Proteomic Analysis of Benzo[a]pyrene-Mediated Bladder Toxicity

Inaugural Dissertation for the Degree of Doctor of Natural Science
Dr. rer. nat.

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Nisha Verma
from Shimla, India
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Die der folgenden Arbeit zugrunde liegenden Experimente wurden in der Arbeitsgruppe ‘Toxicoproteomics’ am Institut für Hygiene und Arbeitsmedizin der Universität Duisburg–Essen, durchgeführt.

1. Gutachter: Prof. Dr. Albert. W. Rettenmeier

2. Gutachter: Prof. Dr. Markus Kaiser

Vorsitzender des Prüfungsausschusses: Prof. Dr. Dr. H. de Groot

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List of Abbreviations

1D  one-dimensional
2D  two-dimensional
2DE  two-dimensional electrophoresis
8-‘OH-dG  8-hydroxydeoxyguanosine
ACN  acetonitrile
AhR  aryl hydrocarbon receptor
AhRE  aryl hydrocarbon responsive element
AKR  aldo-keto reductase
ALDH  aldehyde dehydrogenase
amu  atomic mass unit
ANX  annexin
APS  ammoniumperoxodisulfate
ATP  adenosine triphosphate
AR  aldose reductase
ARNT  aryl hydrocarbon nuclear translocator
Asp  aspartic acid
B[a]P  benzo[a]pyrene
BPDE  benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
BPM  beats per minute
BN  blue native
BSA  bovine serum albumin
BSTFA  $N,O$-bis(trimethylsilyl)trifluoroacetamide
CaM  calmodulin
CAPS  $N$-cyclohexyl-3-aminopropanesulfonic acid
CALR  calreticulin
CAPN  calpain
CBB  Coomassie brilliant blue
CDD  charged-coupled device
cDNA  complementary deoxyribonucleic acid
CHAPS  3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA  α-cyano-4-hydroxycinnamic acid
CLIC  chloride intracellular channel protein
Cys  cysteine
CTSD  cathepsin D
CYP  cytochrome P450
DAPI  4',6-diamidino-2-phenylindole
DAVID  Database for the Annotation Visualization and Integrated Discovery
DMSO  dimethyl sulfoxide
DNA  dexyribonucleic acid
dNTP  deoxynucleotide triphosphate
dNu  deoxynucleotide
DTT  1,4-dithiothreitol
EDTA  ethylenediaminetetraacetic acid
EEF2  elongation factor 2
EGF  epidermal growth factor
EH  epoxide hydrolase
ENU  N-ethyl-N-nitrourea
ETF  electron transfer flavo protein
FAD  flavin adenine dinucleotide
FCS  fetal calf serum
GRAVY  grand average of hydropathy
G6PD  glucose-6-phosphate-1-dehydrogenase
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GC-MS  gas chromatography-mass spectrometry
Glu  glutamic acid
GSH  glutathione
HBSS  Hanks balanced salt solution
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNRNP  heterogeneous nuclear ribonucleoprotein
HSP  heat shock protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IAA</td>
<td>iodoacteamide</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KRT</td>
<td>keratin</td>
</tr>
<tr>
<td>LSM</td>
<td>laser scanning microscopy</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LMNB2</td>
<td>lamin B2</td>
</tr>
<tr>
<td>LIP</td>
<td>labile iron pool</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MFO</td>
<td>mixed-functional oxidase</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MSDB</td>
<td>Mass Spectrometry protein sequence DataBase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>MOWSE</td>
<td>molecular weight search</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>n.a.</td>
<td>not applicable</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NCBInr</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NEDA</td>
<td>naphthyldiamine dihydrochloride</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>OTM</td>
<td>olive tail moment</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PANTHER</td>
<td>Protein Analysis Through Evolutionary Relationships database</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PG</td>
<td>Phen Green(^0)</td>
</tr>
<tr>
<td>PHE</td>
<td>phenanthroline</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMF</td>
<td>peptide mass fingerprinting</td>
</tr>
<tr>
<td>PRDX</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>PTM</td>
<td>posttranslational modification</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PUBEC</td>
<td>pig urinary bladder epithelial cells</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>rcf</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAA</td>
<td>sulfanilamide</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SQ</td>
<td>semiquinone anion radical</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>STRING</td>
<td>Search Tool for the Retrieval of Interacting Genes/Proteins</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>TPT</td>
<td>translationally-controlled tumor protein</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethan</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling</td>
</tr>
<tr>
<td>2D-BN</td>
<td>two-dimensional blue native</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>Uniprot</td>
<td>universal protein</td>
</tr>
<tr>
<td>VDAC2</td>
<td>voltage-dependent anion-selective channel protein 2</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>Vh</td>
<td>volt hours</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>XPC</td>
<td>Xeroderma pigmentosum, complementation group C</td>
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</table>
List of peer-reviewed publication list

The following articles have been produced on the course of this PhD thesis at the Institute of Hygiene and Occupational Medicine, University Hospital Essen, and are partially attached to this thesis:

Published papers


Manuscripts submitted


Published Abstracts


1. Introduction

1.1 Benzo[a]pyrene (B[a]P)

Benzo[a]pyrene (B[a]P) is a highly persistent environmental contaminant, known for its cytotoxic, mutagenic, and carcinogenic properties [1]. B[a]P first attracted wide attention in the 1933’s when it was revealed as the compound responsible for the 19th century incidences of scrotal cancer among workers in the chimney sweep trade of London [2]. Since the discovery of its carcinogenic nature and because of its ability to accumulate in various organs it has become a model compound for many toxicological investigations.

Belonging to the large family of polycyclic aromatic hydrocarbons (PAHs) [3], B[a]P (Figure 1) is listed among the group of chemicals known as particulate polycyclic organic matter that are registered as federal hazardous air pollutants [4]. Based on epidemiological evidence, the International Agency for Research on Cancer (IARC) has classified B[a]P-containing mixtures such as soot, mineral oils, shale-oils, and coal tars as Group 1 carcinogens in humans [5].

![Figure 1: Chemical structure of benzo[a]pyrene.](image)

B[a]P enters the environment from multiple sources including coal–processing waste products, petroleum sludge, asphalt, creosote, and tobacco smoke [6]. The long half-life of B[a]P in soil, water, air, and subsequently our food makes it a persistent contaminant that can be absorbed by the oral, inhalation and dermal routes of exposure [7-9]. Many studies have shown the accumulation of B[a]P in liver, lung, kidney, placenta, and bladder after its uptake from food or aerosols [10-18]. B[a]P that enters the bloodstream is believed to be transported by chylomicrons and lipoproteins [19].
Once inside the body, B[a]P requires activation to reactive primary and secondary metabolites both for mediating or protection against its toxic effects. In particular, its carcinogenic effect is induced by these metabolites that ultimately interact with critical cellular constituents such as DNA. These ultimate carcinogens are usually electrophilic intermediates that attack the nucleophilic sites of vital macromolecules in the cells, thus initiating the process of carcinogenesis. The metabolic activation of B[a]P to DNA-binding carcinogens is catalyzed by cytochrome P450 (CYP). Moreover, once inside the cytosol, B[a]P also initiates the induction of genes for CYP via a ligand-receptor mechanism [20]. The aryl hydrocarbon receptor (AhR) is a cytosolic receptor involved in the transcriptional regulation of drug metabolizing enzymes for which xenobiotics such as 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) and B[a]P act as ligands [21]. B[a]P binding to AhR induces the translocation of the AhR-B[a]P complex into the nucleus, where it interacts with the aryl hydrocarbon nuclear translocator (ARNT) forming the AhR-ARNT heterodimer. This heterodimer then binds with aryl hydrocarbon response elements (AhRE) in the regulatory regions of CYP and forms a positive feedback regulatory loop that sustains the metabolism of B[a]P [22-25] (Figure 2).

**Figure 2:** Pictorial representation of the aryl hydrocarbon receptor (AhR). AhR ( ), a cytosolic receptor protein, is present in the cytoplasm as a heterodimer complex ( ) in an inactive form. B[a]P a ligand to AhR, triggers the “transformation” of the receptor into a DNA-binding protein ( ). The B[a]P-AhR complex translocates into the nucleus and dimerizes with its nuclear partner protein, ARNT ( ). The ligand-AhR-ARNT complex binds specific DNA sequences known as aryl hydrocarbon responsive elements (AhREs) located in the 5’-flanking region of target genes. This provides a platform for recruiting multiple co-activator proteins that increase or decrease gene transcription. Figure adapted from Denison et al., [26].
## 1.2 Metabolism of B[a]P

B[a]P is metabolized to approximately 20 primary and secondary oxidative metabolites and to a variety of conjugates by the activity of phase I and phase II enzymes (Table 1).

**Table 1:** Primary and secondary metabolites of B[a]P.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Types</th>
<th>Enzymes involved</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epoxides</strong></td>
<td>1,2-epoxide</td>
<td>mixed-function oxidases</td>
<td>[27-29]</td>
</tr>
<tr>
<td></td>
<td>2,3-epoxide</td>
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<td>H₂O</td>
<td>[54, 56]</td>
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<td><strong>Tetrols</strong></td>
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<td>non-enzymatic conversion + NADPH/NADH⁺</td>
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<td>triol (7,9,8)</td>
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These enzymes are the initial biological receptors for B[a]P after its ingestion, absorption, and transport into the body [57] [58]. Among all organs, liver contains most enzymes required for the bioactivation of B[a]P [59, 60]. Phase I reactions involve enzymes such as cytochrome P450 mixed-function oxidases (MFOs), epoxide reductases, and epoxide hydrolases, while phase II enzymes include conjugating enzymes such as glutathione transferases, UDP-glucuronyl transferases, and sulfotransferases (Figure 3). The first step of metabolic activation to genotoxic metabolites is initiated by endoplasmic reticulum-based CYPs that introduce oxygen to the parent compound to form the most genotoxic metabolites, the 2,3-, 4,5-, 7,8-, and 9,10-epoxides [29]. These epoxides undergo hydration in the presence of epoxide hydrolase to the corresponding 4,5-, 7,8-, and 9,10-trans-dihydrodiols. B[a]P 7,8-trans-dihydrodiol that has been reported to be carcinogenic to animal cells [56, 61] can be converted by the activity of dihydrodiol dehydrogenase to a catechol that is subsequently autoxidized to B[a]P 7,8-quinone [62].

Other than dihydrodiols, epoxides undergo non-enzymatic rearrangement (NIH shift) to form phenolic intermediates such as 1-, 3-, 6-, 7-, and 9-OH-B[a]P [63, 64]. The 3- and 6-OH phenolic isomers can also be formed by direct hydroxylation [65] and are further involved in the formation of quinones. In the presence of peroxidases or CYP, 6-OH-B[a]P undergoes one-electron oxidation to the toxic radical cation 6-oxy-B[a]P that is further oxidized to 1,6-, 3,6-, or 6,12-quinones via hydroquinone and semiquinone radicals [66, 67]. On the other hand, 3-OH-B[a]P upon incubation with heat-inactivated microsomes can be metabolized to B[a]P 3,6-quinone [66-68]. Quinones are known to cause many cytotoxic effects through the generation of reactive oxygen species (ROS) [69, 70]. Furthermore, the phenols, quinones, and dihydrodiols form conjugates with glucuronic and sulfuric acid or are hydrolyzed to triols and tetrols [32, 65] to form water-soluble intermediates that can be excreted from the body as part of the detoxification process. On the other hand, B[a]P 7,8-dihydrodiol undergoes further oxidation to form 7,8-dihydrodiol-9,10-epoxide that is considered the most important carcinogenic metabolite of B[a]P due to its ability to form adducts with DNA [53, 71].
Figure 3: Representation of steps involved in the metabolism of B[a]P to reactive intermediates by the activity of phase I and phase II enzymes. P450: cytochrome P450; EH: epoxide hydrolase; AKR: aldo-keto reductases; NIH shift: hydroxylation-induced migration. Figure taken from Verma et al., Proteomics 2012, 12, 1731–1755.
1.3 Mechanisms of B[a]P-mediated toxicity

1.3.1 DNA and protein adducts

DNA damage is the important first step in the process of cancer development. B[a]P is a strong genotoxic compound, a property attributed to the ability of some of its metabolites to form DNA adducts. The formation of these adducts results from the presence of reactive sites known as bay region [72]. The bay region is an angular ring formed by fusion of a saturated benzene ring encompassing carbons 9-12 with the active center (α-carbon) at C-10 (Figure 4). These angular rings are prone to easy oxidation or radical ion formation but immune to conjugation and detoxification [73]. B[a]P 7,8-dihydrodiol-9,10-epoxide (BPDE), a compound arising from oxidation of B[a]P 7,8-dihydrodiol at the C-10 position, preferentially forms an adduct with DNA at the N2 position of guanine [53, 71]. These epoxides are capable of undergoing ring opening to form carbonium ions, electrophiles that are highly susceptible to nucleophilic attack by macromolecules such as DNA [63].

Figure 4: Metabolic pathways of B[a]P to different configurational isomers of B[a]P-7,8-diol-9,10-epoxide and formation of DNA adducts. dNu: deoxyribonucleotide, CYP: cytochrome P450; EH: epoxide hydrolase, DNA: deoxyribonucleic acid. Figure adapted from Xue et al., [74].
The CYP enzymes and the microsomal epoxide hydrolase involved in B[a]P metabolism are highly stereoselective. BPDE itself is known to occur in four different isomers, however, (+)-anti-BPDE, derived from (-)-B[a]P 7,8-dihydriodiol has been found to possess greater biological activity than the other three isomers [75] (Figure 4). In addition, B[a]P metabolites are also nucleotide-specific. More than 90% of racemic anti-BPDE target deoxyguanosine residues [76]. However, the K region (carbon 4-5 of B[a]P) metabolite B[a]P 4,5-oxide has also been reported to form adducts with DNA [63]. Other than the 7,8-dihydriodiol-9,10-epoxide and B[a]P 4,5-oxide, 9-OH-B[a]P-4,5-oxide, an intermediate of 9-OH-B[a]P, is also capable of binding to DNA [77].

These adducts when formed cause mutations by inducing changes in the nucleotide sequences due to misincorporation of a nucleotide opposite to the damaged base during DNA replication. The most common transversions associated with B[a]P exposure that cause cancer are G → T and C → A transversions [78, 79]. In fact, most of these transversions are responsible for mutations found in tumor suppressor genes in B[a]P-induced cancers [18]. A deficient repair of these adducts increases the rate of mutations and hence favors carcinogenesis. A number of different studies have revealed the formation of these adducts upon B[a]P exposure, but the effects are dose-, species-, tissue-, and strain-specific [80-82] [83]. Other than DNA adducts, hemoglobin and serum albumin are known to form stable adducts with anti-BPDE. These protein adducts have been suggested as biomarkers of cumulative human PAH exposure (Table 2) [84, 85] because of the long lifespan of these proteins (24 days for albumin and 120 days for hemoglobin).

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Characteristics</th>
<th>Number of subjects</th>
<th>Technique used*</th>
<th>Protein adduct levels</th>
<th>References</th>
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</thead>
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<td>n.a.</td>
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<td>0.70 (0.19-1.55) fmol/mg</td>
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<tr>
<td>Hb-BPDE</td>
<td>PAHs in air ≤4 0 µg/m³</td>
<td>113</td>
<td>HPLC-FLD</td>
<td>24.3 (2.5) fmol/mg</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td>PAHs in air &gt;4 0 µg/m³</td>
<td>93</td>
<td></td>
<td>31.8 (2.3) fmol/mg</td>
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</tr>
<tr>
<td></td>
<td>1-HOP in urine ≤2 0 µg/g creatinine</td>
<td>139</td>
<td></td>
<td>27.2 (2.4) fmol/mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-HOP in urine &gt;2 0 µg/g creatinine</td>
<td>64</td>
<td></td>
<td>27.7 (2.5) fmol/mg</td>
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<tr>
<td>Albumin-BPDE</td>
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<td>23</td>
<td>GC-NCI-MS</td>
<td>0.026 ± 0.047 fmol/mg</td>
<td>[102]</td>
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<tr>
<td></td>
<td>passive smokers</td>
<td>24</td>
<td></td>
<td>0.015 ±0.040 fmol/mg</td>
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<tr>
<td></td>
<td>non-smokers</td>
<td>22</td>
<td></td>
<td>0.016. ± 0.029 fmol/mg</td>
<td></td>
</tr>
<tr>
<td>Albumin-BPDE</td>
<td>smokers</td>
<td>27</td>
<td>GC-NCI-MS</td>
<td>0.042 (0.011) fmol/mg</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td>non-smokers</td>
<td>42</td>
<td></td>
<td>0.019 (0.008) fmol/mg</td>
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<tr>
<td></td>
<td>passive smokers</td>
<td>19</td>
<td></td>
<td>0.021 (0.007) fmol/mg</td>
<td></td>
</tr>
<tr>
<td>Hb-BPDE</td>
<td>smokers</td>
<td>27</td>
<td></td>
<td>0.105 (0.020) fmol/mg</td>
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</tr>
<tr>
<td></td>
<td>non-smokers</td>
<td>42</td>
<td></td>
<td>0.083 (0.024) fmol/mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>passive smokers</td>
<td>19</td>
<td></td>
<td>0.049 (0.007) fmol/mg</td>
<td></td>
</tr>
</tbody>
</table>

* Hb: hemoglobin, BPDE: benzo[a]pyrene 7,8-dihydrodiol-9,10-epoxide, GC/EC/NCI-HRMS: gas chromatography/electron capture/negative chemical ionization high-resolution mass spectrometry, GSTM1: glutathione-S-transferase M1, HPLC: high-performance liquid chromatography, ELISA: enzyme-linked immunosorbent assay, HPLC-FLD: high-performance liquid chromatography with postcolumn fluorescence derivatization, GC-NCI-MS: gas chromatography negative chemical ionization mass spectrometry
1.3.2 Oxidative stress

It was believed for a long time that anti-diol epoxide-DNA adducts formed during B[a]P exposure are solely responsible for the mutagenic events in B[a]P-induced cancer, however, no mechanism could be provided for the formation of 8-hydroxy-dG and thymine glycol from diol epoxide. Soon it was realized that the cells were capable of converting B[a]P to redox-active o-quinones by the activity of dihydrodiol dehydrogenase and peroxidase \([62, 103]\) (Figure 5). It was observed that these enzymes compete with CYPs for trans-dihydrodiol proximate carcinogens \([104, 105]\). In the presence of dihydrodiol dehydrogenase, diols undergo NADP\(^+\)-dependent oxidation to form ketols that further undergo spontaneous rearrangement to catechols \([62]\).

**Figure 5**: Generation of reactive oxygen species by B[a]P via o-quinone. Figure adapted from Xue et al., \([74]\).
Figure 6: Generation of covalent and oxidative DNA adducts with o-quinones. (A, B) represent stable and depurinated adducts. (C, D) represent oxidative-DNA lesions. Figure adapted from Penning et al.,[106].

The catechols thus formed are itself unstable and undergo autooxidation in air. The first one-electron oxidation results in the formation of an o-semiquinone anion radical (SQ) and hydrogen peroxide. The second one-electron oxidation produces the fully oxidized o-quinone and superoxide anion (O2•−) [36]. The resulting o-quinones are highly reactive and are capable of forming both stable and depurinated DNA adducts (Figure 6) [107, 108]. These adducts have the potential to give rise to the G → T transversions as observed in ras and p53 gene mutations. Also, the o-quinone, by undergoing a two-electron non-enzymatic reduction, is capable of reforming the catechol or, by a one-electron enzymatic reduction, reforms the SQ. These events establish a futile redox cycle, which in turn leads to the amplification of ROS. The ROS thus generated are capable of forming 8-hydroxy-dG leading to G → T transversions [109]. Moreover, ROS generation can promote lipid peroxidation and thus the generation of reactive mutagens [110]. Likewise, ROS generation can promote the production of mitogens and the activation of protein kinase C to enhance tumor promotion.
1.3.3 Disturbance of signal transduction pathways

Other than causing DNA damage, B[a]P is also known to interfere with signal transduction pathways, especially those involving calcium. Calcium, a universal second messenger, is involved in the regulation of a wide variety of cellular events, such as muscle contraction, gene expression, neurotransmission, fertilization, motility, hormone secretion, energy metabolism, cell growth, and cell death [111]. B[a]P has been shown to elicit an early and transient increase in intracellular calcium concentration (Ca$^{2+}$) [112]. It has been proposed that the rise in (Ca$^{2+}$) concentration is necessary for the AhR-mediated up-regulation of genes such as CYP or proinflammatory chemokines [113]. Other than that, it also affects cell proliferation and regulation by its profound effects on protein kinase C (PKC) [114]. PKC consists of a group of enzymes involved in controlling the functional activities of other proteins through phosphorylation of the hydroxyl groups of serine and threonine residues on these proteins [115]. B[a]P has been reported to inhibit these enzymes in a time- and concentration-dependent manner [116]. B[a]P is also known to interfere with epidermal growth factor (EGF) signaling cascades [117]. EGF is a membrane-bound tyrosine kinase receptor that primarily activates the Ras-MAPK signaling pathway following receptor autophosphorylation [118]. B[a]P is known to decrease EGF binding and hence affects proliferation and growth in placental cells and mouse fibroblasts [119, 120].

1.4 Objective

As one of the most frequently diagnosed urologic malignancies bladder cancer accounts for approximately ninety percent of cancers of the urinary collecting system (renal pelvis, ureters, bladder, and urethra). Depending upon its occurrence, bladder cancer is further divided into subcategories, such as transitional cell carcinomas, squamous cell carcinomas, adenocarcinomas, small cell carcinomas, and leiomyomas (Pauli et al. 1983). More than 90 % of all bladder cancers are transitional cell carcinomas arising from the cells lining the inside of the hollow organ (uroepithelium), roughly 10-20 % proliferate to the layers beyond the epithelium, thus impairing the prognosis [121]. Although many specific agents have been identified as causal factors of bladder cancer, epidemiological studies indicate that occupational and environmental chemicals are significant determinants in many of the cancer incidences. Cigarette smoking is one of the main known contributors to the development of urinary bladder cancer [122, 123]. Among the many components of cigarette smoke, B[a]P that occurs in amounts of twenty to forty nanogram per cigarette is among the best studied of these compounds, particularly because of its mutagenic and carcinogenic properties [124].
However, epidemiological analyses have not yet documented B[a]P or any other PAH as significant candidates for initiating bladder cancer development. Therefore, the risk that exposure to any of these compounds causes bladder cancer is still uncertain [121, 125, 126].

Thus, the overall objective of this thesis was to investigate the B[a]P-induced effects at the protein level in primary urinary bladder epithelial at non-toxic doses, in an effort to identify proteins and pathways involved in the cellular response to this potential bladder carcinogen.

For the studies primary urinary bladder epithelial cells (PUBEC) from pigs were chosen. These animals share many anatomical and physiological similarities with humans (Table 3) and have been one of the earliest animals used for research purposes.

Table 3: An overview on similarities between pigs and humans. Table taken from Verma et al., Proteomics 2010, 11, 776–779.

<table>
<thead>
<tr>
<th>System</th>
<th>Comparative Anatomy*</th>
<th>Model system</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><strong>Cardiovascular</strong></td>
<td></td>
<td>coronary artery distribution</td>
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<tr>
<td></td>
<td></td>
<td>blood pressure higher in pigs:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>145 - 160/105 mm Hg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>heart rate higher in pigs:</td>
<td>[128]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 - 150 BPM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cardiac output</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pulmonary pressure higher in pigs</td>
<td>[129, 130]</td>
</tr>
<tr>
<td></td>
<td>torus pyloricus</td>
<td>peptic ulcers</td>
<td>[133]</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>branching of mesenteric vesse</td>
<td>intestinal transplant granulomatous enteritis (Crohn's disease)</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td>spiral colon (ascending colon)</td>
<td>cystic fibrosis</td>
<td>[135]</td>
</tr>
<tr>
<td></td>
<td>submucosal glands</td>
<td>chronic bronchitis</td>
<td>[137]</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>striking similarities of glycoprotein composition of submucosal glands</td>
<td>[138]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lung size</td>
<td>cystic fibrosis</td>
<td></td>
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<td>lung functional capacity</td>
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<td>Renal</td>
<td>cranial left kidney</td>
<td>embryonal nephroma (Wilms' tumor)</td>
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<td></td>
<td>urine/plasma osmolar ratio</td>
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<tr>
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<td>von Willebrand's disease</td>
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<td>immunologically mature at 6 months</td>
<td>porcine anaphylaxis</td>
<td>[141]</td>
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<td>major histocompatibility complex (MHC)</td>
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<td>[142]</td>
</tr>
<tr>
<td></td>
<td>clotting mechanism</td>
<td>malignant lymphoma</td>
<td>[143]</td>
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However, factors such as size, cost, ethical, and societal implications often limit the use of this model organism. One cost-effective alternative for this limitation can be the use of organs from slaughtered pigs. Organs from slaughtered pigs represent an unlimited, reliable and inexpensive resource of viable cell material for all fields of applied research. The method for isolating primary bladder epithelia cells for our toxicoproteomic studies was adopted from Guhe et al. [158]. It was applied to achieve the following specific aims:

- To investigate the dynamics of B[a]P uptake and subcellular distribution in primary porcine urinary bladder epithelial cells (PUBEC) by using confocal laser scanning microscopy

- To quantify B[a]P and its metabolites in these cells by spectrofluorometry and gas chromatography-mass spectrometry (GC-MS)

- To establish proteome and phosphoproteome reference maps of PUBEC

- To investigate B[a]P toxicity at the protein level in PUBEC

- To analyze and compare protein complexes as downstream targets of AhR signaling in human bladder epithelial cells (RT4 cell line) exposed to B[a]P and TCDD

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<table>
<thead>
<tr>
<th>System</th>
<th>Comparative Anatomy*</th>
<th>Model system</th>
<th>References</th>
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<tr>
<td>Endocrine</td>
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<td>total pancreatectomy for type I diabetes</td>
<td>[145]</td>
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<tr>
<td></td>
<td></td>
<td>type II diabetes</td>
<td>[147]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GM2 gangliosidosis</td>
<td>[148]</td>
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<tr>
<td></td>
<td></td>
<td>malignant hyperthermia</td>
<td>[149]</td>
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<tr>
<td></td>
<td></td>
<td>obesity</td>
<td>[150, 151]</td>
</tr>
<tr>
<td>Liver</td>
<td>blood supply</td>
<td>liver transplantation</td>
<td>[152]</td>
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<td>metabolic function</td>
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<td>cryptorchidism</td>
<td>[41]</td>
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<td>size of Fallopian tubes</td>
<td>maternal-fetal interaction</td>
<td>[154]</td>
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<td></td>
<td>fetal surgery</td>
<td>[156]</td>
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<tr>
<td></td>
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<td>embryo development</td>
<td>[156, 157]</td>
</tr>
</tbody>
</table>

* Similar anatomy and physiology in pigs and humans unless otherwise specified.
2. Material and Methods

2.1 Cell Culture

2.1.1 Pig urinary bladder epithelial cells (PUBEC)

Primary cultures of porcine urinary bladder epithelial cells were used for the experiments to minimize the heterogeneity of the cellular responses observed when using cell lines. Porcine urinary bladders were obtained immediately after slaughter from a local slaughterhouse. For the isolation of the cells, the method described by Guhe and Föllmann [158] was used with few modifications. For aseptic transfer bladders from slaughtered pigs were transferred in icecold phosphate-buffered saline solution, supplemented with 100 pg/mL streptomycin and 100 U/mL penicillin. Cells were isolated by scraping the inner wall of individual bladders with sterile glass slides. After washing the cells three times with PBS, cells derived from different bladders were pooled and resuspended in serum-free culture medium F-12, supplemented with 146 mg/mL glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.25 µL/amphotericin B, 5 µg/mL transferrin, 10 µg/mL insulin, 0.1 mM non-essential amino acids, 2.7 mg/mL glucose, 1 µg/mL hydrocortisone, and 20 ng/mL epidermal growth factor.

Before culturing the cells, cell number and vitality were determined by the trypan blue exclusion assay. Trypan blue, a vital dye, is negatively charged and thus does not react with the cells, unless the cells are damaged. Therefore, the viable cells exclude the dye, and the dead cells are stained blue. For the assay, 20 µL of cell suspension were mixed with 500 μL of trypan blue solution plus 480 μL of PBS. The solution was thoroughly mixed, and 20 µL of the mixture were used for cell counting by using a hemocytometer plate. Cell numbers within four squares (1 mm²) were recorded. The total number was divided by four and multiplied by 50 (because of the dilution with trypan blue), then multiplied by 10,000 to obtain the number of cells per milliliter. The cell viability was calculated by dividing the total number of viable cells by the total number of viable plus dead cells and multiplying the result by 100. In all cell experiments, starting cultures contained at least 95 % viable cells. The volume of cell suspension corresponding to the number of required cells was calculated and added together with warm F12 medium and cultured in collagen-coated culture flasks for 72 h in a humidified incubator in an atmosphere of 95 % air and 5 % CO₂ at 37 °C before further use.
2.1.2 Evaluation of cell morphology and purity of PUBEC

Cell morphology of isolated epithelial cells was evaluated by using microscopic images. For the experiment 20,000 cells/chamber were seeded onto eight-well chamber slides (BD Falcon, Heidelberg, Germany). After five days of culture, the cells in the chamber slides were taken to prepare microscopic images by using a Leica microscope attached to a charged-coupled device (CDD) camera and the Leica application suite version 3.5.0 software (Leica Microsystems CMS GmbH, Switzerland). Cell purity was evaluated by immunostaining of the cells with the epithelial marker MCA1907T (AbD Serotec, Kidlington, Oxford, UK). MCA1907T is a pan cytokeratin reagent consisting of a cocktail of clone AE1 and clone AE3 that provides the broadest spectrum of reactivity to the 19 known human epidermal keratins and is known to produce positive staining in virtually all epithelial cells. For the analysis, the cells were fixed with icecold ethanol for 15 min at room temperature (RT). The cells were then washed 3 times with PBS (5 min each) and blocked by using 2 % BSA for 30 min at RT in a humidified chamber. After blocking, the cells were again washed three times with PBS for 5 min each. The cells were then incubated with 50 µL mouse anti-keratin (2 µg/mL/chamber) for 45 min at RT under humidified conditions. After the first incubation with the antibody the cells were again washed with PBS and incubated with the second antibody (50 µL/chamber of a 1:50 diluted solution of rabbit antimouse IgG conjugated with Texas red) for another 45 min in a humidified chamber at RT, followed by three times washing with PBS and incubation for 1 min with 4',6-diamidino-2-phenylindole (DAPI) in the dark. The cells were again washed with PBS and placed on a glass slide with mounting medium for observation under the fluorescence microscope (DAPI: absorption at 358 nm, emission at 461 nm; Texas red: absorption at 589 nm, emission at 615 nm).

2.1.3 RT4 cell line

Cells of the human bladder urinary epithelial cell line RT4 were cultured in McCoy’s 5A medium, supplemented with 10 % fetal bovine serum, 0.74 % L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator in an atmosphere of 95 % air and 5 % CO₂ at 37 °C.
2.2 Cell exposure

Shortly before starting cell exposure, cells were checked under the light microscope for normal growth, cell shape, and absence of contaminations. The medium in the flasks were then discarded, and the cells were washed once with warm PBS (37 °C). For cell exposure B[a]P or 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD: used for comparative studies, see chapter 6) were dissolved in dimethyl sulfoxide (DMSO) and added to the fresh medium. Then, the cells were allowed to grow in this medium for another 24 h in a humidified incubator in an atmosphere of 95 % air and 5 % CO\(_2\) at 37 °C.

2.3 Uptake and metabolism

2.3.1 Determination of the time course of B[a]P uptake and subcellular distribution by confocal laser-scanning microscopy

For fluorescence measurements 1.5x10\(^6\) cells/well were seeded onto collagen-coated glass coverslips (6.15 cm\(^2\)) in six-well plates. After three days of culture, cells were exposed to 0.5 µM B[a]P dissolved in DMSO (<0.1 % of final volume) for different time intervals (2, 6, 12, 18, and 24 h). The same percentage of DMSO was used to expose controls in all experiments. At the time of measurement, pig epithelial cells were covered with 0.02 M HEPES-buffered H12 medium. B[a]P uptake and distribution were monitored with a laser-scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an argon laser. The objective lens was a 63 × NA 1.25 plan-neofluar. The pinhole was set at 136 µm, producing confocal optical slices of about 1.0 µm in thickness. Blue fluorescence of B[a]P, excited at 365 nm by using the UV laser power supply at 80 mW, was collected through a 389-470 nm long-pass filter. Three different areas from each chamber at all time periods were scanned by using the above settings. Single-cell fluorescence was determined by setting the region of interest (ROI) manually to the individual cells and for subcellular fluorescence to specific cellular compartments. Image processing and evaluation were performed by using the software of the LSM 510 imaging system. The intensity of B[a]P within the selected ROIs was evaluated and plotted over time. Experiments were carried out in triplicates with four different PUBEC pools prepared as described above.

2.3.2 Quantification of B[a]P uptake by spectrofluorometry

30x10\(^6\) cells in 50 mL culture medium were seeded in collagen-coated 175 cm\(^2\) culture flasks. The cells were exposed to B[a]P after 72 h of cell culture at concentrations of 0.5 µM and
10 µM for 24 h. Cells at confluence were washed four times with PBS, and after every step PBS used for washing was stored for further analysis. Before harvesting the cells, care was taken to remove all of the residual PBS. The cells were collected by scraping them into a 2 mL Eppendorf tube, and the total volume of the cells (200 µL) was adjusted to 2 mL with icecold medium designed to simulate the composition of the cytosol [159].

The composition of “cytosolic medium” was: 40 % FCS, 100 mM KCl, 5 mM Na₂HPO₄, 2 mM MgCl₂, the full amino acid composition contained in Eagle’s minimum essential medium (MEM; from Sigma), 6.85 mM glucose, 1.5 mM lactate, 230 mM citrate, 138 mM pyruvate, 2.99 mM inorganic phosphate, 4 mM ATP, 4.5 mM glutathione (GSH) and 2 mM ascorbate (glutathione and ascorbate solutions freshly prepared), and 10 mM imidazole buffer, pH 7.2. Cells were then homogenized by using a mixer mill with steel grinding balls (MM200, Retsch, Haan, Germany) for 5 min at maximum frequency. After homogenization, the mixture was centrifuged (10 min at 30,000 g, 4 °C), and the protein content of the supernatant was calculated by the Bradford assay. Fluorescence spectra of the homogenate supernatant (Figure 7) were recorded by using a spectrofluorometer (Varian Cary Eclipse, Varian, Palo Alto, CA).
B[a]P was quantified based on the UV excitation and fluorescence emissions at 365 and 405 nm, respectively, by using calibration curves (Figure 8) that were obtained under the same settings as for the sample measurements. The amount of B[a]P released from the cells, i.e., the intracellular B[a]P concentration, was calculated by considering a dilution factor of 10 (total cell volume of 200 µL plus 1,800 µL “cytosolic” medium). PBS used for washing the cultured cells was controlled for remaining B[a]P. A total of eight independent PUBEC pools were prepared for the experiment. Four PUBEC pools were exposed to 0.5 µM B[a]P and the other four to 10 µM B[a]P.

Figure 8: Calibration curves for the determination of intracellular B[a]P concentrations by spectrofluorometry. [A] for cells exposed to 0.5 µM B[a]P; [B] For cells exposed to 10 µM B[a]P.

2.3.3 Quantification of B[a]P uptake by gas chromatography-mass spectrometry (GC-MS)

For GC-MS analysis a 30 m Optima 5 GC column (Macherey-Nagel, Düren, Germany) coupled to a quadrupol mass spectrometer (HP 6890/5973, Agilent Technologies, Waldbronn, Germany) was used. Following preparation of the samples as described above, aliquots of 100 µL each were spiked with 10 µL of a 100 ng/mL solution of B[a]P-d12 in toluene. To extract B[a]P, 100 µL of toluene were added, and the samples were shaken for one hour at room temperature. The organic layer was separated and used for analysis. The GC settings were as follows: injection of 1 µL of the analyte in the splitless mode: injector temperature 270 °C; purge flow of 10 mL/min for 2 min: pressure 0.230 bar (23 kPa). The oven temperature was initially held at 80 °C for 10 min and then increased by 12 °C/min up to 250 °C, where it was maintained for 8 min. After that, the oven was heated by 5 °C/min to 290 °C and kept at that temperature for another 10 min. The temperature of the quadrupole
was set at 150 °C and that of the ion source at 230 °C. A calibration curve (Figure 9) was prepared by spiking a set of B[a]P standard solutions (0.5, 1, 2.5, 5, 7.5, 10, 25 µg/mL), each with 10 µL of a 100 ng/mL solution of B[a]P-d12. The mass spectrometer was operated in the single ion monitoring mode recording the molecular ions at m/z 252 and 264 for the nondeuterated and deuterated B[a]P species, respectively.

![Calibration curve](image)

**Figure 9:** Calibration curve obtained from B[a]P standards (0.5, 1, 2.5, 5, 7.5, 10, 25 µg/mL) spiked with 10 µL of a 100 ng/mL solution of B[a]P-d12. The resulting ratios between the peak areas of the nondeuterated and deuterated B[a]P (F = A_{B[a]P}/A_{B[a]P-d12}) were used for the calculations.

### 2.3.4 Identification of 3-OH-B[a]P in PUBEC by GC-MS

3-OH-B[a]P, a major hydroxylated metabolite of B[a]P, was identified in PUBEC by using the analytical procedure described above for the quantification of the parent compound. A synthetic preparation of 3-OH-B[a]P (Campro Scientific GmbH) was used as reference compound. Following extraction, 90 µL of the derivatizing reagent N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was added to 10 µL of the concentrated analyte. The solution was then heated at 80 °C for 30 min and, subsequently, the whole sample was injected into the GC-MS system for analysis. The mass spectrometer was operated in the single ion monitoring mode recording the molecular ion of the trimethylsilyl (TMS) derivative of 3-OH-B[a]P at m/z 340.
2.4 Proteomic analysis

2.4.1 2D gel electrophoresis

For 2D gel electrophoresis $30 \times 10^6$ cells were seeded in collagen-coated 175 cm$^2$ culture flasks in 50 mL culture medium. The cells were exposed to B[a]P after 72 h of cell culture at a concentration of 0.5 µM. Cells at confluence were harvested and washed three times with ice-cold washing buffer containing 10 µM Tris-HCl and 250 µM sucrose (pH 7.0). The cell pellet was lysed by adding 1 mL of lysis buffer (8 M urea, 2 M thiourea, 2 % CHAPS, 1 % DTT, 0.8 % ampholyte, protease inhibitor cocktail). After ultracentrifugation at 30,000 rpm for 60 min at 4 °C, the supernatant was used for 2D gel electrophoresis. The protein concentration was determined by the Bradford assay. The separation of the proteins was carried out with a GE Health Care IPGphor IEF and an Ettan DALTsix electrophoresis system. 500 µg of whole cell protein was mixed with 450 µL of rehydration buffer (8 M urea, 10 % glycerol, 0.5 % CHAPS, 0.5 % ampholyte, and 0.002 % bromophenol blue). The rehydration step was performed with a precast 24 cm immobilized pH gradient (IPG) strip for 2 h, with subsequent active rehydration for 12 h at a voltage of 30 V. Isoelectric focusing (IEF) was run following a stepwise voltage increase procedure: After starting with 500 V and 1000 V for 1 h each, the voltage was linearly increased to 8000 V over a period of 8 h 20 min. After IEF, the IPG strips were subjected to a two-step equilibration in respective buffers (6 M urea, 30 % glycerol, 2 % SDS, 0.002 % bromophenol blue, and 50 mM Tris/HCl; pH 8) with 1 % DTT (w/v) for the first step, and 2.5 % iodoacetamide (w/v) for the second step. The separation in the second dimension was performed by using 1 mm thick 12.5 % polyacrylamide gels (35 x 45 cm). Electrophoresis was carried out overnight with running conditions of 2 W/gel for 14-16 h.

2.4.2 Precipitation of phosphoproteins

The whole cell lysates prepared for 2D gel electrophoresis were used for precipitation of pig phosphoproteins with a method developed in our laboratory [160]. The precipitation of phosphoproteins was carried out by the addition of 3 µL LaCl$_3$ (1 M) to 1 mg/mL protein sample and 2 min of vortexing, followed by the addition of 3 µL KH$_2$PO$_4$ (2 M). The mixture was vortexed and centrifuged at 2500 rcf at 4 °C for 1 min. The supernatant was decanted, and the pellet was resuspended in 300 µL of a solution of 8 M urea and 1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), followed by centrifugation for 30 s at 2500 rcf. The pellet was washed three times with 300 µL mili-Q water to
remove urea and CHAPS. Finally, a mixture of 25%/75 % 4M imidazole/lysis buffer mixture was used to solubilize the pellet, and the supernatant containing the phosphoproteins was collected by centrifugation for 10 min at 18,000 rcf. The protein concentration was estimated, and 100 µg were used for 2DE gel separation analysis by applying to a 7 cm IPG strip in a stepwise procedure with increasing voltage: S1: step-n-hold 50 V, 50 Vhr; S2: gradient 150 V, 150 Vhr; S3: step-n-hold 300 V, 300 Vhr; S4: gradient 1000 V, 325 Vhr; S5: gradient 5000 V, 4500 Vhr; S6: step-n-hold 5000 V, 2500 Vhr. After IEF, the IPG strips were subjected to a two-step equilibration as described above. The separation in the second dimension was performed by using 1 mm thick 12 % polyacrylamide gels (10 cm x 10 cm) for 2 h.

2.4.3 Coomassie brilliant blue (CBB) staining of gels

Gels were stained according to the Coomassie staining protocol developed in our laboratory [161]. The staining solution was prepared by mixing 5 % aluminium sulfate 14–18 hydrate, 10 % ethanol, 0.02 % of CBB G-250, and 8 % phosphoric acid. The gels were stained for 3 h followed by destaining for 30 min in a solution containing 2 % phosphoric acid and 10 % ethanol. Gels were further allowed to destain in water overnight before being ready for further processing.

2.4.4 Image acquisition and analysis

The stained gels were scanned by using a ScanMaker 9800XL instrument (Microtek International, Inc., Willich, Germany). Spot detection, quantification (rel. % volume), and pattern matching were performed by using the Delta2D v4.0 software (Decodon, Greifswald, Germany). The background was removed from each gel, and image spots were matched, automatically detected, and then manually edited. The total density of each gel image was used to normalize the individual spot volumes to minimize variations between each gel. Each spot volume was normalized as relative percentage of the total volume of all spots present in a gel. To identify the protein spots with a consistent expression in the groups, only spots matching in at least 60 % of the gel images in a group were considered.

2.4.5 In-gel enzymatic digestion

Protein spots of interest were excised, and the gel pieces were washed and dehydrated by incubating them in 50 µL of pure acetonitrile (ACN, 100 %) for 5 min. Following the incubation the acetonitrile was removed, and the spots were digested with 30 µL of trypsin
solution (40 mM ammonium bicarbonate, 3% ACN, 0.1 ng/mL trypsin) at 37 °C for three hours. The digestion was stopped by the addition of 20 µL of 1% trifluoroacetic acid (TFA) and the digested peptides were purified by using C-18 Zip tips according to the manufacturer’s protocol. The first step involved the activation of Zip tips with pure acetonitrile (two times), followed by two times washing with 0.1% TFA solution. Finally, the samples were loaded onto the C18 column by pipetting the samples 7-10 times into these tips. The C18 tips with absorbed peptides were then washed twice with 0.1% TFA solution, before the purified peptides were eluted with matrix solution (10 mg/mL α-cyano-4-hydroxy-cinnamic acid (CHCA) in 70% ACN, 7% water (Milli Q), and 0.3% TFA) onto a MALDI target. The peptides were then allowed to dry for another 5-10 min before their analysis by MALDI-TOF-MS.

2.4.6 MALDI-TOF-MS analysis and protein identification

The protein analysis was performed on a Voyager-DE™ STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.). Trypsin-digested samples were placed on a Mass-Spec-Turbo 192 CHCA Chip (Qiagen, Hilden, Germany). The mass spectrometer was operated in the positive-ion, delayed-extraction (200 ns delay time) reflector mode. The identification of the proteins was performed by peptide mass fingerprinting (PMF) using the Mass Spectrometry protein sequence DataBase (MSDB) and the National Center for Biotechnology Information (NCBInr) protein databases with the special search engine Mascot (http://www.matrixscience.com). Raw data of the peptide masses were queried to the theoretical peptide mass of the entire database of MSDB and NCBInr. The criteria for searching were set with assumptions that the peptides are monoisotopic, oxidized at methionine, and carboxyamidomethylated at cysteine residues. Only one missed trypsin cleavage and a peptide mass tolerance of 100 ppm was allowed for each peptide fragment. For the studies with pig epithelia cells the taxonomy was set to pigs, while for the studies with the RT4 cell line the taxonomy was set to humans. Probability-based molecular weight search (MOWSE) scores were estimated by comparison of the search result against an estimated random match population and was reported as -10*LOG₁₀(P), where P is the absolute probability. Scores greater than 65 were considered statistically significant (p <0.05).

2.4.7 TdT-mediated dUTP-X nick end labeling (TUNEL) assay

Apoptosis, also known as programmed cell death, is a process involving a series of biochemical events leading to specific cell morphology characteristics and ultimately to the
death of cells. One of the peculiar characteristics of late stage apoptosis is the fragmentation of nuclear chromatin, which results in a multitude of 3’-hydroxyl termini of DNA ends. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) is a method that involves the identification of apoptotic cells by labeling the DNA breaks (3’-hydroxyl termini of DNA ends) with fluorescent-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates (dNTP) to the 3’-hydroxyl ends of double- or single-stranded DNA which generates DNA strands with exposed 3’-hydroxyl ends. The apoptotic cells can then be separated from non-apoptotic cells, as these cells do not incorporate much of the F-dUTP because of the absence of exposed 3’-hydroxyl DNA ends.

The assay was performed by using the in situ death detection kit TMR red (Roche Diagnostics GmbH, Germany) according to the manufacturer’s instruction. Briefly, 20,000 cells/chamber were seeded onto eight-well chamber slides (BD Falcon, Heidelberg, Germany). After five days of culture, the cells were exposed to 0.5 µM B[a]P for 24 h. The exposed epithelial cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for one hour at RT. The fixed cells were then washed three times with PBS for 5 min each. After fixation, the cells were permeabilized with freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate. Cells were subsequently labeled with the TUNEL working solution. Apoptotic cells were identified as red fluorescent TUNEL-positive cells by fluorescence microscopy and are given in percentage of the total number of cells as determined by DAPI nuclear staining. The cells were treated with DNAsae as positive control. All experiments (n=6) were carried out in triplicates, and the level of statistical significance relative to control was calculated by using the one tailed t-test (p <0.05).

2.4.8 Western blot

The differentially expressed proteins, screened with 2DE, were confirmed by immune-blotting. A total of 30 µg of total protein was resolved in a 12% polyacrylamide gel. These samples were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Invitrogen GmbH, Germany). After blocking with 5% nonfat dry milk, the membranes were immunoblotted with PMSD4, HSP27, HSP70, Hnrnpa1, VDAC2, and β-actin antibodies at dilutions recommended by the suppliers. Horse radish-conjugated secondary antibodies and a chemiluminescence kit (Invitrogen GmbH, Germany) were used for detection. Protein
expression was visualized by the Versa Doc Imaging System (Bio-Rad Hercules, CA, USA). The intensity of the bands normalized to the band of β-actin was measured by using the UNSCAN-IT automated digital system version 5.1 software (Orem, USA) and given in terms of calculated quantitative fold change with respect to control.

2.4.9 Comet assay

The comet assay, also known as single-cell gel electrophoresis, is a sensitive method for measuring DNA strand breaks in eukaryotic cells. The method involves the lysis of cells embedded in agarose on a slide with a detergent and a high amount of salt, which leads to the formation of nucleoids containing supercoiled loops of DNA, linked to the nuclear matrix. The supercoiled DNA is then electrophoresed at high pH, where looped DNA becomes free and moves towards the anode in structures resembling comets. These structures are then stained and observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks.

The comet assay for the study was performed under alkaline conditions as previously reported [80]. Briefly, cells were exposed to different B[a]P concentrations (0.1 to 10 μM) for 24 h. After exposure, the cells were washed twice and detached by trypsin/ethylenediamine tetraacetic acid (EDTA) treatment for 5 min. The cells were collected by centrifugation, and 20 mL of cell suspension (8000 cells) were mixed with 45 μL of low-melting agarose and quickly pipetted into each of eight wells of a comet slide and allowed to set for 1 h at 4 °C in the dark. The slides were then immersed in prechilled lysis solution (10 mM Tris-HCl, 10 mM EDTA, 2.5 M NaCl, and 30 mM N-laurylsarcosine sodium salt, pH 10; 1 % Triton X and 10 % DMSO were added freshly) for overnight at 4 °C. Following the lysis, alkaline treatment in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, and 10 mM Trizma; pH 13) was carried out for 30 min at 4 °C, and then electrophoresis was run for another 30 min at 4 °C (300 mA, 25 V). After neutralization (400 mM Tris-HCl, pH 7.5) for 60 min, the gels were dehydrated in absolute ethanol for 2 h and stored in the dark to dry completely. Just before image analysis, gels on each slide were stained with SYBR®-Green nucleic acid stain (Invitrogen, Darmstadt, Germany) in the dark for 20 min. A coverslip was placed over the moist gel, and the gels were examined by using the comet assay IV software (Perspective Instruments, UK) and a Leica microscope attached to a CDD camera. Values of the olive tail moment (OTM) were automatically calculated by the software. As a positive control, the cells were treated with 1 mg/mL N-ethyl-N-nitrosourea (ENU). All experiments were carried out in
triplicates, and the level of statistical significance relative to control was calculated by using the t-test (p ≤0.001)

2.4.10 Detection of the mitochondrial membrane potential (MMP)

The mitochondrial potential of pig urinary epithelial cells was measured by use of the fluorescent dye Rodamine123. Rodamine123, a monovalent cationic dye, is used to monitor the mitochondrial function in living cells by its ability to distribute across the mitochondrial inner membrane according to the negative membrane potential [162]. The loss of potential due to early events during apoptosis results in a loss of the dye and, therefore, the fluorescence intensity.

For the determination of the mitochondrial potential, PUBEC at a concentration of 50,000 cells/well were plated in black 96-well plates with a clear bottom for 72 h, followed by exposure to 0.5 \( \mu \)M B[a]P for 2, 6, 12, and 24 h. Cells treated with normal cell culture medium were used as negative controls, while 100 \( \mu \)M of the ionophore valinomycin served as positive control. Following exposure, the cells were washed three times with warm PBS and exposed to 5 \( \mu \)M Rhodamine123 dye for 30 min at 37 °C. Measurements were obtained immediately at excitation and emission wavelengths of 488 and 535 nm by using the Tecan microplate reader (Tecan, Mainz, Germany).

2.5 Two-dimensional Blue Native/SDS-PAGE (2D BN/SDS-PAGE)

2.5.1 Sample preparation and subcellular fractionation of RT4 cells

For organellar enrichment ProteoExtract®, a commercially available subcellular fractionation kit (S-PEK), was applied. By using the kit control cells and cells exposed to B[a]P (0.5 \( \mu \)M) and TCDD (200 pM) were fractionated into four subcellular compartments: cytosol, membrane/organelle proteins, nuclear, and cytosolic fraction (Figure 10). Except for the cytosolic fraction, all other fractions were used for 2D BN/SDS-PAGE.

3.5x10^6 RT4 cells were seeded in 25 cm² culture flasks in 5 mL culture medium. Cells at confluence were harvested, mixed with 1 mL of cold extraction buffer 1 including protease inhibitors, and incubated at 4 °C for 10 min (all incubations were performed on an end-over-end shaker). Insoluble material was sedimented at 1000×g at 4 °C for 10 min, and the resulting supernatant, the cytosolic subproteome, was removed. Then, the whole procedure
was repeated. The pellet was mixed with 1 mL of cold extraction buffer 2 and incubated for 30 min at 4 °C. The insoluble material was sedimented at 6000×g at 4 °C for 10 min. The supernatant, the membrane/organelle subproteome, was removed, and the pellet was mixed with 500 μL of cold extraction buffer 3 including 1.5 μL benzoase to digest DNA. After 10 min of incubation, the insoluble material was sedimented at 7000×g at 4 °C for 10 min, and the supernatant, the nuclear fraction, was removed. The final fraction, the cytoskeletal subproteome, was obtained by resuspending the remaining pellet in 500 μL of extraction buffer 4. Fractions were aliquoted and stored at -80 °C until further use. The buffer composition for the subcellular fractionation, as provided in the kit, was sufficient. Therefore, no external detergent was added to these buffers. To enrich the subcellular fractions and to avoid the large carryover of proteins from one fraction to another, the enrichment step on each fraction was repeated up to three times. These enriched fractions were then used for 2D BN/SDS-PAGE.
Material and Methods

2.5.2 2D BN/SDS-PAGE

Blue Native/SDS-PAGE is a kind of native electrophoresis that helps in high-resolution separation of enzymatically active protein complexes from tissue homogenates or cell fractions. The separation principle relies on binding of CBB G250, which provides negative charges, to the surface of the protein. During migration to the anode, protein complexes are separated according to their molecular mass and/or size, and high resolution is obtained by the decreasing pore size of a polyacrylamide gradient gel. These complexes are then separated.
into the subunits by usual SDS gel electrophoresis (Figure 11). Thus, this technique allows the separation of multiprotein complexes in their native forms.

50 mg of protein sample were mixed with 5 µL of sample buffer (750 mM aminocaproic acid and 5 % (w/v) CBB G-250) and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was then separated on a 4–16 % Bis-Tris polyacrylamide native gradient gel by using the Invitrogen electrophoresis system. The outer chamber was filled with icecold anode buffer (50 mM Bis–Tris, pH 7.0) and the inner chamber with icecold blue cathode buffer (15 mM Bis–Tris, pH 7.0, 50 mM tricine, and 0.02 % CBB G-250). Electrophoresis was performed at 20 mA, 200 V and 10 W for approximately 4 h at 4 °C and stopped when the tracking line of the CBB G-250 dye had left the edge of the gel. The lanes from the first dimension were cut into individual strips, and before the separation in the second dimension, the strips were equilibrated in denaturation buffer (1 % SDS and 1 % iodoacetaamide (IAA)) for 30 min at RT and placed into a 12 % Bis–Tris polyacrylamide gel of the same thickness. The second-dimension run was performed at 150 V, 75 mA, and 5 W, until the blue front migrated out. At the end of the run, the gel was stained with Coomassie, and the spots were picked up for mass spectrometry by applying the protocol described above.
2.5.3 Determination of intracellular chelatable iron by Phen Green™ SK

Phen Green™ SK (PG SK), a metal-sensitive probe, was used for measuring the cellular labile iron content of the cells. The dye can be swiftly loaded into cells via its nonfluorescent acetomethoxy precursor PG SK-AM. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound. The PG SK-loaded cells have a fluorescence component (ΔF) that is quenched by intracellular iron and can be revealed by the addition of a chelator, e.g., phenanthroline. The rise in fluorescence is equivalent to the change in PG SK concentration or to the amount of cellular iron originally bound to PG SK. Thus, the change in PG SK fluorescence intensity is directly proportional to the labile iron pool. (Figure 12)

For the determination of chelatable iron with PG SK, the protocol described by Petrat et al. was used [159]. 2x10⁶ cells/well were seeded onto collagen-coated glass coverslips (6.15 cm²) in six-well plates. After two days of culture, the cells were exposed to 0.5 µM B[a]P or 200 pM TCDD dissolved in DMSO (<0.1 % of final volume) for 24 h. The same percentage of DMSO was used to expose controls in all experiments. The experiment was repeated four times in duplicates. The cells after exposure were washed twice with Hanks balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 0.4 mM MgSO₄, 0.3 mM Na₂HPO₄, and 25 mM Hepes, pH 7.4).
Material and Methods

Figure 12: Measurement of the labile iron pool by using the Phen Green™ SK molecular probe. (A) PG SK-AM, the acetomethoxy derivate of Phen Green™ SK, is a nonfluorescent and membrane-permeable dye. (B) Upon entry into the cells, PG SK-AM is hydrolyzed to give the fluorescent PG SK that is quenched upon binding of iron (Fe). Phenanthroline (PHE), a strong iron chelator, evokes the fluorescence dequenching by competing with PG SK-bound iron. Thus, the rise in fluorescence is equivalent to the change in PG SK concentration or to the amount of cellular iron originally bound to PG SK. Figure adapted from Kakhlon et al., [164].

The cells were loaded with PG SK (20 µM) for 10 min at 37 °C in a CO₂ incubator. After the incubation the cells were washed three times with HBSS buffer and again incubated in 5 mL HBSS buffer at 37 °C in a CO₂ incubator for another 10 min. A laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany), equipped with an argon laser and a helium/neon laser, was used to perform the fluorescence measurements. The objective lens was a 63×NA 1.25 plan-neofluar. The green fluorescence of PG SK, excited at 488 nm by using the argon laser at a power rating of 6.75 mW, was collected through a 505 nm longpass filter. Confocal images (scanning time 30 s) were collected with the power of the argon laser set at 0.7 % after 3 min of PG SK loading. After establishing baseline fluorescence, cellular chelatable iron was removed from PG SK by adding the cell-permeant iron chelator 1,10-phenanthroline (2 mM) to the supernatant, and the images were collected first after 2 min of addition and then after every 10 min for the next 50 min. Full image scans of the cells were determined. Image processing and evaluation were performed by using the software of the
LSM 510 imaging system. Experiments were carried out in triplicates with RT4 cells prepared as described above.

### 2.5.4 Determination of NO with the Griess test

Nitric oxide (NO) is a key biosignaling molecule produced by a family of enzymes known as nitric oxide synthases (NOSs). The Griess method involves the determination of nitrite and nitrate (as a measure of nitric oxide) by formation of a red azo dye upon treatment of a NO$_2^-$ containing sample with the Griess reagent (Figure 13). The reagent is an acidic solution of sulfanilamide and alpha-naphthylamine that undergoes a diazotization reaction with nitrites, forming a red azo dye.

![Figure 13: Mechanism of nitrite detection by using the Griess reagent. Figure taken from Sun et al., [165].](image)

The production of NO was determined by measuring the total nitrite/nitrate (NO$_2^-$/NO$_3^-$) concentration by means of the Griess reaction [165], using a commercially available photometric nitrate test (R-Biopharm, Darmstadt, Germany) with some modifications. 4.0x10$^6$ RT4 cells were seeded in 3 mL culture medium into a 6-well plate. Cells at confluence were exposed to 750 µL of medium containing B[a]P (0.5 and 5 µM) or TCDD
Material and Methods

(200 pM and 1 nM) for 24 h. The medium used for exposure was filtered (Vivaspin 500, MW 30,000, Sartorius Stedim Biotech, Göttingen, Germany) by centrifugation (15,000 × g, 60 min, 4 °C) subsequent to a wash step of the filters with distilled water to remove possible contaminating nitrate. 50 µL of the undiluted filtered sample was mixed with 50 µL phosphate buffer (50 mM, pH 7.4), and after adding 50 µL of a NADPH/FAD solution and 5 µL of nitrate reductase solution (both from the nitrate test kit of R-Biopharm) the mixture was incubated for 20 min at RT. Afterwards, 5 µL of sodium pyruvate solution (12 mg/mL distilled water) and 5 µL lactate dehydrogenase solution (Roche, Mannheim, Germany, diluted 1:10 with distilled water.) were added to this mixture and incubated for another 20 min at RT. Finally, 200 µL of Griess reagent (1 % sulfanilamide plus 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride, 1:1) was added to the mixture, and after 10 min of incubation the absorption was determined at 542 nm. Concentrations in micromoles were then assessed from calibration curves performed from standard solutions of NaNO₂ (10–1000 µM).

2.5.5 Determination of intracellular calcium concentration

Based upon the same principle as the Phen Green molecular probe, Fluo-4/AM and Rhod-2/AM are fluorescence indicators for calcium (Figure 14). Having an absorption spectrum that is compatible with an excitation at 488 nm by argon ion laser sources, Fluo4 exhibits a high calcium-binding affinity and selectivity and a very large increase (>100-fold) of fluorescence intensity in response to calcium binding (Kd = 345 nM). Rhod-2/AM, on the other hand, is a cationic dye with a strong affinity for mitochondria, and thus acts as a selective indicator for mitochondrial calcium. It has its fluorescence excitation and emission maxima at 552 nm and 581 nm, respectively, and upon calcium binding the fluorescence intensity has been reported to increase up to more than 100-fold (Kd= 540 nM).

To study \([\text{Ca}^{2+}]_i\) modulations by B[a]P and TCDD, two \(\text{Ca}^{2+}\)-sensitive dyes were used: Fluo-4/AM (1.4 µM) to observe changes of \([\text{Ca}^{2+}]_i\), and Rhod-2/AM (3.6 µM) to determine the changes of \(\text{Ca}^{2+}\) within the calcium stores. 2x10⁶ cells/well were seeded onto collagen-coated glass coverslips (6.15 cm²) in six-well plates. After two days of culture, cells were exposed to 0.5 µM B[a]P or 200 pm TCDD dissolved in DMSO (<0.1 % of final volume) for 24 h. The same percentage of DMSO was used to expose controls in all experiments. The experiment was repeated four times in duplicates. The cells after exposure were washed twice with HBSS (137 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 1.2 mM KHPO₄, 25 mM glucose, 0.1 mM Na₂-EDTA, 0.01% pluronic acid).
0.4 mM MgSO$_4$, 0.3 mM Na$_2$HPO$_4$ and 25 mM Hepes, pH 7.4). Afterwards, the cells were incubated with a HBSS solution containing Fluo-4/AM (1.4 µM) and Rhod-2/AM (3.6 µM) for 30 minutes at RT, followed by incubation for another 30 min at 37 °C. The dye solution was then removed and the cells were incubated for additional 30 min at 37 °C in 3 mL HBSS buffer supplemented with 2.5 mM probenecid (500 mM stock solution in 1 N NaOH).

For measurement, the coverslips were washed once with warm HBSS (37 °C) and placed in a coverslip holder. The cells were overlaid with 3 mL probenecid-supplemented HBSS buffer, and the fluorescence was measured on a confocal laser scanning microscope equipped with an argon laser and a helium/neon laser. The green fluorescence of Fluo-4/AM was excited at 488 nm by using the argon laser and was collected through a 505 nm longpass filter, whereas the red fluorescence of Rhod-2/AM, excited at 543 nm (by using the helium/neon laser), was collected through a 560 nm longpass filter. Three different areas from each chamber at all time periods were scanned by using the above settings. Full image scans of cells were determined. Image processing and evaluation were performed by using the software of the LSM 510 imaging system. All experiments were carried out in triplicates.
2.6 Bioinformatic analysis

The Protein Analysis Through Evolutionary Relationships database (PANTHER) was used to elucidate the molecular function, biological process, and signaling pathway associated with each individual protein (http://panther.appliedbiosystems.com/) [166]. The subcellular location annotation of the proteins was predicted by using the clustering program (http://david.abcc.ncifcrf.gov/) called Database for the Annotation Visualization and Integrated Discovery (DAVID) [167].
3. Exposure of PUBEC to B[a]P: In vitro uptake, intracellular concentration, and biological response†

3.1 Objective

A requirement for B[a]P to mediate its carcinogenic potential is the ability of the particular tissue to incorporate the chemical and to subsequently metabolize it to reactive intermediates. Although a basic phenomenon, the simple mechanism behind B[a]P uptake is still largely unknown. Moreover, the presence of B[a]P metabolites in urine has been used as gauge for PAH exposure in environmental toxicology for a long time, yet no information is available on the ability of bladder tissue for B[a]P uptake. Investigations in humans and in animal models suggest that the urothelium may be exposed to B[a]P both by its diffusion from the capillaries in the lamina propria and by its uptake from urine.

Therefore, the aim of the present study was to investigate the dynamics of B[a]P uptake and its subcellular distribution in bladder epithelial tissue. To analyze these processes in a cell type that closely reflects the native function within the living system, the experiments were performed with primary urinary epithelial cells from pigs (PUBEC). As an eutherian mammal, pigs share many similarities with humans. PUBEC have previously been used for many in vitro studies and are known to maintain all functions specific to bladder epithelial cells [80, 168-173].

3.1.1 Morphologies and purity of PUBEC

Isolated porcine urothelial cells maintain all morphological structures typical of the urothelium. Microscopic images were made after five days of cell culture to evaluate the morphology of isolated epithelial cells from pig bladders. The cultured cells formed a monolayer with many morphological polarities resembling the epithelium in vivo (Figure 15A). Moreover, immunostaining of isolated cells with MCA1907T, a pan cytokeratin reagent, also confirmed the epithelial characteristics of these cells (Figure 15B).

† Verma et al., Archives of Toxicology, 2012, (doi 10.1007/s00204-012-0899-y)
Figure 15: Establishment and characterization of porcine bladder epithelial cell culture: (A) Pig bladder epithelial cells were seeded onto collagen-coated plates and cultured for seven days. The cultured cells formed a monolayer and showed many morphological structures typical of urothelial cells. (B) Epithelial cell marker expression of cultured bladder epithelial cells for cell morphology and purity. (a) Positive staining shown in red; (b) DAPI was used to stain nucleus blue; (c) merged image of positively stained epithelial cells and DAPI-stained nucleus. Figure taken from Verma et al., Electrophoresis 2011, 32, 3600–3611.

3.1.2 Analysis of B[a]P uptake and its subcellular distribution

The time-dependent increase of the intracellular amount of B[a]P was monitored by using confocal laser scanning microscopy. (Figure 16) demonstrates the uptake of PUBEC expose to 0.5 μM B[a]P for different time intervals. As compared to nonexposed control cells, a time-dependent increase in the cellular fluorescence intensity of B[a]P-exposed cells was observed, however, no significant uptake was measured during the initial 2 h of exposure. Only after six hours of exposure an initial cellular uptake of B[a]P occurred, which increased linearly with time but with substantial variation among the different cell pools.
Results

Figure 16: Box plots representing the time-dependent increase in B[a]P fluorescence of different PUBEC pools exposed to 0.5 µM B[a]P. The fluorescence was monitored by using laser-scanning microscopy ($\lambda_{\text{excitation}} = 365$ nm; $\lambda_{\text{emission}} = 389-405$ nm). The boxes encompass the 25th and 75th percentiles. Horizontal bars inside the box represent the median. Whiskers extend to the highest and lowest levels that are not outliers. (•) Outliers representing 1.5 box lengths from the 25th or 75th percentile. (♦) Outliers representing 3 box lengths from the 25th or 75th percentile. Figure taken from Verma et al., Archives of Toxicology 2012.

Analysis of the subcellular distribution revealed a substantial B[a]P uptake by the plasma membrane as compared to other cellular compartments. A significant increase of cell membrane fluorescence intensity was recorded over the time period of 24 h (Figure 17A, B). In addition, a slight but significant increase in fluorescence intensity was observed in the cytosol and nucleus (Figure 17C, E).
3.1.3 Quantification of intracellular B[a]P concentration by spectrofluorometry and GC-MS

By exploiting the fluorescence properties of B[a]P, the quantification of the intracellular concentration was carried out by spectrofluorometry, and the results were further verified by GC-MS. By using an *ex situ* calibration method, the intracellular B[a]P concentration was
Results

determined spectrofluorometrically as a function of the released fluorescence in pig epithelial cells exposed to 0.5 mM or 10 µM B[a]P for 24 h. For the *ex situ* calibration B[a]P concentrations were plotted vs. their fluorescence. The emission intensity linearly depended on the B[a]P concentration. The intracellular concentration of PUBEC pools (n = 4) exposed to 0.5 µM B[a]P ranged from 7.28 µM to 35.70 µM, whereas the concentration of B[a]P in cells (four other PUBEC pools) exposed to 10 µM B[a]P ranged from 29.90 µM to 406.64 µM. At both exposure concentrations considerable variation in intracellular B[a]P accumulation was observed (Table 4).

Table 4: Spectrofluorometric quantification of B[a]P concentrations in pig urinary bladder epithelial cells. PUBEC pools were incubated with 0.5 µM (A) and 10 µM B[a]P (B) for 24 h, respectively, and then homogenized. B[a]P standards (0.1–2.5 µM) were dissolved in cytosolic medium (pH 7.2, 37 °C), designed to simulate the composition of the cytosol as described in the text. Measurements were performed by using an excitation wavelength of 365 nm and an emission wavelength of 405 nm. Figure obtained from Verma et al., *Archives of Toxicology* 2012.

<table>
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<th>Sample</th>
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<th>Intracellular concentration (µM)</th>
<th>Accumulation factor (%)</th>
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<td>PUBEC pool 12</td>
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<td>406.64</td>
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Figure 18: (A) A representative single ion chromatogram obtained from the supernatant of a PUBEC pool exposed to 0.5 µM B[a]P for 24 h. The deuterated B[a]P (—) elutes earlier than the non-deuterated compound (—). (B) GC-MS separation of the B[a]P metabolite 3-OH-B[a]P in unexposed samples (—) and cells exposed to 0.5 µM (—) and 10 µM (—) B[a]P for 24 h (retention time 37.80 min). Figure obtained from Verma et al., Archives of Toxicology 2012.
By using B[a]P d-12 as internal standard, the intracellular B[a]P concentrations of the individual PUBEC pools as obtained by spectrofluorometry were further verified by GC-MS analysis. The intracellular B[a]P concentrations were measured by means of a calibration curve prepared by measuring the B[a]P standards in the presence of a constant B[a]P-d12 amount. The deuterated and the non-deuterated B[a]P differ from each other in their elution time: B[a]P eluted after 28.87 min and B[a]P d-12 after 28.78 min (Figure 18A). The resulting ratios between the peak areas of the nondeuterated and deuterated B[a]P (F=AB[a]P/AB[a]P-d12) were used for the mathematical calculations. Thus, as observed from the experiment based on laser scanning microscopy, also these experiments point at distinct batches of PUBEC with strongly different properties in B[a]P uptake.

### 3.1.4 Formation of 3-OH-B[a]P in PUBEC

In PUBEC, the expression of CYP involved in the metabolism of B[a]P has recently been demonstrated [172]. To prove the actual formation of B[a]P metabolites in PUBEC, cells exposed to 0.5 and 10 µM B[a]P were analyzed by GC-MS. By using a commercially available standard of 3-OH-B[a]P, this major phenolic BaP intermediate could indeed be identified for the first time (Figure 18B). However, because of the unavailability of a deuterated reference compound, it could not be quantified. Nevertheless, it appeared from the peak areas of the metabolite in the exposed samples that a considerably larger quantity of 3-OH-B[a]P was formed in PUBEC exposed 10 µM B[a]P as compared to PUBEC exposed to 0.5 µM B[a]P.
4. Proteome and phosphoproteome maps of PUBEC‡

4.1 Objective

The majority of bladder cancers is transitional cell carcinomas and is thought to be caused to a significant part by the exposure to chemical carcinogens. In recent years, major advances in the culture of urinary bladder-derived cells have been achieved, and various urothelial in vitro models have been developed. Since culturing primary human tissues is still a difficult task, often because of an insufficient availability of cells required to perform large-scale studies, the culture of animal urothelial cells is used as an alternative. Beyond that, cell lines derived from transformed cells may be applied in specific investigations. With the advent of high-throughput and multiplex proteomic technologies in recent years, a variety of cell models and transformed cell lines have been utilized in an effort to achieve a better understanding of bladder biology, in particular of bladder carcinogenesis. However, information regarding the expression of proteins in normal bladder tissue is essentially missing as yet. Therefore, the aim of the present study was to develop a 2D map of proteins expressed in normal bladder cells that can be used as a reference map for studying alterations in urothelial biology.

4.2 2DE analysis and identity assignment

Inasmuch as the porcine model bears some remarkable similarities with humans, it is important to recognize that there are some differences between the two species, too, which may lead to a divergent response to a certain experimental regime. For reducing the variability and interindividual differences, pooling of PUBEC from several pigs has been suggested [171]. To reduce the effects of polymorphism as often observed in pigs, cells from a minimum of 30 pig bladders were pooled. The cells were cultured, and the extracted proteins were used for subsequent 2DE separation. The experiment was repeated eight times (n=8), and the gel triplicates of eight different protein extracts representing eight different groups were used for establishing the proteome map. The expression of more than 1000 spots with molecular masses ranging from 10 to 150 kDa and pI values ranging from 4 to 10 were observed. To determine the significantly expressed proteins in the groups, the gels were compared by using the Delta2D v4.0 software. Only protein spots with an expression variance of no more than ±1.2 in the samples (eventually 150 proteins) from different gels were further

‡ Verma et al., Electrophoresis 2011, 32, 3600–3611
considered for inclusion in the proteome map. A representative fused gel image obtained after matching eight different protein lysates is shown in (Figure 19) in which all spots identified as consistently expressed are marked by numbered arrows.

Figure 19: Representative 2DE gel image of PUBEC. 450 μg of proteins from a whole cell extract were separated and stained with Coomassie brilliant blue. Numbered spots were identified by mass spectrometry after in-gel digestion of proteins by trypsin. Figure taken from Verma et al., Electrophoresis 2011, 32, 3600–3611.

For assigning the identity of significantly expressed proteins as analyzed by MALDI-TOF-MS, protein peptide mass matching was performed on Mascot by searching the MSDB and NCBInr protein databases with the taxonomy pig. Out of the total of 150 proteins the identity could only be assigned to 61 % (92 proteins) of the protein spots analyzed (Table 5). 39 % of the spots remained unidentified, although utilizable MS data were collected. To overcome the problem of poor sequence representation for proteomic identification, the pig proteins were matched against the protein equivalents of other species such as human, rat, and mouse. By that approach the rate of identification was considerably increased, and the identity of another 28 spots could be clarified, making the total of identified proteins to 80 % (120 proteins). Twenty percent of spots remained unidentified, which might be due to common technical problems such as spot overlapping or incorrect mass identification.
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<td>-0.209</td>
<td>Sus scrofa</td>
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<tr>
<td>142</td>
<td>Proteasome subunit beta type 3</td>
<td>PDDC1</td>
<td>29.50/5.69</td>
<td>58.51/6.48</td>
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<td>0.107</td>
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<tr>
<td>143</td>
<td>Keratin, type II cytoskeletal 8</td>
<td>BAG2</td>
<td>53.70/5.52</td>
<td>58.24/6.25</td>
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<td>4</td>
<td>27</td>
<td>-0.297</td>
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<tr>
<td>145</td>
<td>Valosin-containing protein isoform 3</td>
<td>VCP</td>
<td>89.93/5.31</td>
<td>83.42/4.38</td>
<td>300</td>
<td>32</td>
<td>32</td>
<td>-0.346</td>
<td>Sus scrofa</td>
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<tr>
<td>146</td>
<td>Beta-enolase*</td>
<td>ENO3</td>
<td>47.44/8.05</td>
<td>53.54/6.88</td>
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<td>30</td>
<td>-0.209</td>
<td>Sus scrofa</td>
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<tr>
<td>147</td>
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<td>61.04/5.71</td>
<td>50.97/5.95</td>
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<td>30</td>
<td>-0.165</td>
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<tr>
<td>148</td>
<td>Lamin-A/C*</td>
<td>LMNA</td>
<td>74.40/6.73</td>
<td>76.89/6.56</td>
<td>80</td>
<td>5</td>
<td>25</td>
<td>-0.677</td>
<td>Sus scrofa</td>
</tr>
<tr>
<td>149</td>
<td>Lamin-A/C*</td>
<td>LMNA</td>
<td>74.40/6.73</td>
<td>76.89/6.63</td>
<td>188</td>
<td>24</td>
<td>40</td>
<td>-0.677</td>
<td>Sus scrofa</td>
</tr>
</tbody>
</table>

Gene name, protein name, GRAVY value, theoretical molecular weight and pI entries in Uniprot Database for each protein identified by MALDI-TOF-MS. Sequence coverage, peptide matches, score, and reference organism entries in the Mascot search engine. If multiple spots were identified as the same protein, the protein is marked with an asterisk (*)
4.3 Physicochemical properties of identified proteins

For the 120 proteins identified, physicochemical properties such as hydrophobicity (GRAVY value), pI, and molecular weight were examined. The analysis of pI distribution revealed that the pI of the identified proteins varied between 4 and 10. Proteins with theoretical pI values higher than 9.6 were not detected. The molecular mass of 70% of the identified proteins varied between 20 kDa and 80 kDa. Only one protein with a molecular mass higher than 100 kDa was identified (Figure 20). One of the most likely limitations for the identification of low-molecular proteins is associated with the staining method. Yet with the staining method developed in our laboratory that is able to stain protein in amounts as low as 2 ng [161], a few lower molecular mass proteins could also be stained and identified (spot no-130-132).

![Figure 20: Theoretical isoelectric point (pI) (A) and molecular weight (MW) distribution (B) of proteins from pig bladder epithelial cells in relation to the number of identified proteins. Figure taken from Verma et al., Electrophoresis 2011, 32, 3600–3611.](image)

For displaying the hydropathic character of a protein, grand average of hydropathy values (GRAVY) as annotated in the Swiss-Prot database were determined according to the method described by Kyte and Doolittle [174]. For the 120 proteins identified, the GRAVY values ranged from -1.021 to +0.107. In the range from +2 to -2, a positive GRAVY score indicates...
a hydrophobic nature of the protein, while a negative GRAVY score points to a hydrophilic property. Most of the proteins of the proteome map were hydrophilic, which is expected as the proteins identified by use of 2DE gels are generally hydrophilic. However, four protein spots with positive values were also observed (heat shock 70 kDa protein 5, partial (spot no-36), D-3-phosphoglycerate dehydrogenase (spot no-45), mitochondrial ATP synthase, H⁺ transporting F1 complex beta subunit (spot no-50), and proteasome subunit beta type 3 (spot no-142).

4.4 Functional and subcellular annotation of proteins

The identified proteins represent multiple gene families and functions. To get an initial overview on the structural, trophic, metabolic, and signaling conditions of the tissue, the identified proteins were analyzed with the PANTHER classification system. This system grouped the identified proteins into four different functional annotations, namely biological functions, molecular functions, protein pathways, and protein classes (Figure 21). The molecular classification of these proteins revealed that they were involved in twelve different metabolic pathways: ion channel activity (4 %), transporter activity (1 %), translation (1 %), transcriptional regulatory activity (2 %), enzyme regulatory activity (5 %), catalytic activity (26 %), motor activity (3 %), receptor activity (4 %), antioxidant activity (1 %), structural molecular activity (23 %), and binding activity (30 %). The identified proteins were grouped into twenty-two different pathways by the annotation software: ATP synthesis (2 %), apoptosis signaling pathway (7 %), angiogenesis (2 %), Alzheimer disease-presenilin pathway (4 %), integrin signaling pathway (4 %), inflammation mediated by chemokine and cytokine signaling pathway (4 %), EGF receptor signaling pathway (7 %), Parkinson’s disease (11 %), PI3 kinase pathway (2 %), cytoskeleton regulation by Rho GTPase (7 %), PDGF signaling pathway (2 %), nicotinic acetylcholine receptor signaling pathway (4 %), cadherin signaling pathway (4 %), serine glycine biosynthesis (2 %), Huntington’s disease (7 %), p53 pathway (2 %), VEGF signaling pathway (2 %), glycolysis (7 %), FGF signaling pathway (4 %), TCA cycle (4 %), and ATP synthesis (2 %).

The analysis of biological functional annotation revealed that the identified proteins were involved in diverse arrays of processes including 14 proteins (20.3 %) in cell communication, 28 proteins (40.6 %) in cellular processes, 11 proteins (15.9 %) in transport, 13 proteins (18.8 %) in cellular component organization, 2 proteins (2.9 %) in apoptosis, 8 proteins (11.6 %) in system processes, 1 protein (1.4 %) in reproduction, 11 proteins (15.9 %) in
results to stimuli, 16 proteins (23.2%) in developmental processes, 3 proteins (4.3%) in the generation of precursor metabolites and energy, 4 proteins (5.8%) in cell cycle, 13 proteins (18.8%) in immune system processes, 2 proteins (2.9%) in cell adhesion, and a maximum of 36 proteins (52.2%) in metabolic processes.

**Figure 21:** Ontological classification of the identified proteins according to the PANTHER prediction (4A). The proteins were classified according to their (A) biological functions, (B) molecular functions, (C) pathways, and (D) protein classes. Figure taken from Verma et al., Electrophoresis 2011, 32, 3600–3611.

Meanwhile, the protein classes’ annotation categorized the proteins into nineteen different groups, among which 13% of proteins were grouped as cytoskeleton proteins, 5% as transporter proteins, 3% as transmembrane receptor regulatory/adaptor proteins, 8% as transferases, 6% as oxidoreductases, 3% as lyases, 8% as nucleic acid binding proteins, 5% as signaling molecules, 5% as enzyme modulators, 3% as calcium-binding proteins, 3% as transfer/carrier proteins, 2% as membrane traffic proteins, 11% as chaperons, 10% as structural proteins, 3% as isomerases, 2% as receptor proteins, 2% as extracellular matrix proteins, and 5% as proteases.

For detailed subcellular annotation of the identified proteins, a functional annotation database clustering program (http://david.abcc.ncifcrf.gov/) called Database for the Annotation Visualization and Integrated Discovery (DAVID) was used [167] (Figure 22). According to
Results

the program 31.6% of proteins were grouped as cytosolic, 22.8% as cytoskeleton-related, 12.3% as mitochondrial, 12.3% as cytoplasmic/membrane-associated, 12.2% as ribonucleoprotein complex, 5.2% as endoplasmic, and 3.5% as nuclear proteins.

**Figure 22:** Subcellular location of the identified proteins according to DAVID prediction. Figure taken from Verma et al., *Electrophoresis* 2011, 32, 3600–3611.
4.5 Phosphoproteome profiling of PUBEC

By using the lanthanum precipitation method for phosphoprotein enrichment, 33 phosphoproteins were identified, with minimal contamination by non-phosphopeptides (Table 6). The identified phosphoproteins exhibited many single or multiple phosphorylation sites. 

Gene ontology examination of the phosphoproteins revealed 21% proteins associated with cellular processes, 17% involved in metabolic processes, 13% in cell communication, 10% in transport, 8% in cellular component organization, 11% in developmental processes, 4% in system processes, 3% in responses to stimuli, 3% in immune system processes, 6% in cell cycle, 3% in generation of precursor metabolites and energy, and 1% in apoptosis (Figure 23).

**Figure 23:** Classification of the identified phosphoproteins from PUBEC based on their predicted biological function. Figure taken from Verma et al., *Electrophoresis* 2011, 32, 3600–3611.
Table 6: List of phosphoproteins identified in PUBEC after precipitation by La$^{3+}$ ions. Table taken from Verma et al., Electrophoresis 2011, 32, 3600–3611.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein Name</th>
<th>Gene name</th>
<th>Phosphorylation sites</th>
<th>Phosphorylated amino acid</th>
</tr>
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<td>Filamin-B</td>
<td>FLNB</td>
<td>18</td>
<td>S, T, Y</td>
</tr>
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<td>YWHAZ</td>
<td>5</td>
<td>S, T</td>
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<tr>
<td>P06753</td>
<td>Tropomyosin alpha-3 chain isoform 2</td>
<td>TPM3</td>
<td>3</td>
<td>S, T, Y</td>
</tr>
<tr>
<td>Q6QAQ1</td>
<td>Actin, cytoplasmic 1</td>
<td>ACTB</td>
<td>8</td>
<td>T, Y</td>
</tr>
<tr>
<td>P08758</td>
<td>Annexin A5-like, partial</td>
<td>ANXA5</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>P83686</td>
<td>NADH-cytochrome b5 reductase 3-like</td>
<td>CYB5R3</td>
<td>2</td>
<td>S, T, Y</td>
</tr>
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<td>P19620</td>
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<td>ANXA2</td>
<td>11</td>
<td>S, T, Y</td>
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<tr>
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<td>33</td>
<td>S, T, Y</td>
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<td>NADPH-cytochrome P450 reductase</td>
<td>POR</td>
<td>1</td>
<td>Y</td>
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<td>Importin subunit beta-1-like</td>
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<td>S</td>
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<td>Alpha-actinin-4-like</td>
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<td>CYB5R3</td>
<td>1</td>
<td>Y</td>
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<tr>
<td>P04574</td>
<td>Calpain small subunit 1*</td>
<td>CAPNS1</td>
<td>1</td>
<td>S</td>
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<tr>
<td>O75665</td>
<td>Oral-facial-digital syndrome 1 protein-like</td>
<td>OFD1</td>
<td>2</td>
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<td>3</td>
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<td>3</td>
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<td>VIM</td>
<td>31</td>
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<td>CALR</td>
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<td>P34935</td>
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<td>Vinculin</td>
<td>VCL</td>
<td>9</td>
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<td>P38646</td>
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<td>P31946</td>
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<td>S</td>
</tr>
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<td>P60174</td>
<td>Triosephosphate isomerase 1</td>
<td>TPII</td>
<td>2</td>
<td>S</td>
</tr>
</tbody>
</table>

*Uniprot accession number

bPhosphorylation site and phosphorylated amino acid entries in Uniprot Database for each protein identified by mass spectrometry.

Proteins marked with an asterisk (*) with entries for phosphorylation site and phosphorylated amino acids (S: serine, T: thymine, Y: tyrosine) in GeneCards V3 Database.
5. Toxicoproteomic analysis of apoptotic pathway in B[a]P-exposed PUBEC

5.1 Objective

Epidemiologic studies have indicated that, in addition to genetic factors and chronic irritation, occupational and environmental chemicals are significant determinants in many of the incidences of bladder cancer. In most populations tobacco smoking is the main known contributor to this malignancy: Among the many components of cigarette smoke, B[a]P which occurs in amounts of twenty to forty nanograms per cigarette is by far the best studied of these compounds and is known for its mutagenic and carcinogenic properties. However, epidemiological analyses have not yet documented B[a]P or any other PAH as significant candidates for initiating bladder cancer development. Therefore, the risk that exposure to any of these compounds causes bladder cancer is still uncertain. While genomic studies have helped in understanding the role of gene expression changes induced by B[a]P, the analysis at the protein level is a must to reveal the impact of these changes on cellular functions. The prime aim of this study was to analyze the early protein expression changes induced by B[a]P at low doses by using 2DE and MALDI-TOF-MS analysis in order to identify proteins/pathways involved in the cellular response to B[a]P-mediated toxicity. This is the first proteomic study using primary urinary epithelial cells for investigating the effects of B[a]P exposure on urothelial cell biology.

5.2 2DE analyses of protein expression in control and B[a]P-exposed cells

To closely reflect the epithelial cell conditions in vivo, cultured PUBEC derived from pigs were used to study the effects on B[a]P-induced toxicity, particularly in respect to its potential role in bladder cancer development. Proteins from five whole cell lysates obtained from each group of B[a]P-exposed and control cells were subjected to 2DE. Over 1000 protein spots were quantitatively identified by using the image analysis software Decodon 4.0. The comparison between the B[a]P-exposed and control groups indicated a more than twofold expression change of 40 proteins. Representative fused gels of control and B[a]P-exposed cell extracts are shown in
Results

(Figure 37, see annex I) in which all spots identified as consistently differentially regulated are marked by numbered arrows. Due to the “poor” peptide mass database for pigs, only 25 proteins could be identified by MALDI-TOF-MS (Table 7). Among these 13 proteins (52%) were up-regulated while 11 proteins (44%) were down-regulated. Next, the biological networks of the identified proteins were examined by using the search tool STRING 9 (Search Tool for the Retrieval of Interacting Genes/Proteins). The identified proteins were searched with the corresponding Swiss-Prot access number for their exact gene counterpart in STRING 9. The gene counter parts were uploaded to the STRING pathway analysis software. Interestingly, the software associated the differentially expressed proteins with a network that contains three nodes of interest involving TP53, XPC, and splicing genes (Figure 24).

5.3 Induction of DNA repair proteins and determination of DNA damage

Xeroderma pigmentosum, complementation group C, also known as XPC, is a protein that is involved in the recognition of bulky DNA adducts in nucleotide excision repair. One of the metabolite of B[a]P, 7,8-dihydrodiol-9,10-epoxide (BPDE), is known to form such adducts. Biological network pathways analysis using the STRING pathway analysis software showed significant correlations between four differentially regulated proteins (RAD23A, RAD23B, PMSD5, and PMSD4) and the XPC protein system. The up-regulation of these proteins points towards DNA damage in the urothelial cells during exposure to B[a]P for 24 h and is in agreement with the results obtained by the comet assay (see section-5.4)
Table 7: Proteins with altered expression (≥2) after B[a]P exposure compared to controls.

<table>
<thead>
<tr>
<th>ID</th>
<th>Accession No.</th>
<th>Gene name</th>
<th>Protein name</th>
<th>MOWSE score</th>
<th>Peptide matches</th>
<th>Regulation</th>
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<td>RNH1</td>
<td>Ribonuclease inhibitor</td>
<td>122</td>
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<td>up-regulated</td>
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<tr>
<td>ID02</td>
<td>Q32YV9</td>
<td>PMSD4</td>
<td>Proteasome 26S subunit non-ATPase 4</td>
<td>95</td>
<td>13</td>
<td>up-regulated</td>
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<tr>
<td>ID03</td>
<td>A1BPP7</td>
<td>ACTB</td>
<td>Beta actin</td>
<td>69</td>
<td>22</td>
<td>up-regulated</td>
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<tr>
<td>ID04</td>
<td>F1SGG2</td>
<td>KRT8</td>
<td>Keratin 8</td>
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<td>13</td>
<td>up-regulated</td>
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<tr>
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<td>Q6W6X2</td>
<td>PMSD5</td>
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<tr>
<td>ID09</td>
<td>Q9MZ15</td>
<td>VDAC2</td>
<td>Voltage-dependent anion selective channel protein 2</td>
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<tr>
<td>ID11</td>
<td>A3EX84</td>
<td>LGALS3</td>
<td>Lectin galactoside binding soluble 3</td>
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<tr>
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</table>

1 Internal ID number, 2 Gene name and protein name in Uniprot Database for the each protein identified by MALDI-TOF-MS, 3 Peptides matches and entries in the Mascot search engine, 4 Protein scores greater than 66 are considered significant (p < 0.05)
Figure 24: Potential protein-protein interactions of all differentially expressed protein species (p <0.05) associated with B[a]P exposure as suggested by the STRING 9 database and web resources. Uniprot gene names were loaded into the STRING tool (http://string-db.org/) and analyzed by using the standard settings (medium confidence, network depth 1, no additional white nodes). The color of the connecting lines between two protein species encodes the source of the information: experimental data (rose), databases (light blue), co-expression data (black), co-occurrence data (dark blue), and text mining. (green) The nodes of interest are marked in colored boxes.
5.4 Verification of DNA damage in PUBEC exposed to 0.5 µM B[a]P

Results obtained by the comet assay indicating DNA damage in B[a]P-treated pig epithelial cells are shown in (Figure 25). The cells in culture were exposed to B[a]P in concentrations ranging from 0.1 to 10 µM for 24 h. The experiments were repeated three times with different PUBEC pools. The application of the comet assay revealed the presence of nuclear DNA forming tail-like structures in a concentration-dependent manner. The mean value of the olive tail moment (OTM) of the control cells was 0.35 ± 0.03 after 24 h of B[a]P exposure, while the mean values of the OTMs of the cells exposed to higher concentrations (5 or 10 µM B[a]P) were 1.72 ± 0.07 ($p < 0.001$) and 1.13 ± 0.08 ($p < 0.001$), respectively, indicating a significant level of DNA damage. The mean values of the OTMs of the bladder epithelial cells exposed to 0.5 µM B[a]P also showed a statistically significant increase when compared to control cells.

Figure 25: Concentration-dependent increase in B[a]P-induced DNA damage evaluated with the comet assay. (A) Alkaline comet assay images of individual bladder epithelial cells with various degrees of DNA damage: a) unexposed cells, b) positive control (1 mg/ml N-ethyl-N-nitrosourea (ENU), c) 0.5 µM B[a]P. (B) PUBEC pools were incubated with increasing concentrations of B[a]P (0.1-10 µM) for 24 h, and ENU was used as positive control. As indicator for DNA strand breaks, the olive tail moment is expressed as mean ± standard deviation obtained in three independent experiments with pooled PUBEC cultures. The level of significance relative to controls was determined by using the t-test (***, $p < 0.001$).
5.5 B[a]P induced changes expression of proteins involved in apoptosis

Notably, many of the identified proteins were associated with another very interesting network involving TP53 (Figure 24). Other than its role in genome stability, senescence, DNA repair, and cell cycle arrest, TP53 is known to play a major role in the induction of apoptosis. PUBEC exposed to B[a]P showed a differential expression of Cathepsin D, VDAC 2, HSP27, and HSP70, proteins known to be involved in the intrinsic mitochondrial death receptor pathway. To find out whether B[a]P exposure indeed induced mitochondria-associates apoptosis, changes in mitochondrial potential were assessed and the TUNEL test was employed.

5.6 Analysis of mitochondrial alterations following B[a]P exposure

In order to validate whether the mitochondrial dysfunction was involved in B[a]P-induced apoptosis, the mitochondrial membrane potential (MMP) was measured in PUBEC by using Rhodamine 123. As indicated by the decrease of fluorescence intensity of Rhodamine 123 (Figure 26), the MMP was significantly impaired at a B[a]P concentration of 0.5 µM after exposure times of 2, 6, and 12 h, with the maximum impairment being observed after 2 h (p < 0.001 in all experiments). However, the MMP was nearly reconstituted after 24 h of exposure.

![Figure 26: Determination of the MMP of PUBEC by using the monovalent cationic dye Rhodamine 123. Cells were cultured on collagen-coated 96-well plates with a clear bottom and were exposed to 0.5 µM B[a]P for different time periods (2, 6, 12, and 24 h). Valinomycin (100 µM) was used as positive control, while as negative control cells were only exposed to cell culture medium. Rhodamine 123 (5 µM) was applied for 30 min. The measurements were performed by using an ELISA plate reader (λ_{excitation} = 488; λ_{emission} = 535 nm). A significant decrease in MMP in a time-dependent manner was observed. The data were presented as mean ± standard deviation of three independent experiments with pooled PUBEC cultures. The level of significance relative to the positive control was determined by using the t-test (***p < 0.001).](image-url)
5.7 Application of western blot and TUNEL assay for analysis of apoptosis in PUBEC

Next, the validation of the p53-regulated apoptotic proteins modulated in response to B[a]P exposure was sought. The TUNEL assay and immunochemical quantification were used to confirm these findings. The percentage of apoptotic PUBEC was determined by counting the number of positively stained pig epithelial cells and total epithelial cells in a ten-field (Figure 27A). The number of TUNEL-positive cells noticeably increased in the presence of 0.5 µM B[a]P (n = 6 independent experiments, p <0.05), whereas apoptotic cells were barely detectable in control epithelial cells (Figure 27B).

Figure 27: Analysis of pig urinary epithelial cell apoptosis by the TUNEL assay. Control and B[a]P-exposed (0.5 to 10 µM) PUBEC pools (n = 6) were subjected to the TUNEL assay. (A) A TUNEL-positive control was obtained by incubation with DNase I. In control cells exposed to DMSO (<0.1 %), apoptotic cells were hardly detected. However, a marked increase in TUNEL-positive cells was observed in B[a]P-exposed (0.5-10 µM, 24 h) epithelial cells. The white bars represent 50 µm. (B) Box plots (median and interquartile range) show the level of apoptosis in PUBEC pools exposed for 24 h to different concentrations of B[a]P compared to controls. –ve represents PUBEC unexposed to B[a]P, while +ve represents positive controls obtained by incubation of PUBEC with DNase I. The level of significance relative to control was determined by using the t-test (**p <0.001, *p <0.05).
Results

Figure 28: Immunoblot analysis of differentially expressed proteins. (A) Western blot analysis of total protein extracts (30 mg of protein) from three independent lysates from PUBEC exposed to 0.5 µM B[a]P and from controls was carried out on a 12% SDS gel. Actin was used as loading control. (B) Densitometric analysis of gel bands after western blot analysis. The bars represent the mean of three SEM of gel band density determined in B[a]P-exposed and control PUBEC. The level of significance relative to control was determined by using the t-test (**p <0.001, *p <0.05).

The expression of apoptosis-related proteins identified by MALDI-TOF-MS was determined by using immunoblot analysis. CTSD, VDAC 2, HSP27, and HSP70 were chosen for analysis, as these proteins play a key role in the intrinsic mitochondrial apoptotic pathway. The analysis confirmed the increased abundance of Cathepsin D and VDAC 2 and a decreased expression of proteins such as HSP27 and HSP7 in PUBEC exposed to 0.5 µM B[A]P when compared to control cells. These findings are consistent with the results obtained by 2DE and MALDI-TOF-MS (Figure 28).
6. Blue Native PAGE analysis of B[a]P- and TCDD-exposed cells

6.1 Objective

Multiprotein complexes are vital supramolecular assemblies involved in the regulation of various intracellular processes and signaling pathways. Most molecular processes in the cell result from interactions of different protein complexes. Therefore, a complete understanding of the composition and interactions of these complexes is crucial for the understanding of the molecular mechanisms mediated by these complexes. As detailed in the introduction, B[a]P is a ligand for the aryl hydrocarbon receptor complex (AhR). The same holds true for 2,3,7,8-dibenzo-p-dioxin (TCDD), another environmental contaminant. AhR is a cytosolic receptor complex involved in the transcriptional modulation of cell growth and differentiation and, most importantly, in the transcriptional regulation of drug metabolizing enzymes such as cytochrome P450 (CYP). These enzymes help in the detoxification of PAH and other xenobiotics and are also known to affect other cellular processes by interaction with other protein complexes. While a lot of information is available on the interaction of these xenobiotics with the AhR complex, much less is known in regard to their possible effects on other protein complexes.

The understanding of these complexes and the possible interactions between them can shed some light into the possible mechanism of B[a]P-mediated toxicity. Therefore, the present work was carried out to identify and characterize multiprotein complexes by using Blue Native PAGE (2D BN/SDS-PAGE) to elucidate the network of protein-protein interactions that regulate protein functions and hence the toxicity of B[a]P and TCDD. 2D BN/SDS-PAGE permits the separation of multiprotein complexes under native conditions and thus maintains the intact complex. For the enrichment of the complex, subcellular fractionation was performed. 2D BN/SDS-PAGE and subcellular fractionation form an ideal partnership when it comes to the enrichment and analysis of intracellular organelles and of low-abundant multiprotein complexes. Nevertheless, the concurrent study on the effects of B[a]P and TCDD was performed to find out whether the protein expression profiles are similar or different in response to two different classic inducers of the AhR pathway.
6.2 Subcellular fractionation and 2D BN/SDS-PAGE analyses of fractionated samples for proteome map generation

For organellar enrichment, the commercially available subcellular fractionation kit ProteoExtract® (Calbiochem, Darmstadt, Germany) was applied. By using this kit, the control cells and cells exposed to B[a]P (0.5 µM) or TCDD (200 pM) were fractionated into four subcellular compartments: cytosol, membrane/organelle proteins, nuclear, and cytosolic fraction. The method was able to enrich proteins to a certain degree, but a few carryovers from one fraction to another were observed. The proteins from these fractionated samples were then used for 2D BN/SDS-PAGE electrophoresis.

To have an overview about the protein complexes of the RT4 cell line, a suborganellar proteome map was generated by analyzing the complexes of unexposed cells of different fractions by 2D BN/SDS-PAGE analyses. In the gels obtained from five different preparations approx. 200 protein spots per fraction were detected, among which only those spots were identified by MALDI-TOF-MS which showed a significant expression of ±1.3 in all gels. A total of 64 protein spots were identified in the cytosolic fraction, 65 protein spots for the membrane and organelle fraction and 55 protein spots for the nuclear fraction (Table 9-11, see annex II). Interestingly, the identified proteins were enriched; however, some proteins were carried over between the fractions, but with very low inter-experimental variation.

6.2.1 Protein complexes of cytosolic fraction

As discussed for 2D-BN/SDS-PAGE gels [163], the resulting gels showed typical patterns for BN-PAGE experiments, i.e. monomeric proteins within a certain hyperbolic diagonal field, whereas the components of stable protein complexes (subunits of the same protein) arranged in an vertical line under this diagonal field.

By using the above mentioned criteria, MALDI-TOF-MS analysis of this fraction revealed among others many typical cytosolic proteins (Table 9, see annex II), such as proteins of the proteasome complex (including both α and β subunits, Figure 29, protein spots ID-5 to ID-18), proteins of the lactate dehydrogenase (LDH) enzyme complex (Figure 29, protein spot ID 24-29), and the dimeric enzyme complex phosphoglycerate mutase (PGAM, Figure 29, protein spot ID 61-63). LDH consists of A and B chains, both of which were identified in the cytosolic fraction. Along with these proteins many proteins involved in glucose metabolism.
(phosphoglycerate kinase, isocitrate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, UDP-glucose 6-dehydrogenase) were also identified.

**Figure 29:** Representative 2D BN/PAGE gel of the cytosolic fraction of RT4 cells. The gels were stained with Coomassie brilliant blue after 4-12 \% BN-PAGE and 12 \% SDS-PAGE separation followed by identification of the protein spots by MALDI-TOF-MS. The ID number, protein name, molecular weight, Mascot score, and peptide match is listed in Table 9.

### 6.2.2 Protein complexes of the membrane/organelle fraction

The 2D BN/SDS-PAGE analyses of this fraction revealed many mitochondria-related proteins, which include proteins of the respiratory chain ATP synthase complex (alpha and beta subunits of F1- ATP synthase, **Figure 30**, protein spot ID 60-62) and the alpha and beta subunits of the electron transfer flavoprotein complex (ETFA and ETFB). Also, many mitochondrial enzymes, such as mitochondrial malate dehydrogenase 2, NADPH:adrenodoxin oxidoreductase, mitochondrial glutamate dehydrogenase 1, mitochondrial 60 kDa heat shock protein, and NADP(+)-dependent isocitrate dehydrogenase, were also identified. Other than mitochondrial complexes, a few proteins of the endoplasmic reticulum (cyclophilin B complexed with cyclosporine and calreticulin (**Figure 30**, protein spot ID 24-25) and of the cytoskeleton (actin-binding ARP2/3 complex, **Figure 30**, protein spot ID no 7-12) were also detected (**Table 10, see annex II**).
Results

Figure 30: Representative 2D BN/PAGE gel of the membrane/organelle fraction of RT4 cells. The gels were stained with Coomassie brilliant blue after 4-12 % BN-PAGE and 12 % SDS-PAGE separation followed by identification of the protein spots by MALDI-TOF-MS. The ID number, protein name, molecular weight, Mascot score, and peptide match is listed in Table 10.

6.2.3 Protein complexes of the nuclear fraction

In the nuclear fraction several proteins belonging to the nuclear matrix (lamins), nucleosome (histone core complex proteins), and proteins related to DNA processing (DNA polymerase α, HnRNPs) were detected (Table 11, see annex II). Lamins exist as a homodimer consisting of lamin A and lamin C. Of these two, lamin A along with a wide range of cytoskeletal proteins (cytokeratin 19, 7, 8, and 17) were identified. Other than structural proteins, proteins of the histone core complex were found. This complex exists as an octamer occurring at the center of a nucleosome core particle. It consists of two copies of each of the four core histone proteins (H2A, H2B, H3, and H4), and as tetramer of two copies of both H3 and H4 complexed with two H2A/H2B dimers. In the samples both monomers and dimers of histone proteins were found separated in close proximity to each other. Additionally, many proteins
involved in DNA processing, such as subunits of Pol I complex, HnRNP/splicing factor (Heterogeneous nuclear ribonucleoprotein, Serine/arginine-rich splicing factor 9, and Heterogeneous nuclear ribonucleoproteins A2/B1 isoform A2), ribosomal protein (60s ribosomal protein L10a), DNA/RNA-binding proteins (ATP-dependent RNA helicase A, RNA-binding protein 4 isoform 3, DNA topoisomerase 2-beta, and DNA2-like helicase), and transcription factors (Homeobox B7) were also identified.

6.3 2D BN/SDS-PAGE analyses of fractionated samples of B[a]P-and TCDD-exposed cells

After establishing the proteome map of protein complexes in our cell model (RT4 cell line), 2D BN/SDS-PAGE analyses was applied to reveal alterations in these complexes after exposure to B[a]P or TCDD. The cells exposed to 0.5 μM B[a]P or 200 pM TCDD and control cells were fractionated as described in the Materials and Methods chapter. After fractionation 2D BN/SDS-PAGE analyses of cytosolic, membrane/organelle, and nuclear fraction were carried out for B[a]P- and TCDD-exposed and for control cells. To reveal differential expressions of protein complexes in control cells and cells exposed to B[a]P or TCDD, gels from five different experiments for all three fractions were compared by using the Decodon software (as described in the Materials and Methods chapter). Only those proteins were considered significant which showed an expression change of ±2. The entire process of analysis with the Decodon software was repeated individually for all three fractions exposed to B[a]P or TCDD and the control samples. In total, more than 200 spots were found in each fraction, with a differential expression of 19 proteins in the cytosolic fraction, of 23 proteins in the membrane/organelle fraction, and of 8 proteins in the nuclear fraction for TCDD-exposed cells, while 15 proteins in the cytosolic fraction, 21 proteins in the membrane/organelle fraction, and 18 proteins in the nuclear fraction were differentially expressed in the B[a]P-exposed cells (Table 11.1-11.6, see annex III). Representative images of 2D BN/SDS-PAGE of all three fractions are shown in (Figure 36-38, see annex IV). Protein spots identified as proteins with statistically significant expression differences between control and exposed cells were marked.

6.4 Alterations in calcium- and iron-containing proteins

MALDI-TOF-MS analysis of the differentially expressed proteins yielded many proteins involved in calcium and iron homeostasis in both TCDD- and B[a]P-exposed cells (Table 8).
Results

Table 8: List of calcium- and iron-associated proteins with altered expression (≥2) after B[a]P or TCDD exposure compared to controls

Identified calcium-containing proteins differentially regulated in control and TCDD-exposed RT4 cells. Representative images of 2D BN/SDS-PAGE marked with differentially expressed proteins are shown in Figure 37 and Figure 38, annex IV

<table>
<thead>
<tr>
<th>Spot ID no</th>
<th>gene name</th>
<th>protein name</th>
<th>subcellular fraction</th>
<th>regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID 231</td>
<td>CALM1</td>
<td>calmodulin</td>
<td>cytosol</td>
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</tr>
<tr>
<td>ID203</td>
<td>S100A2</td>
<td>protein S100-A2</td>
<td>cytosol</td>
<td>3.8684</td>
</tr>
<tr>
<td>24</td>
<td>ANXA10</td>
<td>annexin A10</td>
<td>membrane/organelle proteins</td>
<td>1.02979</td>
</tr>
<tr>
<td>25</td>
<td>ANXA5</td>
<td>annexin A5</td>
<td>membrane/organelle proteins</td>
<td>1.34865</td>
</tr>
<tr>
<td>16</td>
<td>GSN</td>
<td>gelsolin isoform b</td>
<td>membrane/organelle proteins</td>
<td>1.08039</td>
</tr>
</tbody>
</table>

Identified calcium-containing proteins differentially regulated in control and B[a]P-exposed RT4 cells. Representative images of 2D BN/SDS-PAGE marked with differentially expressed proteins are shown in Figure 38, annex IV

<table>
<thead>
<tr>
<th>Spot ID no</th>
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<th>protein name</th>
<th>subcellular fraction</th>
<th>regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>PPIB</td>
<td>cyclophilin B</td>
<td>membrane/organelle proteins</td>
<td>1.74984</td>
</tr>
<tr>
<td>43</td>
<td>PPIA</td>
<td>cyclophilin A</td>
<td>membrane/organelle proteins</td>
<td>3.41278</td>
</tr>
<tr>
<td>30</td>
<td>ANXA10</td>
<td>annexin A10</td>
<td>membrane/organelle proteins</td>
<td>-3.95467</td>
</tr>
<tr>
<td>19</td>
<td>CALR</td>
<td>calreticulin</td>
<td>membrane/organelle proteins</td>
<td>2.15407</td>
</tr>
</tbody>
</table>

Identified iron-containing proteins differentially regulated in control and TCDD-exposed RT4 cells. Representative images of 2D BN/SDS-PAGE marked with differentially expressed proteins are shown in in Figure 37 and Figure 38, annex IV

<table>
<thead>
<tr>
<th>Spot ID no</th>
<th>gene name</th>
<th>protein name</th>
<th>subcellular fraction</th>
<th>regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID172</td>
<td>HEBP2</td>
<td>heme-binding protein 2</td>
<td>cytosol</td>
<td>2.03805</td>
</tr>
<tr>
<td>ID1755</td>
<td>FTH1</td>
<td>ferritin</td>
<td>cytosol</td>
<td>-2.68431</td>
</tr>
<tr>
<td>ID5,6</td>
<td>TFRC</td>
<td>transferrin receptor protein 1</td>
<td>membrane/organelle proteins</td>
<td>1.35857</td>
</tr>
</tbody>
</table>

Particularly in the TCDD-exposed cells a unique cluster of three protein spots was observed (Figure 31) that was missing on the gels of the B[a]P- and the nonexposed cells. Two out of three protein spots were identified as calmodulin and protein S100-A2. Modulations of calcium- and iron-containing proteins upon exposure to TCDD or B[a]P have been reported before, however, none of the studies till date has suggested that a possible interaction between these two protein systems might exist.
In a few recent studies the role of calmodulin (CaM) in AhR-dependent and -independent genomic responses after exposure to these compounds have been indicated. Other researchers reported a TCDD-mediated impairment of cellular iron homeostasis due to a changed ferritin content associated with transferrin (TfR-1) induction. Based upon these studies and the expression of both calcium- and iron-containing proteins observed in the TCDD- and B[a]P-exposed samples, it is hypothesized that these two protein systems might interact via the nitric oxide synthase enzyme. Accordingly, a rapid increase in intracellular calcium ion concentration occurs upon exposure to TCDD or B[a]P, which activates the major calcium sensor CaM. CaM further activates the nitric oxide synthase, which in turn modulates the labile iron pool through nitric oxide production (details of this hypothesis is given in the discussion section of the thesis). The following experiments were carried out to prove this hypothesis.

**6.5 Analysis of intracellular calcium changes after B[a]P and TCDD exposure**

To monitor whether exposure to B[a]P or TCDD for 24 h alters the intracellular calcium concentration the fluorescence indicators Fluo-4 and Rhod-2 were used. The fluorescence measurement with Fluo-4 revealed an increase of intracellular calcium by 21% in the B[a]P-exposed cells, whereas an elevated level of 27% was found in the TCDD-exposed cells. The
determination of calcium in internal calcium stores with Rhod-2 showed significantly raised levels for both exposure groups. The B[a]P-exposed cells exhibited a 12% higher calcium level than that of controls, while in the TCDD-exposed group an increase of 30% was observed compared to control cells (Figure 32). In some studies a transient increase in $[\text{Ca}^{2+}]_i$ that occurs 2–5 min after the addition of an apoptosis-inducing agent have been observed. However, such an increase in both B[a]P- or TCDD-exposed cells was not found in the present experiments.

Figure 32: Alterations of cellular calcium homeostasis after exposure to B[a]P or TCDD. Cells were cultured on collagen-coated glass coverslips and loaded with Fluo-4/AM (1.4 µM) and Rhod-2 AM (3.6 µM) for 30 minutes at room temperature, followed by incubation for another 30 min at 37 °C after exposure to 200 pM TCDD for 24 h. The fluorescence was recorded by using a laser-scanning microscope (Fluo 4/AM, $\lambda_{\text{excitation}} = 488$; $\lambda_{\text{emission}} = 505$ nm, Rhod-2/AM, $\lambda_{\text{excitation}} = 543$; $\lambda_{\text{emission}} = 560$ nm). [A] Fluorescence images of control, TCDD- and B[a]P-exposed cells loaded with Fluo-4 and Rhod-2. [B] Statistical analysis of intracellular calcium levels following treatment of RT4 cells with 0.5 µM B[a]P or 200 pM TCDD. Data are expressed as percentage of calcium levels found in unexposed cells, arbitrarily set to 100%. The results correspond to the mean ±S.D of four independent experiments. The white bar represents 50 µm.
6.6 Effects of B[a]P and TCDD on the labile iron pool (LIP) and the possible role of calcium

To prove whether the changes in the expression of proteins involved in iron homeostasis led to alterations in the LIP, the chelatable iron pool of the RT4 cell line was determined by using the fluorescent probe Phen Green™ SK (PG SK). Control cells and the cells exposed to 0.5 µM B[a]P or 200 pM TCDD for 3 h and 24 h, respectively, were loaded with PG SK for the analysis. In the cells exposed to TCDD for both 3 h and 24 h, the LIP increased moderately compared to control cells (about 7 % and 13 %, respectively), whereas no effect on the cellular LIP was observed in B[a]P-exposed cells (Figure 33). To analyze whether the calcium sensor protein calmodulin (CaM) was responsible for the TCDD-induced increase in LIP, the cells were blocked with the CaM antagonist W-7 (10 µM), followed by treatment of the cells with 200 pM TCDD for 3 h and 24 h. During the initial three hours, W-7 inhibited the TCDD-mediated increase of LIP, and to a lesser extent this effect was also observed in the cells exposed for 24 h. These results demonstrated that CaM was activated by TCDD and that it was somehow modulating the iron content of the cells.
Figure 33: Measurement of the chelatable iron pool by PG SK. For confocal images cells were cultured on glass coverslips and loaded with 20 mM PG SK (10 min, 37 °C) after exposure to 200 pM of TCDD for 24 h. Cellular fluorescence was excited at 488 nm by using a laser scanning microscope (LSM 510) equipped with an argon laser. For emission, a 505 nm longpass filter was used. Cellular microfluorographs of (A) TCDD-exposed cells and (B) cells after dequenching with 1,10-phenanthroline. The white bar represents 50 µm. The changes in intracellular iron was determined by the differences in cell fluorescence of control against [C] B[a]P- or [D] TCDD-exposed cells as described in Material and Methods. Data are expressed as percentage of iron levels found in unexposed cells, arbitrarily set to 100 %. The results correspond to the mean ±S.D of four independent experiments. The level of significance relative to control was determined by using the t-test (**p <0.0001).

6.7 Possible involvement of nitric oxide (NO) in calmodulin-modulated iron content of cells

Finally, to analyze whether the changes in LIP of the cells was mediated by nitric oxide synthase (NOS) via calmodulin, the NO content of the cells was determined first. The measurement of the nitrite and nitrate concentrations (as a measure of NO) with the Griess regent revealed an increase of NO in B[a]P-exposed cells by 153 % (compared to control) and in TCDD-exposed cells by 96 % (compared to control) (Figure 34). The experiments were also performed at higher concentrations of B[a]P (5 µM) and TCDD (1000 pM). Here, NO production increased considerably at both concentrations (111 % and 78 %, respectively) when compared to that in control cells.
Figure 34: NO production after exposure to TCDD or B[a]P in normal and W-7-inhibited RT4 cells. 4.0x10^6 cells were exposed to TCDD or B[a]P alone or in a co-treatment with 10 µM W-7 (for 10 min). After 24 h of exposure, the medium was used for the determination of the NO content by using the Griess assay. Data are expressed as percentage of NO levels found in unexposed cells, arbitrarily set to 100%. The results correspond to the mean ±S.D of four independent experiments. The level of significance relative to control was determined by using the t-test (***p <0.001, *p <0.05).

Also, as calmodulin is a key enzyme required for NOS activity, CaM was blocked by using its antagonist W-7. W-7 decreased the basal levels of NO both in (200 pM) TCDD- and (0.5 µM) B[a]P-exposed RT4 cells by 50% and 122%, respectively (p <0.05, Figure 34) when compared to non–inhibited cells. These results suggest that CaM is a necessary signal for NOS that, in turn, is capable of regulating iron metabolism by producing NO.
7. Discussion

Benzo[a]pyrene (B[a]P), a five-ring polycyclic aromatic hydrocarbon, is a well-recognized environmental pollutant found in coal tar, automobile exhaust fumes (especially from diesel engines), in smoke resulting from the combustion of organic material (including cigarette smoke), and in charbroiled food. It is known to cause lung, breast, and cervical cancer; whether it plays a role in bladder cancer development is still discussed. The ubiquitous presence of B[a]P in the environment and its potential involvement in the most important urologic malignancy was the rationale to investigate how and to what extent it is taken up by urothelial cells and which are the effects induced by this environmental chemical, especially at the protein level. A well-established primary urinary bladder epithelial cell model (PUBEC), isolated from bladders of freshly slaughtered pigs were used for the major part of the studies. In addition, to investigate B[a]P-induced effects on protein complexes (by Blue Native PAGE analysis), the human urinary bladder epithelial cell line RT4 was utilized.

7.1 Uptake and subcellular distribution of B[a]P

As in many other instances of carcinogenic compounds, considerable attention has been given so far to the genotoxic effects of B[a]P and to its metabolic activation to reactive intermediates. In contrast, essentially no information has been gathered on its uptake and subcellular distribution, processes that necessarily precede all other biological events occurring after the exposure. Nevertheless, as the presence of small quantities of non-metabolized B[a]P in addition to that of its hydroxylated metabolites in urine has been demonstrated and because B[a]P is a highly lipophilic compound, the possibility that it is taken up by the urothelium cannot be ignored. In this thesis, the pattern of cellular uptake and distribution of B[a]P in pig urinary epithelial cells is reported for the first time.

In many studies the fluorescent properties of B[a]P, resulting from the multi-fused aromatic rings with delocalized $\pi$ bonds, have been utilized to monitor the mixed-function oxygenase activity by applying flow cytometry, laser cytometry, and confocal microscopy [175-177]. Here, the uptake of B[a]P by pig bladder epithelial cells was analyzed by using confocal microscopy. This technique did not only allow to study and visualize the intracellular distribution of B[a]P but also helped in minimizing the photobleaching of B[a]P due to unwanted collateral non-confocal irradiation. In the cells exposed to 0.5 $\mu$M B[a]P (for 2, 6, 12, 18, and 24 h), the first indication of cellular uptake of B[a]P was obtained after 6 h of
Discussion

exposure (Figure 16), while no signs of B[a]P uptake were apparent after the initial 2 h (data not shown). Also, in contrast to studies with other cell lines in which a saturation of B[a]P uptake was observed as early as 5 min and 4 h, respectively [41, 178], there was no evidence of approaching uptake saturation in PUBEC during the entire exposure period of 24 h. Nevertheless, B[a]P uptake was markedly different in the various PUBEC pools used for the study (Figure 16). The variation in uptake kinetics of B[a]P in PUBEC seems to reflect the situation in humans where a wide variation in the toxic response to B[a]P and other PAH is observed.

As reported for other cell types [179], B[a]P rapidly distributes into the cell membrane (Figure 17A). This rapid uptake of B[a]P can be attributed to its highly lipophilic character and to the wide range of lipoproteins contained in the plasma membrane. It has been speculated that these proteins facilitate the rapid uptake of B[a]P into the cell membrane [180]. A small but significantly higher amount of B[a]P compared to control cells was also found in the cytosol and nucleus of PUBEC (Figure 17B). The uptake of B[a]P by these cell compartments has been reported in a few other studies [181]. There are some indications that B[a]P is not only metabolized by cytosolic enzymes but also in the nuclear compartment. It has been shown in various studies that monooxygenases/hydroxylases are not only active in the cytosol but are also present in the nuclear envelope [182-184].

After having analyzed the time-dependent uptake and subcellular distribution of B[a]P by using confocal microscopy, spectrofluorometric and GC-MS techniques were applied to quantitatively determine the uptake by PUBEC exposed to low (0.5 µM) and high (10 µM) B[a]P concentrations. An ex situ calibration method was applied for which standard solutions of B[a]P in a cytosolic medium (as described in the Materials and Methods chapter) were prepared to obtain a homogenous solution and to mimic the intracellular environment. B[a]P does not form homogenous solutions with various organic solvents. Similar to the initial uptake studies, two distinct subpopulations of PUBEC were found with regard to the intracellular B[a]P concentrations: In cells exposed to 0.5 µM B[a]P, the intracellular B[a]P concentrations ranged from 7.28 µM to 10.75 µM in one subpopulation and from 23.17 µM to 35.07 µM in the other one. The corresponding ranges of values for the two subpopulations of cells exposed to 10 µM B[a]P were 29.9 µM to 48.31 µM and 373.72 µM to 406.64 µM, respectively. An increased metabolic turnover originating in an increased induction of B[a]P-metabolizing enzymes may be the reason for the lower intracellular B[a]P concentrations in one of the PUBEC subpopulations. PUBEC have been shown to express inducible
cytochrome P450 isoenzymes, which is of particular relevance since these enzymes contribute to the activation of B[a]P to carcinogenic metabolites [171, 172, 185, 186]. The formation in PUBEC of 3-OH-B[a]P, one of the major B[a]P metabolites, is reported for the first time. 3-OH-B[a]P has often been used as biomarker for assessing internal exposure to PAH [187, 188]. GC-MS analysis revealed that this metabolite is present in all of the B[a]P-exposed PUBEC (Figure 18B). It is the first reported proof that oxygenated B[a]P metabolites are actually formed in this cell model.

In summary, the results of the uptake studies indicate that urinary bladder epithelial cells are able to incorporate B[a]P, which may come in contact with the epithelium either directly during urinary excretion or by diffusion from the capillaries in the lamina propria. The uptake of B[a]P by these cells and their ability to metabolize this chemical to reactive intermediates strongly supports the hypothesis that B[a]P may act as a urinary bladder carcinogen. Moreover, the uptake differences found in the studies may be one of the reasons for the varying carcinogenic response to PAH exposure observed in humans [189].

### 7.2 Development of a 2DE proteome map of PUBEC

Historically, the use of cell lines is well established in proteomic research. However, the most important disadvantage of note while using a cell line is the loss of some critical physiological traits that are inherent in the process of stable cell line creation [190]. Urinary bladders from freshly slaughtered pigs were used for generating the first 2DE reference proteome map of bladder epithelial cells as a valuable reference resource for comparative studies [191]. In total, 1000 protein spots with a molecular weight of 20-100 kDa and isoelectric points from 4-10 were detected by using a colloidal Coomassie brilliant blue staining protocol developed in our laboratory. 120 of these spots were identified by tryptic digest and subsequent MALDI-TOF-MS analysis of the peptides (Table 5). During Mascot analysis for protein identification, many proteins were not identified even though mass spectra of high quality were recorded. It became apparent that the available pig database is still incomplete and that homologous sequences from other species are not sufficiently identical to achieve a high enough score to produce a match [192]. The PANTHER classification revealed a broad range of biological processes in which the identified proteins were involved (Figure 35). Proteins taking part in biotransformation, energy metabolism, structural, signaling, and stress pathways were identified even under basal conditions. Of considerable interest were proteins associated with cell stress and detoxification (GSTP1, PRDX2, HSP60, HSP71, HSP70, HSP90, and HSP27).
GSTP1 and PRDX2 are proteins involved in detoxification processes (through redox regulation), and have emerged as significant candidates for cancer biomarker development [193-195]. Seven proteins related to carbohydrate metabolism were identified, which is of great interest for studying bladder-related diseases as there are distinct carbohydrate profiles in normal and tumor tissues [196]. Other than these, many cytoskeletal proteins (KRT7, KRT17, KRT10, KRT19) were identified which do not only provide structure stability to the cells but have also been implicated in malignant transformation via a differential regulation of the actin-based cytoskeleton [197].

Precipitation, a conventional and economical method of enriching proteins of interest, was used for isolating phosphoproteins. Phosphorylation is a posttranslational modification principally of serine, threonine, or tyrosine residues of proteins. Enrichment of phosphoproteins is essential because of their low abundance. Many methods involving enhancement of phosphoproteins by precipitation with calcium and barium phosphate have been suggested [198-200]. Often these methods are tedious and time-consuming, and the amount of precipitated proteins is not sufficient to be separated on gels. In our laboratory, a method for the enrichment of phosphoproteins by precipitation with lanthanum ions (La³⁺) prior to 2DE separation and mass spectrometric identification was developed [160]. Because of its strong electropositive properties and its preference for oxygen-containing anions, lanthanum ions form very tight complexes with most common biological ligands such as carboxyl and phosphate groups [201]. By using this method, 31 phosphoproteins were identified in PUBEC (Table 6). Phosphorylation of many calcium-containing proteins (calumenin, clathrin, calpain, and calreticulin) was observed in these cells. Among them, the differential regulation of calreticulin (CALR) has been reported in several cancerous tissues such as breast, colon, and liver tumors [202-204]. CALR has also been discussed in many recent studies as a suitable marker for the diagnosis of bladder cancer [205, 206]. Other than that of calreticulin, the phosphorylation of clathrin, a protein involved in the transactivation of p53 target proteins [207-210], was observed. Moreover, three proteins of the electron transport chain (NADH-cytochrome b5 reductase 3, NADPH-cytochrome P450 reductase, cytochrome b5), required by the bladder for the energy-demanding maintenance of urine storage and removal, were discovered [211]. Collectively, with the current work a comprehensive 2DE proteome map along with a phosphoproteome map of healthy bladder epithelial cells were reported for the first time. The map can prove as a useful source of information for bladder cancer studies and especially for studies involving pigs (as model system) where information is still very scarce.
Discussion

7.3 Toxicoproteomic analysis of B[a]P-mediated toxicity in PUBEC

After the 2DE map of PUBEC had been established, these cells were used for analyzing the protein-protein interactions involved in B[a]P-mediated toxicity. After B[a]P exposure for 24 h approx. 40 proteins showed significant changes in expression levels. Twenty-five of these proteins were successfully identified by MALDI-TOF-MS analysis (Table 7). When the biological networks of the identified and differentially expressed proteins were analyzed by using the STRING software, three nodes of interest involving TP53, XPC, and splicing genes were indicated (Figure 24). Four of the differentially associated proteins (RAD23A, RAD23B, PMSD5, and PMSD4) were related to the xeroderma pigmentosum, complementation group C (XPC) protein system. XPC is a part of the core incision complex of the mammalian nucleotide excision repair (NER) system that is involved in the elimination of a wide variety of DNA lesions, mostly of carcinogen-induced bulky DNA adducts related to tobacco smoke [212, 213]. B[a]P, one of the components of tobacco smoke, is known to cause DNA damage [214]. The sequence of events that occur during DNA repair can be divided into recognition, unwinding, incision, and repair synthesis [215]. RAD23, PMSD5, and PMSD4 play a crucial role in DNA repair [216] and are up-regulated after B[a]P exposure complexed with RAD4, another nuclear protein. The RAD4/RAD23 complex is involved in the recognition of DNA adducts [217]. The up-regulation of these proteins suggests that B[a]P exposure for 24 h leads to DNA damage in bladder epithelial cells. These findings were supported by the concentration-dependent increase in olive tail moments as determined by the comet assay. A significant increase (p <0.001) was observed even at a low B[a]P exposure concentration (Figure 25). These findings were also in agreement with the results of the metabolism studies, where the formation of 3-OH–B[a]P, a likely precursor of genotoxic B[a]P metabolites, was demonstrated in PUBEC exposed to 0.5 and 10 µM B[a]P. Overall, the synopsis of the results obtained by the proteomic, genotoxicity, and metabolism studies substantiate the hypothesis that B[a]P is capable of causing DNA damage in bladder epithelial cells by generation of ultimate carcinogenic intermediates [218].

Interestingly, for the proteins differentially regulated in B[a]P-exposed PUBEC, one node of interest involving TP53 was observed. One of the most dramatic responses to p53 activation is the induction of apoptosis [219]. DNA damage induces cell cycle arrest that as well as apoptosis is related to the mutation status of p53 [220]. A lack of functional p53 inactivates the G1 checkpoint, and the cell proceeds to the G2/M phase arrest notwithstanding DNA
damage. The G2/M phase arrest is only transient, however, and the cell proceeds in the presence of the damaged DNA to an unscheduled premature mitosis that develops into a mitotic catastrophe and to apoptosis [221]. Two p53-induced apoptotic pathways have been proposed: the intrinsic mitochondrial and the extrinsic death receptor pathway [222]. Generally, the cells committed to die via p53-dependent apoptosis typically follow the intrinsic apoptotic mitochondrial pathway. The STRING pathway analysis indicated that four of the differentially regulated proteins (VDAC2, CTSD, HSP27, and HSP70) were related to this pathway. To validate these findings, the mitochondrial membrane potential (MMP) of PUBEC exposed to 0.5 µM B[a]P for different time periods was measured (Figure 26). The MMP decreased within 2 h of exposure, however, in contrast to the previous reports, it was nearly re-established after an exposure period of 24 h, thus indicating that B[a]P-mediated apoptosis is not entirely dependent on the intrinsic mitochondrial apoptotic pathway [223].

Consistent with the 2DE data, western blot analysis also revealed an up-regulated expression of VDAC2 and CTSD, while the expression of HSP27 and HSP70 was down-regulated (Figure 28) HSP27 and HSP70 are known for their anti-apoptotic regulation. Both proteins, when present in abundance, suppress mitochondrial damage and nuclear fragmentation, and hence apoptosis [224, 225]. Since the expression of both proteins was decreased in PUBEC after B[a]P exposure, they may be responsible for the B[a]P-mediated apoptosis in PUBEC. On the other hand, while the up-regulation of CTSD is in support of the observed B[a]P-induced apoptosis, the up-regulation of VDAC2 is not. In addition to its role as a porin ion channel protein, it is also known to be involved in apoptotic signaling. The down-regulation of VDAC2 leads to mitochondrial membrane permeabilization and to apoptosis [226]. The observed up-regulation of this protein, as also supported by the results of the MMP measurements points towards the struggle of the cells to avoid cell death. Similar results were reported for 5L rat hepatoma cells exposed to TCDD [227]. The exact role of VDAC2 in mitochondrial apoptosis remains controversial. On the one hand, this protein has been implicated in forming an open pore through which cytochrome c and other proteins can be released from the mitochondrial intermembrane space to the cytosol to induce apoptosis [228]. On the other hand, it has been suggested to form a closed pore that promotes the permeabilization of the MMP by completely block the flux of metabolites [229]. In either case, further studies are required to elucidate the precise role of VDAC2 in xenobiotic-induced apoptosis.
7.4 2D BN/SDS-PAGE analysis for the identification of protein complexes involved in B[a]P toxicity

Interactions between different protein complexes are responsible for many biological processes such as cell-cell signaling, cell cycling, folding, and transport [230]. These diverse structures operate to boost signaling efficiency, ensure specificity, and increase sensitivity of the biochemical circuitry [231]. There are a variety of conventional (liquid chromatography, ultracentrifugation, and sucrose density gradient centrifugation) and non-conventional (co-immunoprecipitation, epitope-tagging, tandem affinity purification, and GST-pulldown methods available for the isolation of these multiprotein complexes [232]. However, most of these techniques often separate a population of multiprotein complex assemblies. To isolate individual complexes, further separation is required which can be achieved by 2D BN/SDS-PAGE. This technique involves the use of the anionic dye Coomassie brilliant blue instead of an ionic detergent to introduce the negative charge on the multiprotein complex. As a result, the multiple protein complexes are separated due to the sieving effect of the polyacrylamide gel, but the protein-protein interaction is still retained [233].

This approach was used to analyze the multiprotein complex interactions in the cells exposed to B[a]P and TCDD, two classic inducers of the AhR pathway, in an effort to investigate potentially alternate cellular mechanisms that may respond to these two xenobiotics. As mentioned above, B[a]P itself is nontoxic but is converted to reactive DNA adduct-forming metabolites by the AhR-induced cytochrome P450 enzyme system [234]. Unlike B[a]P, TCDD is not a substrate of this enzyme. The toxicity of this xenobiotic is related to its ability to initiate the metabolism of other toxic compounds (such as B[a]P) and to its slow detoxification from the body [235].

Before the multiprotein complexes were separated by 2D BN/SDS-PAGE, a subcellular fractionation of the exposed cells (0.5 μM B[a]P or 200 pM TCDD) was carried out by using a commercially available fractionation kit. The subcellular fractionation of the samples is necessary, because the multiprotein complexes are distributed in different subcellular compartments (such as mitochondria, Golgi apparatus, plasma membrane, and others) in addition to their presence in the cytoplasm. Subcellular fractionation leads to a concentration of these multiprotein complexes, otherwise they are difficult to identify. By using the kit, control, B[a]P- and TCDD-exposed RT4 cell lysates were separated into cytosolic proteins, membrane/organelle proteins, and nuclear proteins. Prior to the use of these fractions for
differential 2D BN/SDS-PAGE analysis, a proteome map of different fractions of non-exposed RT4 cells was generated.

MALDI-TOF-MS analysis revealed many typical protein complexes and monomeric proteins specific for these compartments (Table 9, Table 10, Table 11 see annex II). These proteins were classified according to the GO annotation of the PANTHER software and were found to be associated with a broad range of protein classes (Figure 35).

**Figure 35:** Ontological classification of the proteins present in different subcellular fractions by using the PANTHER software. The corresponding gene names of the proteins identified by MALDI-TOF-MS analysis were searched in the Uniprot database. The gene names were then uploaded into the PANTHER search engine that assigned the identified proteins to different protein classes.
After the proteome map had been generated, the gels obtained from different fractions of the exposed samples were compared with the respective control gels. By MALDI-TOF-MS analysis of the differentially regulated proteins, the expression of many calcium-containing (calmodulin, protein S100-A2, annexin A10, annexin A5, gelsolin isoform b) and iron-containing (ferritin, heme-binding protein 2, transferrin receptor protein 1) proteins were identified in TCDD-exposed subcellular fractions (Table 8), along with others proteins. Also, the expression of a unique cluster of three protein spots was observed in the cytosolic fraction of TCDD-exposed cells that was absent in control and B[a]P-exposed fractions. Two of these three protein spots were identified as calmodulin and protein S100A. Furthermore, a few calcium-containing proteins (cyclophilin B, cyclophilin A, and calreticulin) were identified in B[a]P-exposed fractions.

The modulation of intracellular calcium and iron homeostasis upon TCDD and B[a]P exposure has been observed in several studies [236-239]. The induction of CYP1A1 by AhR agonists has been shown to be abolished by inhibition of calcium movements [240, 241]. However, the mechanisms behind these interactions were largely unknown until recently, where a few studies indicated a role of the Ca\(^{2+}\)/CaM/CaMKI\(\alpha\) pathway in AhR-dependent and -independent genomic responses [242-244]. The observed expression of calmodulin in TCDD-exposed samples is also in support of these findings. In another current study a TCDD-mediated impairment of the cellular iron homeostasis associated with a changed ferritin content coupled to TfR-1 induction was reported [245]. Similar differences in the expression of iron-containing proteins (ferritin (FTH1), heme-binding protein 2 (HEBP2), transferrin (TfR-1)) were found in the TCDD-exposed samples (Table 11.1-11.2, see annex II).

Apart from this, studies indicate a cross-link between calcium and nitric oxide signaling via nitric oxide synthases (NOSs) [246, 247]. NOSs are a family of enzymes consisting of a bi-domain structure: the N-terminal oxygenase and the C-terminal reductase domain [248-250]. These enzymes are involved in the production of NO from L-arginine for which they require calmodulin binding to its C-terminal reductase domain. By that, electrons could be transferred from the reductase domain to the oxygenase domain which subsequently generate NO and citrulline as products by oxygenation of arginine [251, 252]. NO, a very important signaling molecule, is the key mediator for a wide variety of physiological processes including vasodilation, neurotransmission, and platelet aggregation [253]. Other than these functions, its
role in Fe metabolism has also been recognized [254]. Increased production of NO species in cells exposed to TCDD or BaP has been reported [255, 256]

Based upon these studies and the observed differential expression of both calcium- and iron-containing proteins, the following hypothesis was developed (Figure 36). Upon exposure to TCDD or B[a]P, a rapid increase in intracellular calcium ion concentration occurs. CaM, the major sensor for these calcium ions, becomes active and binds to the constitutive forms of NOS.

![Figure 36](image)

**Figure 36**: Pictorial representation of the hypothesis proposed for the interactions between calcium and iron. (A) Exposure to B[a]P/TCDD causes elevation of intracellular calcium which activates calmodulin (CaM). Activated CaM, along with the translocation of the aryl hydrocarbon receptor (AhR) for the induction of cytochrome P450 (CYP450) release, is also capable of causing conformation changes in nitric oxide synthase (NOS). Electrons are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There, they interact with the heme iron at the active site to catalyze the reaction of oxygen with L-arginine, generating citrulline and NO as products. NO by interacting with iron clusters of IRP1 stimulates its RNA-binding activity resulting in an increase in transferrin (TfR1) mRNA levels and a decrease in ferritin (FTH1) synthesis. The changed ferritin and TfR1 content impairs the cellular iron homeostasis, ultimately leading to significant changes in the labile iron pool (LIP).

This enzyme undergoes global conformational changes that enhance the rate of NO production. NO, except for causing cytotoxic effects, is also capable of modulating Fe-containing proteins such as the iron regulatory proteins IRPA1 and IRPA2 by direct coordination to the Fe centers of IRPA1 [254, 257]. The activated IRPA1 can promote the up-
regulation of transferrin by binding to the iron regulatory element in 3'UTR of TfR mRNA, whereas by binding to iron response element (IRE) in the 5'UTR of ferritin mRNA blocks the translation of ferritin. This ultimately leads to significant changes in the labile iron pool and hence in cellular toxicity [258, 259].

To prove this hypothesis at least in part, the intracellular calcium changes upon exposure to TCDD and B[a]P were monitored by using the fluorescence probes Fluo-4 and Rhod-2. As detailed in the results section, the transient increase in intracellular calcium concentration reported by several groups [113, 242, 243, 260, 261] was not observed during the exposure. The first changes in intracellular calcium levels were observed only after an exposure to these xenobiotics for 24 h. An intracellular calcium level elevated by 27 % was found in the TCDD-exposed samples, while a 21 % increase was observed in B[a]P-exposed cells (Figure 32). A sustained increase in intracellular calcium concentration involving cytochrome P450-mediated metabolism of xenobiotics with elevating intracellular activity was observed in some studies [179, 262, 263]. It was reported that the dihydrodiol and dihydrodiol-epoxide metabolites of B[a]P were more efficient in the up-regulation of intracellular calcium than the parent compound [254, 255 [264]. It is possible that alterations of calcium homeostasis in RT4 cells also depend on cytochrome P450-associated mechanisms: metabolic reactions in the case of B[a]P and changes in redox cycle in the case of TCDD. Moreover, it has also been reported that calmodulin is capable of performing its essential functions independent of intracellular calcium mobilization [265, 266]. In addition, the observed increases in Rhod-2 fluorescence intensity by 30 % in the TCDD-exposed RT4 cells and by 12 % in the B[a]P-exposed cells indicate an uptake of calcium from extracellular sources. The increased calcium levels in these stores may be responsible for the biological processes, which could result in cell death as observed by previous 2D proteomic studies on RT4 cells in our laboratory [267].

To find out, if the differential regulation of iron-containing proteins such as transferrin and ferritin leads to an alteration in iron homeostasis, the LIPs of B[a]P- and TCDD-exposed cells were analyzed by using the fluorescent dye PG SK. The measurements were performed after exposure periods of 3 h and 24 h, respectively, to ascertain a time-dependent effect. In B[a]P-exposed cells, the LIP was not affected after either time period, however, the LIP of TCDD-exposed cells was increased by 7 % and 13 %, respectively, after 3 h and 24 h of exposure (Figure 33). An AhR-mediated cellular iron load after exposure to TCDD has been observed in a few studies. In fact, it has been demonstrated that iron potentiates both hepatic porphyria and TCDD toxicity in susceptible mice in an oxidative process involving a disturbed activity
of iron regulatory proteins [236]. Moreover, significant changes in the expression of genes related to heme metabolism and iron homeostasis that were associated with liver injury were found in mice exposed for two or five weeks to TCDD [238, 268]. The changes in LIP as observed in RT4 cells are in support of these findings, although these changes were not as strong as reported by other groups, presumably because of the low TCDD concentrations of 200 pM used in the studies. Furthermore, the cells were blocked with the CaM antagonist W-7 to determine whether CaM was modulating the LIP. While the TCDD-mediated increase in LIP was completely inhibited in W-7-treated cells exposed to TCDD for 3 h, this inhibition was reverted in cells exposed for 24 h (Figure 33). The increase in intracellular calcium as observed after 24 h of TCDD exposure may be responsible for this effect. The experiment proved at least in part that CaM exerts some modulating effects on the LIP of the cells.

As discussed earlier, CaM is also capable of triggering the production of NO via the induction of conformational changes in NOS. The NO thus produced is capable of modulating the LIP of the cells by alternating the expression of the iron-containing proteins FTH1 and TfR-1. A quantitative measurement of NO is hard to perform as it has a half-life of only approx. 6 s [269]. However, NO is rapidly oxidized to NO$_2^-$ and NO$_3^-$, which provides a means to indirectly measure NO production. Therefore, the increased NO$_2^-$ production as measured by using the Griess assay was indicative of an NO release triggered by treatment of the cells with TCDD or B[a]P. The analyses revealed a substantial increase in NO production which amounted to 196% in the cells exposed to B[a]P and to 253% in the TCDD-exposed cells (Figure 34). Decreased basal levels of NO both in TCDD- and B[a]P-exposed RT4 cells after blocking the cells with W-7 indicated that the observed effects were CaM-controlled and thus a further support of the above hypothesis.
8. Summary

The major objective of this study was to evaluate whether the environmental contaminant benzo[a]pyrene B[a]P, one of the most important polycyclic aromatic hydrocarbons (PAH), is capable of mediating DNA damage in urinary bladder epithelial cells and hence potentially bladder carcinogenesis. To pursue this goal, effects of B[a]P on urinary bladder epithelial cells were investigated by applying a proteomic approach with the purpose of identifying proteins and pathways involved in B[a]P toxicity. First, the ability of bladder epithelial cells for B[a]P uptake and metabolism was determined. Secondly, a proteome map of primary porcine urinary bladder epithelial cells (PUBEC), the cell model used in the majority of the studies, was established as basis for comparative investigations. In the same model, investigations on time- and concentration-dependent expression changes of proteins after B[a]P exposure followed. The proteins were separated by using 2D gel electrophoresis and identified by MALDI-TOF-MS analysis in these studies. Finally, to elucidate mechanisms by which B[a]P mediates its toxicity, signaling pathways were studied in RT4 cells by using Blue Native PAGE analysis. Besides offering some insights into B[a]P-mediated toxic effects, the studies also point towards the possibility of bladder cancer development induced by B[a]P exposure.

B[a]P is a ubiquitous environmental pollutant formed during the combustion of fossil fuels, grilling, barbecuing, and smoking of food. Although much information is available on the carcinogenic properties of B[a]P, the mechanism by which this chemical is taken up by cells is still not known. In Chapter 3 of this thesis, attempts were made to investigate the dynamics of B[a]P uptake, subcellular distribution, and metabolism in PUBEC. It was found that exposure to 0.5 µM B[a]P led to an increase in intracellular concentration of B[a]P in bladder epithelial cells in a time-dependent manner but without approaching saturation. Also, a marked difference in B[a]P uptake was observed among various PUBEC pools used for the studies. Subcellular partitioning studies of B[a]P by using confocal microscopy revealed that a significant amount of B[a]P accumulated in the cell membrane of PUBEC, while only a slight but significant increase in B[a]P fluorescence intensity was observed in the cytosol and nucleus. Quantification of B[a]P uptake by bladder epithelial cells by spectrofluorometric and gas chromatographic-mass spectrometric analysis yielded intracellular concentrations ranging from 7.28 µM to 35.07 µM in cells exposed to 0.5 µM B[a]P and from 29.9 µM to 406.64 µM in cells exposed to 10 µM B[a]P. The formation of 3-OH-B[a]P in all of the B[a]P-exposed PUBEC determined by GC-MS analysis demonstrated for the first time that oxygenated B[a]P.
metabolites are actually formed in this cell model. These results indicate that bladder epithelial cells are capable of a strong accumulation and metabolic activation of B[a]P and suggest that B[a]P may act as a bladder cancer-inducing chemical. Also, the differences in B[a]P uptake by the various PUBEC pools is an explanation for the inter-individual variation in PAH toxicity as observed in humans.

Urinary bladder epithelial cells (also known as transitional epithelial cells) are the innermost cells of the bladder which are involved in accommodating the fluctuation of liquid volume in this organ and also help to protect it against caustic/toxic effects of urine. These cells are also the first ones to come in contact with urinary toxicants and thus account for 90 % of bladder cancers known as transitional cell carcinomas. As a prerequisite for proteomic studies, the first reference proteome and phosphoproteome maps of porcine bladder epithelia cells were generated by applying 2DE gel electrophoresis. This is discussed in Chapter 4. A total of 120 selected protein spots were identified by MALDI-TOF-MS analysis, among which 31 phosphoproteins were enriched by using a method based on the precipitation with lanthanum ions (La\textsuperscript{3+}). All identified proteins were bioinformatically annotated according to their physiochemical characteristic, subcellular location, and function. Most of the proteins were distributed in an area of pI 4-10 and a molecular mass range between 20 kDa and 100 kDa. The 2DE map with the complete range of expressed proteins, especially with information about phosphoproteins, provides a valuable resource for comparative proteomic analysis of normal and pathological conditions affecting the bladder function.

The studies described in Chapter 5 of the thesis deal with a series of events leading from DNA damage to apoptosis that were investigated by using a proteomic approach. 2DE gel electrophoresis mapped the differences between cells exposed to 0.5 µM B[a]P and control cells. Twenty-five differentially expressed proteins involved in DNA repair, mitochondrial dysfunction, and apoptosis were identified by MALDI-TOF-MS analysis. A concentration-dependent increase in DNA damage was observed after an exposure period of 24 h. The expression of VDAC2, CTSD, HSP27, and HSP70 indicated towards the intrinsic apoptotic mitochondrial pathway, although the analysis of mitochondrial dysfunction pointed towards an alternate pathway of cell death: The mitochondrial membrane potential (MMP), although disturbed during the initial exposure period, was nearly retained after 24 h of B[a]P treatment. In conclusion, the studies indicated DNA damage caused by B[a]P at low concentrations during an exposure period of 24 h and also shed light on a possible apoptotic mechanism induced by DNA damage.
Studies on protein-protein interactions involved in B[a]P toxicity are described in Chapter 6. A comparative analysis of proteomic complexes involving the two AhR ligands B[a]P and TCDD was carried out by using 2D BN/SDS-PAGE. For the enrichment of the protein complexes, a subcellular fractionation of unexposed cells and cells exposed to B[a]P and TCDD was carried out. BN/SDS-PAGE of these fractions revealed an effective separation of protein species and complexes of various origins, including mitochondria, plasma membrane, and intracellular compartments. The major differences in the protein maps obtained from samples of control cells and cells exposed to B[a]P and TCDD, respectively, concerned the expression of many calcium- and iron-containing proteins. On the basis of these findings, the intracellular calcium content of cells exposed to TCDD and B[a]P was evaluated, revealing an increase only after 24 h of exposure but with no transient elevation. The cells exposed to TCDD also showed an alteration in the labile iron pool (LIP) of the cells, but no such changes were observed in B[a]P-exposed cells. The increase in the LIP was strongly inhibited by the calmodulin (CaM) antagonist W-7 (10 µM). These results point towards a possible interaction between the iron and calcium signaling of the cells. The analysis of nitric oxide generation by using the Griess assay revealed a substantial increase in NO content of both B[a]P- and TCDD-exposed cells. Also in these cells, the basal NO generation was inhibited when the cells were blocked with the CaM antagonist W-7. The results led to the conclusion that alterations in calcium and iron homeostasis upon exposure to TCDD and B[a]P is linked by NO that is produced by CaM-activated nitric oxide synthase (NOS). The NO thus produced by interacting with the iron centers of IRPAs modulated the activity of TfR1 and FTH1 which in turn changed the LIP of the cells and hence the toxicity. Although some new mechanistic insights into the mechanisms of B[a]P- and TCDD-induced toxicity were provided by these studies, further investigations are still required for the validation of these initial results.
9. References


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References


References


10. Annex

10.1 Annex I - Representative 2D-gel image after B[a]P exposure

Figure 37: Representative 2D-gel image representing the differentially expressed proteins after B[a]P (0.5 µM) exposure. 450 µg of proteins from whole cell extract of control and B[a]P-exposed cells were separated by IEF (pH 3-10) and 12 % SDS-PAGE, and the resulting two dimensional protein arrays were detected by applying a Coomassie brilliant blue protocol developed in our labatory. By using the Delta2D v4.0 image analysis software, 40 proteins (numbered spots) were identified as differentially expressed. For assigning the identity of these proteins as identified by MALDI-TOF-MS, protein peptide mass matching was performed on Mascot by searching the MSDB and NCBIInr protein databases with the taxonomy pig. The proteins identified by MALDI-TOF-MS are listed in Table 7.
### 10.2 Annex II – Protein complexes of subcellular fractions of RT4 cells identified by using MALDI-TOF-MS

Table 9: Mass spectrometric data of proteins identified in the cytosolic fraction of RT4 cells, used for the generation of a proteome map (see map with ID numbers in Figure 29).

<table>
<thead>
<tr>
<th>Uniprot accession no^a</th>
<th>Spot no^c</th>
<th>Protein name^a</th>
<th>Gene name^a</th>
<th>Theoretical molecular weight / pI^a</th>
<th>Score^b</th>
<th>Peptide match^b</th>
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a  Gene name, protein name, theoretical molecular weight, and pI entries in Uniprot database for the each protein identified by MALDI-TOF-MS.
b  Score and peptides matches in Mascot search engine.
c  Experimental ID numbers.
*  If multiple spots were identified as the same protein, the protein is marked with an asterisk.
Table 10: Mass spectrometric data of proteins identified in the membrane/organelle fraction of RT4 cells, used for the generation of a proteome map (see map with ID numbers in Figure 30).

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a Gene name, protein name, theoretical molecular weight, and pI entries in Uniprot database for each protein identified by MALDI-TOF-MS.
b Score and peptides matches in Mascot search engine.
c Experimental ID numbers.
* If multiple spots were identified as the same protein, the protein is marked with an asterisk.
### Table 11: Mass spectrometric data of proteins identified in the nuclear fraction of RT4 cells, used for the generation of a proteome map.

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<td>ID-54</td>
<td>Plectin isoform 1e</td>
<td>PLEC</td>
<td>531790.73/5.74</td>
<td>89</td>
<td>15</td>
</tr>
<tr>
<td>P62805</td>
<td>ID-55</td>
<td>Histone H4</td>
<td>HIST1H4A</td>
<td>11367.34/11.36</td>
<td>101</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gene name, protein name, theoretical molecular weight, and pI entries in Uniprot database for each protein identified by MALDI-TOF-MS.

<sup>b</sup> Score and peptides matches in Mascot search engine.

<sup>c</sup> Experimental ID numbers.

* If multiple spots were identified as the same protein, the protein is marked with an asterisk.
### Table 11: List of proteins differentially expressed in the cytosolic fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 200 pM TCDD for 24 h.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Score</th>
<th>Peptide match</th>
<th>Up or down regulated</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID9</td>
<td>FLNB</td>
<td>Filamin B</td>
<td>416</td>
<td>26</td>
<td>down</td>
<td>-3.22999</td>
</tr>
<tr>
<td>ID1755</td>
<td>FTH1</td>
<td>Chain A, recombinant human H Ferritin, K86q mutant, soaked with Zn</td>
<td>83</td>
<td>27</td>
<td>down</td>
<td>-2.68431</td>
</tr>
<tr>
<td>ID34</td>
<td>NPEPPS</td>
<td>Aminopeptidase puromycin sensitive, isoform CRA_a</td>
<td>326</td>
<td>43</td>
<td>up</td>
<td>1.07601</td>
</tr>
<tr>
<td>ID203</td>
<td>S100A2</td>
<td>Protein S100-A2</td>
<td>73</td>
<td>53</td>
<td>up</td>
<td>3.8684</td>
</tr>
<tr>
<td>ID231</td>
<td>CALM1</td>
<td>Chain A, trapped intermediate of calmodulin</td>
<td>71</td>
<td>31</td>
<td>up</td>
<td>2.13139</td>
</tr>
<tr>
<td>ID12</td>
<td>FASN</td>
<td>Fatty acid synthase</td>
<td>465</td>
<td>26</td>
<td>down</td>
<td>-2.16001</td>
</tr>
<tr>
<td>ID82</td>
<td>RBBP7</td>
<td>Histone-binding protein RBBP7</td>
<td>127</td>
<td>43</td>
<td>up</td>
<td>3.65179</td>
</tr>
<tr>
<td>ID148</td>
<td>ANP32A</td>
<td>ANP32A protein</td>
<td>160</td>
<td>49</td>
<td>up</td>
<td>1.75938</td>
</tr>
<tr>
<td>ID216</td>
<td>KRT10</td>
<td>Keratin 10</td>
<td>144</td>
<td>29</td>
<td>up</td>
<td>2.15201</td>
</tr>
<tr>
<td>ID68</td>
<td>TCP1</td>
<td>Chaperonin containing TCP1, subunit 3</td>
<td>93</td>
<td>26</td>
<td>up</td>
<td>2.32344</td>
</tr>
<tr>
<td>ID162</td>
<td>CLTA</td>
<td>Clathrin light chain A isoform a</td>
<td>112</td>
<td>32</td>
<td>up</td>
<td>3.28302</td>
</tr>
<tr>
<td>ID172</td>
<td>HEBP2</td>
<td>Heme-binding protein 2</td>
<td>86</td>
<td>37</td>
<td>up</td>
<td>2.03805</td>
</tr>
<tr>
<td>ID580</td>
<td>TCP1</td>
<td>Chaperonin containing TCP1, subunit 3</td>
<td>151</td>
<td>46</td>
<td>up</td>
<td>2.17571</td>
</tr>
<tr>
<td>ID205</td>
<td>ARPC4</td>
<td>Actin-related protein 2/3 complex subunit 4 isoform a</td>
<td>94</td>
<td>46</td>
<td>down</td>
<td>-2.45396</td>
</tr>
<tr>
<td>ID117</td>
<td>KRT2</td>
<td>Keratin, type II cytoskeletal 2 epidermal</td>
<td>93</td>
<td>26</td>
<td>up</td>
<td>2.28700</td>
</tr>
<tr>
<td>ID1756</td>
<td>IQGAP1</td>
<td>Ras GTPase-activating-like protein IQGAP1</td>
<td>288</td>
<td>26</td>
<td>down</td>
<td>-2.04209</td>
</tr>
<tr>
<td>ID1757</td>
<td>IQGAP1</td>
<td>Ras GTPase-activating-like protein IQGAP1</td>
<td>324</td>
<td>29</td>
<td>down</td>
<td>-1.72557</td>
</tr>
<tr>
<td>ID291</td>
<td>TSG101</td>
<td>Chain B, Tsg101(Uev) domain In complex with ubiquitin</td>
<td>83</td>
<td>61</td>
<td>up</td>
<td>3.12498</td>
</tr>
<tr>
<td>ID229</td>
<td>KRT16</td>
<td>Keratin, type I cytoskeletal 16</td>
<td>127</td>
<td>47</td>
<td>up</td>
<td>1.75034</td>
</tr>
</tbody>
</table>

1: Experimental ID number.
2: Gene name and protein name entries in Uniprot database.
3: Score and peptides matches in Mascot search engine.
4: The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
Table 11.2: List of proteins differentially expressed in the membrane/organelle fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 200 pM TCDD for 24 h.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Score</th>
<th>Peptide match</th>
<th>Up or down regulated</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>NPEPPSL1</td>
<td>PREDICTED: aminopeptidase puromycin sensitive isoform 2</td>
<td>250</td>
<td>26</td>
<td>up</td>
<td>1.13788</td>
</tr>
<tr>
<td>37</td>
<td>ACTB</td>
<td>ACTB protein</td>
<td>169</td>
<td>51</td>
<td>up</td>
<td>2.63077</td>
</tr>
<tr>
<td>24</td>
<td>ANXA10</td>
<td>Annexin A10</td>
<td>129</td>
<td>47</td>
<td>up</td>
<td>1.02979</td>
</tr>
<tr>
<td>25</td>
<td>ANXA5</td>
<td>Annexin A5</td>
<td>142</td>
<td>46</td>
<td>up</td>
<td>1.34865</td>
</tr>
<tr>
<td>23</td>
<td>AKR1B1</td>
<td>Chain A, fidarestat bound to human aldose reductase</td>
<td>201</td>
<td>59</td>
<td>up</td>
<td>2.02283</td>
</tr>
<tr>
<td>26</td>
<td>ANP32A</td>
<td>Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A</td>
<td>166</td>
<td>48</td>
<td>up</td>
<td>2.30848</td>
</tr>
<tr>
<td>12</td>
<td>UBA1</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>325</td>
<td>40</td>
<td>up</td>
<td>1.01613</td>
</tr>
<tr>
<td>15</td>
<td>KRT9</td>
<td>Cytokeratin 9</td>
<td>103</td>
<td>50</td>
<td>up</td>
<td>1.30835</td>
</tr>
<tr>
<td>16</td>
<td>GSN</td>
<td>Gelsolin isofrm b</td>
<td>96</td>
<td>23</td>
<td>up</td>
<td>1.08039</td>
</tr>
<tr>
<td>17</td>
<td>PDIA4</td>
<td>Protein disulfide-isomerase A4 precursor</td>
<td>303</td>
<td>45</td>
<td>up</td>
<td>1.18829</td>
</tr>
<tr>
<td>19</td>
<td>ERO1LB</td>
<td>Endoplasmic reticulum oxidoreductin 1</td>
<td>132</td>
<td>43</td>
<td>up</td>
<td>1.72305</td>
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<tr>
<td>18</td>
<td>CHRN1B</td>
<td>Cholinergic receptor, nicotinic, epsilon, isoform CRA_a</td>
<td></td>
<td></td>
<td>up</td>
<td>1.49665</td>
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<tr>
<td>20</td>
<td>GLUD1</td>
<td>Chain A, structure Of human glutamate dehydrogenase-apo form</td>
<td>245</td>
<td>51</td>
<td>up</td>
<td>1.16010</td>
</tr>
<tr>
<td>21</td>
<td>SHMT2</td>
<td>Serine hydroxymethyltransferase, mitochondrial isoform 3</td>
<td>139</td>
<td>43</td>
<td>down</td>
<td>-2.27026</td>
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<tr>
<td>42</td>
<td>EIF3A</td>
<td>Eukaryotic translation initiation factor 3, subunit A</td>
<td>135</td>
<td>19</td>
<td>down</td>
<td>-2.14621</td>
</tr>
<tr>
<td>3</td>
<td>NME2</td>
<td>Nm23 human nucleoside diphosphate kinase B complexed with Gdp</td>
<td>161</td>
<td>78</td>
<td>down</td>
<td>-1.09288</td>
</tr>
<tr>
<td>5</td>
<td>TFRC</td>
<td>Transferrin receptor protein 1</td>
<td>91</td>
<td>17</td>
<td>up</td>
<td>1.35857</td>
</tr>
<tr>
<td>6</td>
<td>TFRC</td>
<td>Transferrin receptor protein 1</td>
<td>113</td>
<td>24</td>
<td>up</td>
<td>1.35857</td>
</tr>
<tr>
<td>7</td>
<td>KRT1</td>
<td>Keratin 1</td>
<td>83</td>
<td>23</td>
<td>up</td>
<td>1.15404</td>
</tr>
<tr>
<td>33</td>
<td>PSMB9</td>
<td>Proteasome (prosome, macropain) subunit, beta type, 1</td>
<td>90</td>
<td>51</td>
<td>down</td>
<td>-1.42373</td>
</tr>
<tr>
<td>34</td>
<td>PRDX1</td>
<td>Peroxiredoxin-1</td>
<td>77</td>
<td>49</td>
<td>down</td>
<td>-1.93899</td>
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<tr>
<td>40</td>
<td>PSMA3</td>
<td>Proteasome (prosome, macropain) subunit, alpha type, 3</td>
<td>78</td>
<td>45</td>
<td>down</td>
<td>-2.34886</td>
</tr>
</tbody>
</table>

1: Experimental ID number.
2: Gene name and protein name entries in Uniprot database.
3: Score and peptides matches in Mascot search engine.
4: The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
Table 11.3: List of proteins differentially expressed in the nuclear fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 200 pM TCDD for 24 h.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Score</th>
<th>Peptide match</th>
<th>Up or down regulated</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID-359867</td>
<td>MYH9</td>
<td>Myosin-9</td>
<td>166</td>
<td>20</td>
<td>up</td>
<td>3.57206</td>
</tr>
<tr>
<td>ID-359670</td>
<td>HIST1H1E</td>
<td>Histone H1.4</td>
<td>79</td>
<td>36</td>
<td>up</td>
<td>2.45941</td>
</tr>
<tr>
<td>ID-359484</td>
<td>POLI</td>
<td>Human polymerase iota Ubm2- ubiquitin complex</td>
<td>83</td>
<td>77</td>
<td>up</td>
<td>6.25469</td>
</tr>
<tr>
<td>ID-359483</td>
<td>TSG101</td>
<td>Chain B, Tsg101(Uev) domain in complex with ubiquitin</td>
<td>73</td>
<td>61</td>
<td>up</td>
<td>3.05263</td>
</tr>
<tr>
<td>ID-359479</td>
<td>PHB</td>
<td>Prohibitin</td>
<td>89</td>
<td>42</td>
<td>up</td>
<td>2.10602</td>
</tr>
<tr>
<td>ID-359476</td>
<td>VDAC1</td>
<td>Porin 31HM</td>
<td>121</td>
<td>58</td>
<td>up</td>
<td>1.89122</td>
</tr>
<tr>
<td>ID-359470</td>
<td>RPL10A</td>
<td>60S ribosomal protein L7</td>
<td>91</td>
<td>35</td>
<td>up</td>
<td>2.35149</td>
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<tr>
<td>ID-359480</td>
<td>SRSF9</td>
<td>Serine/arginine-rich splicing factor 9</td>
<td>90</td>
<td>42</td>
<td>up</td>
<td>2.83745</td>
</tr>
</tbody>
</table>

1. Experimental ID number.
2. Gene name and protein name entries in Uniprot database.
3. Score and peptides matches in Mascot search engine.
4. The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
Table 11-4: List of proteins differentially expressed in the cytosolic fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 0.5 μM B[a]P for 24 h.

<table>
<thead>
<tr>
<th>Spot no1</th>
<th>Gene name2</th>
<th>Protein name2</th>
<th>Score3</th>
<th>Peptide match3</th>
<th>Up or down regulated</th>
<th>Regulation4</th>
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<tr>
<td>ID22326</td>
<td>PFN1</td>
<td>Profilin-1</td>
<td>147</td>
<td>58</td>
<td>down</td>
<td>1.87683</td>
</tr>
<tr>
<td>ID22471</td>
<td>TBCD</td>
<td>Tubulin-specific chaperone d, isoform CRA_c</td>
<td>161</td>
<td>24</td>
<td>up</td>
<td>1.88427</td>
</tr>
<tr>
<td>ID22486</td>
<td>VCP</td>
<td>Transitional endoplasmic reticulum ATPase</td>
<td>234</td>
<td>46</td>
<td>up</td>
<td>1.92522</td>
</tr>
<tr>
<td>ID22491</td>
<td>ALDH1L1</td>
<td>10-formyltetrahydrofolate dehydrogenase</td>
<td>423</td>
<td>51</td>
<td>up</td>
<td>2.1961</td>
</tr>
<tr>
<td>ID22515</td>
<td>KRT2</td>
<td>Keratin, type II cytoskeletal 2 epidermal</td>
<td>68</td>
<td>17</td>
<td>down</td>
<td>-2.29904</td>
</tr>
<tr>
<td>ID22516</td>
<td>CCT2</td>
<td>T-complex protein 1 subunit beta isoform 2</td>
<td>131</td>
<td>44</td>
<td>up</td>
<td>2.12253</td>
</tr>
<tr>
<td>ID22545</td>
<td>IDH1</td>
<td>Heterodimeric R132h mutant of human cytosolic NADP(+) dependent isocitrate dehydrogenase in complex with NADP and isocitrate</td>
<td>240</td>
<td>53</td>
<td>down</td>
<td>-1.81488</td>
</tr>
<tr>
<td>ID22596</td>
<td>NME2</td>
<td>Nucleoside diphosphate kinase B isoform a</td>
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<td>down</td>
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<tr>
<td>ID22613</td>
<td>FABP4</td>
<td>Human adipocyte fatty acid binding protein</td>
<td>91</td>
<td>59</td>
<td>up</td>
<td>4.13207</td>
</tr>
<tr>
<td>ID24295</td>
<td>PREP</td>
<td>Prolyl endopeptidase</td>
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<td>44</td>
<td>up</td>
<td>2.25443</td>
</tr>
<tr>
<td>ID24958</td>
<td>ATIC</td>
<td>Bifunctional purine biosynthesis protein PURH</td>
<td>203</td>
<td>50</td>
<td>up</td>
<td>-2.04968</td>
</tr>
<tr>
<td>ID25983</td>
<td>LDHA</td>
<td>Lactate dehydrogenase A variant</td>
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<td>31</td>
<td>down</td>
<td>-1.20712</td>
</tr>
<tr>
<td>ID22033</td>
<td>PRDX6</td>
<td>Peroxiredoxin-6</td>
<td>92</td>
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<td>up</td>
<td>2.25032</td>
</tr>
<tr>
<td>ID22510</td>
<td>TCP1</td>
<td>T-complex protein 1 subunit alpha isoform a</td>
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<td>31</td>
<td>up</td>
<td>3.59029</td>
</tr>
<tr>
<td>ID26273</td>
<td>PRDX6</td>
<td>Peroxiredoxin-6</td>
<td>153</td>
<td>64</td>
<td>down</td>
<td>-2.27703</td>
</tr>
</tbody>
</table>

1. Experimental ID number.
2. Gene name and protein name entries in Uniprot database.
3. Score and peptides matches in Mascot search engine.
4. The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
Table 11.5: List of proteins differentially expressed in the cytosolic fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 0.5 μM B[a]P for 24 h.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Score</th>
<th>Peptide match</th>
<th>Up or down regulated</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP2-06</td>
<td>CLTC</td>
<td>Clathrin heavy chain 1 isoform 6</td>
<td>342</td>
<td>27</td>
<td>up</td>
<td>1.73274</td>
</tr>
<tr>
<td>BaP2-15</td>
<td>PDIA4</td>
<td>Protein disulfide-isomerase A4 precursor</td>
<td>329</td>
<td>47</td>
<td>up</td>
<td>2.39697</td>
</tr>
<tr>
<td>BaP2-23</td>
<td>UGDH</td>
<td>UDP-glucose 6-dehydrogenase isoform 1</td>
<td>167</td>
<td>48</td>
<td>up</td>
<td>1.82155</td>
</tr>
<tr>
<td>BaP2-26</td>
<td>ALDOA</td>
<td>Fructose 1,6-bisphosphate aldolase complexed with fructose 1,6-bisphosphate</td>
<td>273</td>
<td>66</td>
<td>down</td>
<td>-1.82195</td>
</tr>
<tr>
<td>BaP2-30</td>
<td>ANXA10</td>
<td>Annexin A10</td>
<td>82</td>
<td>30</td>
<td>down</td>
<td>-3.95467</td>
</tr>
<tr>
<td>BaP2-33</td>
<td>TPM3</td>
<td>Tropomyosin alpha-3 chain isoform 3</td>
<td>103</td>
<td>45</td>
<td>down</td>
<td>-2.92923</td>
</tr>
<tr>
<td>BaP2-39</td>
<td>PPIB</td>
<td>Cyclophilin B complexed with cyclosporin</td>
<td>116</td>
<td>57</td>
<td>up</td>
<td>1.74984</td>
</tr>
<tr>
<td>BaP2-43</td>
<td>PPIA</td>
<td>Cyclophilin A complexed with dipeptide Gly-Pro</td>
<td>85</td>
<td>67</td>
<td>up</td>
<td>3.41278</td>
</tr>
<tr>
<td>BaP2-47</td>
<td>PFN1</td>
<td>Profilin complexed with An L-Pro10-iodotyrosine peptide</td>
<td>113</td>
<td>58</td>
<td>up</td>
<td>1.76324</td>
</tr>
<tr>
<td>BaP2-07</td>
<td>UBA1</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>279</td>
<td>36</td>
<td>up</td>
<td>2.67377</td>
</tr>
<tr>
<td>BaP2-16</td>
<td>P4HA1</td>
<td>Procollagen-proline, 2-oxoglutarate 4-dioxygenase</td>
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<td>57</td>
<td>up</td>
<td>7.82073</td>
</tr>
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<td>BaP2-18</td>
<td>UBA1</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
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<td>34</td>
<td>down</td>
<td>-1.7525</td>
</tr>
<tr>
<td>BaP2-19</td>
<td>CALR</td>
<td>Calreticulin precursor variant</td>
<td>125</td>
<td>43</td>
<td>up</td>
<td>2.15407</td>
</tr>
<tr>
<td>BaP2-20</td>
<td>GPI</td>
<td>Glucose-6-phosphate isomerase isoform 1</td>
<td>123</td>
<td>27</td>
<td>down</td>
<td>-3.0432</td>
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<td>BaP2-22</td>
<td>ATP5A1</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1</td>
<td>182</td>
<td>33</td>
<td>up</td>
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<td>47</td>
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<td>Bap2-31</td>
<td>GNPDA1</td>
<td>Glucosamine-6-phosphate isomerase 1</td>
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<td>64</td>
<td>down</td>
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<td>UBA1</td>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
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<td>37</td>
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<td>Bap2-42</td>
<td>PPIA</td>
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<td>up</td>
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<td>Keratin, type I cytoskeletal 9</td>
<td>108</td>
<td>32</td>
<td>up</td>
<td>2.62558</td>
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</tbody>
</table>

1: Experimental ID number.
2: Gene name and protein name entries in Uniprot database.
3: Score and peptides matches in Mascot search engine.
4: The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
Table 11.6: List of proteins differentially expressed in the nuclear fraction of RT4 cells identified by MALDI-TOF-MS after exposure to 0.5 µM B[a]P for 24 h.

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<tr>
<th>Spot no</th>
<th>Gene name</th>
<th>Protein name</th>
<th>Score</th>
<th>Peptide match</th>
<th>Up or down regulated</th>
<th>Regulation</th>
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<td>KRT19</td>
<td>Keratin, type I cytoskeletal 19</td>
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<td>5</td>
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<td>Heterogeneous nuclear ribonucleoprotein A1</td>
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<td>42</td>
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<td>6</td>
<td>VDAC1</td>
<td>Porin 31HM [human, skeletal muscle membranes</td>
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<td>7</td>
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<td>8</td>
<td>PHB</td>
<td>Prohibitin</td>
<td>77</td>
<td>38</td>
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<td>9</td>
<td>NDUFV1</td>
<td>NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial precursor</td>
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<td>Chain X, E2-ubiquitin-Hect</td>
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<td>60S ribosomal protein L10a</td>
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<td>Ubiquitin-conjugating enzyme E2 K</td>
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<td>27</td>
<td>DHX9</td>
<td>Nuclear DNA helicase II</td>
<td>152</td>
<td>19</td>
<td>up</td>
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</tbody>
</table>

1: Experimental ID number.
2: Gene name and protein name entries in Uniprot database.
3: Score and peptides matches in Mascot search engine.
4: The values represent the ratio of the relative spot volume of treated and control cells as determined by using Delta2D v4.0 software.
10.4 Annex IV – Representative 2D BN/PAGE gel images after exposure to B[a]P and TCDD

Figure 38: 2D BN/PAGE gels representing proteins differentially expressed in the cytosolic fraction of RT4 cells after 24 h exposure to [A] TCDD (200 pM) or [B] B[a]P (0.5 µM). 75 µg of proteins from cytosolic fraction of control, B[a]P- and TCDD-exposed cells were separated by 4-12 % BN-PAGE and 12 % SDS-PAGE. The resulting protein arrays were detected by applying a Coomassie brilliant blue protocol developed in our laboratory. By using the Delta2D v4.0 image analysis software, numbered spots with arrows were identified as differentially expressed. For assigning the identity of these proteins, protein peptide mass matching was performed on Mascot by searching MSDB and NCBInr protein databases with the taxonomy homo sapiens. The proteins identified by MALDI-TOF-MS are listed in Table 11.1 (TCDD) and 11.4 (B[a]P).
Figure 39: 2D BN/PAGE gels representing proteins differentially expressed in the membrane/organelle fraction of RT4 cells after 24 h exposure to [A] TCDD (200 pM) or [B] B[a]P (0.5 µM). 75 µg of proteins from cytosolic fraction of control, B[a]P- and TCDD-exposed cells were separated by 4-12 % BN-PAGE and 12 % SDS-PAGE. The resulting protein arrays were detected by applying a Coomassie brilliant blue protocol developed in our laboratory. By using the Delta2D v4.0 image analysis software, numbered spots with arrows were identified as differentially expressed. For assigning the identity of these proteins, protein peptide mass matching was performed on Mascot by searching MSDB and NCBIinr protein databases with the taxonomy *homo sapiens*. The proteins identified by MALDI-TOF-MS are listed in Table 11.2 (TCDD) and 11.5 (B[a]P).
Figure 40: 2D BN/PAGE gels representing proteins differentially expressed in the nuclear fraction of RT4 cells after 24 h exposure to [A] TCDD (200 pM) or [B] B[a]P (0.5 µM). 75 µg of proteins from cytosolic fraction of control, B[a]P- and TCDD-exposed cells were separated by 4-12 % BN-PAGE and 12 % SDS-PAGE. The resulting protein arrays were detected by applying a Coomassie brilliant blue protocol developed in our laboratory. By using the Delta2D v4.0 image analysis software, numbered spots with arrows were identified as differentially expressed. For assigning the identity of these proteins, protein peptide mass matching was performed on Mascot by searching MSDB and NCBI protein databases with the taxonomy Homo sapiens. The proteins identified by MALDI-TOF-MS are listed in Table 11.3 (TCDD) and 11.6 (B[a]P).
10.5 Annex V - Cell culture media, reagents, and instrumentation

Medium for PUBEC

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<thead>
<tr>
<th>Medium</th>
<th>Suppliers</th>
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<tr>
<td>472 ml (500 – 28 mL) F-12 medium</td>
<td>C.C. Pro GmbH, Oberdorla, Germany</td>
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<tr>
<td>Supplements</td>
<td></td>
</tr>
<tr>
<td>2.5 ml Amphotericin (1.25 µg/mL)</td>
<td>C.C. Pro GmbH, Oberdorla, Germany</td>
</tr>
<tr>
<td>2.5 ml L-Glutamine (146 µg/mL)</td>
<td>PAA GmbH, Cölbe, Germany</td>
</tr>
<tr>
<td>2.5 mL Transferrin (5 µg/mL)</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>5.0 mL NEAA (0.1 mM)</td>
<td>GIBCO, Darmstadt, Germany</td>
</tr>
<tr>
<td>5.0 mL Insulin (10 µg/mL)</td>
<td>PAA GmbH, Cölbe, Germany</td>
</tr>
<tr>
<td>5.0 mL Glucose (2.7 mg/mL)</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>5.0 mL Penicillin-streptomycin (100 U/100 µg/mL)</td>
<td>GIBCO, Darmstadt, Germany</td>
</tr>
<tr>
<td>0.5 mL Hydrocortisone (1 µg/mL)</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>50 µL Epidermal growth factor (20 ng/mL)</td>
<td>Sigma, Taufkirchen, Germany</td>
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Medium for RT4 cell line

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<tr>
<th>Medium</th>
<th>Suppliers</th>
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<tr>
<td>441.3 ml (500 – 58.7 mL) McCoy’s medium</td>
<td>C.C. Pro GmbH, Oberdorla, Germany</td>
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<tr>
<td>Supplements</td>
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<tr>
<td>3.7 ml L-Glutamine (1.5 mM)</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
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<td>5.0 mL Penicillin-streptomycin (100 U/100 µg/mL)</td>
<td>GIBCO, Darmstadt, Germany</td>
</tr>
<tr>
<td>50 mL Fetal bovine serum (10 %)</td>
<td>GIBCO, Darmstadt, Germany</td>
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### Instrumentation

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<th>Equipment</th>
<th>Supplier</th>
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<td>Amersham Bioscience, Freiburg, Germany</td>
</tr>
<tr>
<td>Ettan DALTsix electrophoresis system</td>
<td></td>
</tr>
<tr>
<td>IPG-Stripe (3-10 NL)</td>
<td></td>
</tr>
<tr>
<td>Isoelectric focusing system</td>
<td></td>
</tr>
<tr>
<td>Gel-Custer</td>
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<tr>
<td>MALDI-TOF-MS (Voyager-STR)</td>
<td>Applied Biosystems, Foster City (USA)</td>
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<tr>
<td>Mass Spec Turbo 192 CHCA Chip</td>
<td>Qiagen, Hilden</td>
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<td>Centrifuges: Allegra 6R</td>
<td>Beckman Coulter, Krefeld, Germany</td>
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<tr>
<td>Allegra X15R</td>
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<td>Ultracentrifuge L8-M</td>
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<tr>
<td>Scanner (ScanMaker 9800XL)</td>
<td>Mikrotek, Willich, Germany</td>
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<td>Genios Multi-Detection Microplate Reader</td>
<td>Tecan, Mainz, Germany</td>
</tr>
<tr>
<td>Confocal microscope</td>
<td>LSM 510, Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Gas Chromatograph</td>
<td>HP 6890/5973, Agilent Technologies, Waldbronn, Germany</td>
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<tr>
<td>Leica DMLB, Fluorescence Microscope</td>
<td>Bensheim, Germany</td>
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<tr>
<td>Spectrofluorometer</td>
<td>Varian Cary Eclipse, Varian, Palo Alto, CA</td>
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### Software

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<th>Version</th>
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<td>Delta2D</td>
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<td>Decodon, Greifswald, Germany</td>
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<td>Matrix Science, London, England</td>
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<td>Comet assay IV</td>
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<td>Perspective Instruments, UK</td>
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<tr>
<td>Leica application suite</td>
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<td>Leica Microsystems CMS GmbH, Switzerland</td>
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## Kits and dyes used for the study

### Kit

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<th>Kit</th>
<th>Cat no</th>
<th>Supplier/Location</th>
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<td>Subcellular Proteome Extraction Kit</td>
<td>539790, ProteoExtract&lt;sup&gt;®&lt;/sup&gt;, Merck KGaA, Darmstadt, Germany</td>
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### Dyes

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<th>Supplier/Location</th>
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<tr>
<td>Calcein, AM</td>
<td>C3100MP, Molecular Probes&lt;sup&gt;®&lt;/sup&gt;, Invitrogen, Darmstadt, Germany</td>
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<tr>
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<td>Rhod-2/AM</td>
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<tr>
<td>Rhodamine 123</td>
<td>R302, Molecular Probes&lt;sup&gt;®&lt;/sup&gt;, Invitrogen, Darmstadt, Germany</td>
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### Chemicals

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<td>Sucrose</td>
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Annex

Kits and dyes used for the study

<table>
<thead>
<tr>
<th>Kit</th>
<th>Cat no</th>
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<tr>
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### Dyes

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<tr>
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<td>PK-CA707-50024, PromoKine, Heidelberg, Germany</td>
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<tr>
<td>Rhodamine 123</td>
<td>R302, Molecular Probes&lt;sup&gt;®&lt;/sup&gt;, Invitrogen, Darmstadt, Germany</td>
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<tr>
<td>Bromphenol blue</td>
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<tr>
<td>Acrylamid-Bis solution (30 % Acrylamid, 0,4 % Bis)</td>
<td></td>
<td>BioRad, München, Germany</td>
</tr>
<tr>
<td>Penicillin-streptomycin</td>
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</tr>
<tr>
<td>Trypsin (cell culture)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo[alpyrene (B[a]P)</td>
<td></td>
<td></td>
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<tr>
<td>Iodacetamide (IAA)</td>
<td></td>
<td>GE-Healthcare, München, Germany</td>
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<tr>
<td>Phosphate-buffered saline</td>
<td></td>
<td></td>
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<tr>
<td>1-Butanol</td>
<td></td>
<td></td>
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<tr>
<td>Coomassie brilliant blue G 250</td>
<td></td>
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<tr>
<td>Ethanol</td>
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<tr>
<td>Phosphoric acid 85 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluoroacetic acid (TFA)</td>
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<td></td>
</tr>
<tr>
<td>Fetal calf serum (FCS)</td>
<td></td>
<td>PAA Laboratories, Pasching, Austria</td>
</tr>
<tr>
<td>Dry strip cover fluid (oil for IEF)</td>
<td></td>
<td>Pharmacia Biotech, Uppsala, Sweden</td>
</tr>
<tr>
<td>Aluminium sulfate</td>
<td></td>
<td></td>
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<tr>
<td>Complete™, Protease Inhibitor Cocktail tablets</td>
<td></td>
<td>Roche, Mannheim, Germany</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td></td>
<td>SERVA, Heidelberg, Germany</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td></td>
<td></td>
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<tr>
<td>Ammonium bicarbonate</td>
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<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
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<tr>
<td>Ethylene diamine tetraacetate (EDTA)</td>
<td></td>
<td>Sigma, Taufkirchen, Germany</td>
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<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Chemicals</td>
<td>Supplier</td>
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<tr>
<td>-----------------------------------------------</td>
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<tr>
<td>Thiourea</td>
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<tr>
<td>Trypsin (proteomic grade)</td>
<td></td>
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<tr>
<td>Tris(hydroxymethyl)-aminomethan (Tris)</td>
<td>Sigma, Taukirchen, Germany</td>
<td></td>
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<tr>
<td></td>
<td>USB Corporation, Cleveland, USA</td>
<td></td>
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<tr>
<td>Urea</td>
<td>USB Corporation, Cleveland, USA</td>
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<tr>
<td>3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)</td>
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</tr>
</tbody>
</table>
10.6 Annex VI - Curriculum Vitae

Dr. rer. Nat. Nisha Verma
M.Sc. (Biotechnology), M.Phil. (Biotechnology),
PhD in Natural Sciences

CORRESPONDENCE:
Removed for reasons of data protection

PUBLICATIONS

Published / Accepted for publication


Submitted

Abstracts


Oral* / Poster** Presentations


2. **Verma N. (2011) Protein complexes as downstream targets of AhR signaling and the possible role of calcium and iron in TCDD mediated toxicity. 17 Workshop of...**
Annex

Zell- und Gewebeschädigung: Mechanismen, Protektion und Therapie, Xanthen, Germany.


Conferences* / Workshops** Attended

1. **Membrane proteomics by Blue Native PAGE** (26-28\textsuperscript{th} May, 2010) Hannover University, Germany.

2. **Workshop on protein analysis of tissues** (06-07\textsuperscript{th} March, 2009) Helmholtz Zentrum, München, Germany.

3. **Nanotechnology & Biotechnology** (26-29\textsuperscript{th} March, 2006) Assocham global knowledge millennium summit-IV, New Delhi, India.

4. **Strategy for sustainable development of Himachal Pradesh with special emphasis on industry, tourism, Biotech & IT** (6-7\textsuperscript{th} November, 2006) Shimla, India.

5. **Entrepreneurship development programme in Biotechnology** (11-15\textsuperscript{th} June, 2007) Shimla, India.
RESEARCH EXPERIENCES

   Research Advisors: Prof. A.W. Rettenmeier & Dr. S. Schmitz-Spanke, Institute of Hygiene and Occupational Medicine, University Hospital Essen, Essen, Germany

2. M.Phil. Thesis title: Isolation and screening of Glutaminase producing microbes
   Research Advisors: Dr. Duni Chand, Department of Biotechnology, H.P. University, Shimla.

   Supervisor: Dr. I.D. Garg, Head of the Department, Department of Plant Pathology, Central Potato Research Institute (CPRI), Shimla-171001.

PROJECT COMPLETED


SPECIAL TRAININGS

1. Deutsch I (A1/1) language course (completed). Institute für Optional Studien, Universität Duisburg-Essen, Germany (September 2008 to February 2009).
2. Training on Tools & Techniques used in Biotechnology. Department of Experimental Medical Education, Post Graduate Institute of Research and Education, Chandigarh (PGIMER), India (June-2003 to July 2003).
10.7 Annex VII - Acknowledgement

It gives me immense pleasure to express my heartiest thanks and deep sense of gratitude to my esteemed guides Prof. Rettenmeier and Dr. Schmitz-Spanke for their intellectual stimulation, expert guidance, continued encouragement, and constructive suggestions, throughout the course of this investigation. I am extremely grateful to them for their keen interest, ever available help and unstained support which were a source of great inspiration and helped me a lot in conducting this work successfully.

I owe my deepest thank to all the members of our working group. I would specially like to thank Fr. Rehn, Fr. Stempelmann, Fr. Jeske, Fr. Biermann, and Hr. Diedrich for their technical support.

I am also thankful to Dr. Mosel, Prof. Dopp and all the non-technical staff of Institute for Hygiene and Occupational medicine for their cooperation throughout my work.

I extend my sincere thanks to Prof. de Groot and Dr. Petrat, Institute of Physiological Chemistry for opening the door of their institute for me. I specially would like to thank Dr. Petrat for his excellent scientific guidance. His exhaustive knowledge about iron metabolism and expertise in confocal microscopy has supported me throughout this work. I would also like to thank Dr. Effenberger-Neidnicht for introducing me to the Griess assay.

I also wish to express my sincere appreciation to Prof. Iliakis, Institute of Radiation Biology, for letting me use the facilities of his institute. I would specifically like to thank Dr. Mladenov for his technical assistance.

Special thanks go to my colleague and friend Mario, for all the help, all the work we’ve done together and all the great scientific and nonscientific debate we had. PhD work would never have been so much exciting without him.

Thanks go to Ricarda, Jessica, Heeda and Anna for your friendship and great environment during my stay in the department. I would particularly like to thank Ricarda for your valuable friendship, long walks, many relaxing moments, and enjoyable conversations.

I am also thankful to my friends Kunal, Aashish, Neri, Satyender, Savita, Rohit, Aparaná, Janapriya, Pooja, Stefan, and Deepa for many enjoyable evenings and a wonderful time in Germany. I felt like home with all you people around.

My dearest thanks are due to my revered parents, loving sisters and brother for their affection, inspiration, and moral support.

Last but not the least I fall short of words to thank my husband, my best friend Shree for giving me the confidence to explore my research interests and for believing in me. I am intellectually indebted to his ideas and our conversations. None of this could have happened without him. This thesis is dedicated to him.
10.8 Annex VIII - Declaration

Erklärung:

Essen, den 4. September 2012 ..................................................
(Prof. A.W. Rettenmeier)

Erklärung:
Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den 4. September 2012 ..................................................
(Nisha Verma)

Erklärung:

Essen, den 4. September 2012 ..................................................
(Nisha Verma)