen Und, J. Strelau, C. Laliberte, D. R. Kaplan, K. Unsicker, J Cell Biol. 165 (2004) 357-369.

[112] A. J. Williamson, B. C. Dibling, J. R. Boyne, P. Selby, S. A. Burchill, J Biol.Chem. 279 (2004) 47912-47928.

[113] Y. Liu, E. G. Shepherd, L. D. Nelin, Nat.Rev Immunol 7 (2007) 202-212.

[114] S. Tanabe, C. Bodet, D. Grenier, J Endotoxin.Res. 13 (2007) 219-226.

[115] R. A. Lockshin Z. Zakeri, Int J Biochem.Cell Biol. 36 (2004) 2405-2419.

[116] D. Tang, H. Okada, J. Ruland, L. Liu, V. Stambolic, T. W. Mak, A. J. Ingram, J Biol.Chem. 276 (2001) 30461-30466.

[117] Z. Andrysik, M. Machala, K. Chramostova, J. Hofmanova, A. Kozubik, J. Vondracek, Toxicol.Appl.Pharmacol. 211 (2006) 198-208.

[118] G. R. Dubyak, Adv.Physiol.Educ. 28 (2004) 143-154.

[119] L. F. Diaz, M. Chiong, A. F. Quest, S. Lavandero, A. Stutzin, Cell Death.Differ. 12 (2005) 1449-1456.

[120] M. G. Gutierrez, S. S. Master, S. B. Singh, G. A. Taylor, M. I. Colombo, V. Deretic, Cell 119 (2004) 753-766.

[121] L. A. Kubasiak, O. M. Hernandez, N. H. Bishopric, K. A. Webster, Proc.Natl.Acad.Sci.U S A 99 (2002) 12825-12830.

[122] Z. Hruban, Environ.Health Perspect. 55 (1984) 53-76.

[123] R. Lullmann-Rauch, Front.Biol. 48 (1979) 49-130.

[124] J. S. Prince, C. Kohen, E. Kohen, J. Jimenez, Z. Brada, Tissue Cell 25 (1993) 103-110.

[125] H. Katabuchi, S. Yih, T. Ohba, K. Matsui, K. Takahashi, M. Takeya, H. Okamura, Med Electron.Microsc. 36 (2003) 253-262.

[126] S. Yonehara, Cell Struct.Funct. 28 (2003) 1-2.

[127] X. Saelens, N. Festjens, E. Parthoens, I. Vanoverberghe, M. Kalai, F. van Kuppeveld, P. Vandenabeele, J Cell Biol. 168 (2005) 545-551.

[128] H. J. Kim, K. W. Lee, M. S. Kim, H. J. Lee, J Nutr.Biochem. 19 (2008) 459-466.

[129] Z. Andrysik, J. Vondracek, M. Machala, P. Krcmar, L. Svihalkova-Sindlerova, A. Kranz, C. Weiss, D. Faust, A. Kozubik, C. Dietrich, Mutat.Res. 615 (2007) 87-97.