Differential Cell Type-Specific Transcriptional Regulation of the *CYP1A1* Gene

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Vorgelegt von Magdalena Adamska
geboren in Kepno

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ABBREVIATIONS

ABCD	avidin-biotin complex on DNA
AHR	aryl hydrocarbon receptor
AHRR	AHR repressor
AP	alkaline phosphatase
APS	ammonium persulfate
ARNT	aryl hydrocarbon nuclear translocator
ATP	adenosine triphosphate
B[a]P	benzo[a]pyrene
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bHLH	basic-helix-loop-helix
BSA	bovine serum albumine
BTE	basic transcription element
cAMP	cyclic adenosine monophosphate
CYP	cytochrome P-450-dependent monooxygenase
CYP1A1	cytochrome P450 1A1
CYP1A1	gene coding for CYP1A1 enzyme
db-cAMP	N^6,$O^{2'}$-dibutyryl cyclic adenosine-3',5'-monophosphate
DMSO	dimethylsulfoxide
DRE	dioxin responsive element
DTT	dithiothreitol
EDTA	ethylendiamine N,N,N,N-tetraacetic acid
EMSA	electromobility shift assay
EROD	ethoxyresorufin-*o*-deethylase
FCS	fetal calf serum
GC-rich	guanosine/cytosine-rich
GST	Glutathione S-transferase
H89	*N*-[2-(*p*-bromocynamylamino)ethyl]-5-isoquinolinesulfonamide
HAH	halogenated aromatic hydrocarbon

HAK	halogenierten aromatischen Kohlenwasserstoffen
HAT	histone acetyltransferase
HDAC	histone deacetylase
Hsp	heat shock protein
mSin3	mammalian homolog of yeast Sin3
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrasolium bromide; thiazolyl blue
NaBu	sodium butyrate
NBT	4-nitro blue tetrazolium chloride
NCoR	nuclear receptor corepressor
NF-1	nuclear factor 1
NLS	nuclear localization signal
NRE	negative regulatory element
PAH	polycyclic aromatic hydrocarbon
PAS	stems from the names of the PER, ARNT, SIM proteins
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PVDV	polyvinylidene fluoride
RLU	relative luciferase units
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMRT	silencing mediator for retinoid and thyroid hormone receptor
Sp1	specific protein 1
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'-tetramethylethylene diamine
TF	transcription factor
TSA	trichostatin A
UGT	uridine diphosphate glucuronosyltransferase
XME	xenobiotic metabolizing enzyme
XRE	xenobiotic responsive elements

1 SUMMARY

Cytochrome P450 1A1 (CYP1A1) monooxygenase plays an important role in the metabolism of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and halogenated polycyclic aromatic hydrocarbons (HAHs). Oxidation of these compounds converts them to the metabolites that subsequently can be conjugated to hydrophilic endogenous entities e.g. glutathione. Derivates generated in this way are water soluble and can be excreted in bile or urine, which is a defense mechanism. Besides detoxification, metabolism by CYP1A1 may lead to deleterious effects since the highly reactive intermediate metabolites are able to react with DNA and thus cause mutagenic effects, as it is in the case of benzo(a) pyrene (B[a]P).

CYP1A1 is normally not expressed or expressed at a very low level in the cells but it is inducible by many PAHs and HAHs e.g. by B[a]P or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Transcriptional activation of the *CYP1A1* gene is mediated by aryl hydrocarbon receptor (AHR), a basic-helix-loop-helix (bHLH) transcription factor. In the absence of a ligand AHR stays predominantly in the cytoplasm. Ligand binding causes translocation of AHR to the nuclear compartment, its heterodimerization with another bHLH protein, the aryl hydrocarbon nuclear translocator (ARNT) and binding of the AHR/ARNT heterodimer to a DNA motif designated dioxin responsive element (DRE). This process leads to the transcriptional activation of the responsive genes containing DREs in their regulatory regions, e.g. that coding for CYP1A1. TCDD is the most potent known agonist of AHR. Since it is not metabolized by the activated enzymes, exposure to this compound leads to a persisting activation of AHR resulting in diverse toxic effects in the organism.

To enlighten the molecular mechanisms that mediate the toxicity of xenobiotics like TCDD and related compounds, the AHR-dependent regulation of the *CYP1A1* gene was investigated in two cell lines: human cervix carcinoma (HeLa) and mouse hepatoma (Hepa).

Study of AHR activation and its consequence concerning expression of the CYP1A1 enzyme confirmed the TCDD-dependent formation of the AHR/ARNT complex on DRE leading to an increase of the *CYP1A1* transcription in Hepa cells. In contrast, in HeLa cells formation of the AHR/ARNT heterodimer and binding of a protein complex containing AHR and ARNT to

DRE occurred naturally in the absence of TCDD. Moreover, treatment with TCDD did not affect the AHR/ARNT dimer formation and binding of these proteins to DRE in these cells. Even though the constitutive complex on DRE exists in HeLa, transcription of the *CYP1A1* gene was not increased. Furthermore, the CYP1A1 level in HeLa cells remained unchanged in the presence of TCDD suggesting repressional mechanism of the AHR complex function which may hinder the TCDD-dependent mechanisms in these cells. Similar to the native, the mouse *CYP1A1*-driven reporter constructs containing different regulatory elements were not inducible by TCDD in HeLa cells, which supported a presence of cell type specific *trans*-acting factor in HeLa cells able to repress both the native *CYP1A1* and *CYP1A1*-driven reporter genes rather than species specific differences between *CYP1A1* genes of human and rodent origin.

The different regulation of the AHR-mediated transcription of *CYP1A1* gene in Hepa and HeLa cells was further explored in order to elucidate two aspects of the AHR function: (I) mechanism involved in the activation of AHR in the absence of exogenous ligand and (II) factor that repress function of the exogenous ligand-independent AHR/ARNT complex.

Since preliminary studies revealed that the activation of PKA causes an activation of AHR in Hepa cells in the absence of TCDD, the PKA-dependent signalling pathway was the proposed endogenous mechanism leading to the TCDD-independent activation of AHR in HeLa cells. Activation of PKA by forskolin or db-cAMP as well as inhibition of the kinase by H89 in both HeLa and Hepa cells did not lead to alterations in the AHR interaction with ARNT in the absence of TCDD and had no effect on binding of these proteins to DRE. Moreover, the modulators of PKA did not influence the CYP1A1 activity in these cells in the presence and in the absence of TCDD. Thus, an involvement of PKA in the regulation of the *CYP1A1* Gen in HeLa cells was not evaluated in the course of this study.

Repression of genes by transcription factors bound to their responsive elements in the absence of ligands has been described for nuclear receptors. These receptors interact with protein complex containing histone deacetylase (HDAC), enzyme responsible for the repressional effect. Thus, a participation of histone deacetylase in the transcriptional modulation of *CYP1A1* gene by the constitutively DNA-bound AHR/ARNT complex was supposed.

Inhibition of the HDAC activity by trichostatin A (TSA) or sodium butyrate (NaBu) led to an increase of the *CYP1A1* transcription in the presence but not in the absence of TCDD in Hepa and HeLa cells. Since amount of the AHR and ARNT proteins remained unchanged upon treatment of the cells with TSA or NaBu, the transcriptional upregulation of *CYP1A1* gene was not due to an increased expression of the regulatory proteins. These findings strongly suggest an involvement of HDAC in the repression of the *CYP1A1* gene.

Similar to the native human *CYP1A1* also the mouse *CYP1A1*-driven reporter gene transfected into HeLa cells was repressed by histone deacetylase since the presence of TSA or NaBu led to an increase in the reporter activity. Induction of reporter gene did not require a presence of the promoter or negative regulatory regions of the *CYP1A1* gene. A promoter-distal fragment containing three DREs together with surrounding sequences was sufficient to mediate the effects of the HDAC inhibitors suggesting that the AHR/ARNT binding to its specific DNA recognition site may be important for the *CYP1A1* repression.

Histone deacetylase is recruited to the specific genes by corepressors, proteins that bind to the transcription factors and interact with other members of the HDAC complex. Western blot analyses revealed a presence of HDAC1 and the corepressors mSin3A (mammalian homolog of yeast Sin3) and SMRT (silencing mediator for retinoid and thyroid hormone receptor) in both cell types, while the corepressor NCoR (nuclear receptor corepressor) was expressed exclusively in HeLa cells. Thus the high inducibility of *CYP1A1* in Hepa cells may be due to the absence of NCoR in these cells in contrast to the non-responsive HeLa cells, where the presence of NCoR would support repression of the gene by histone deacetylase. This hypothesis was verified in reporter gene experiments where expression constructs coding for the particular members of the HDAC complex were cotransfected in Hepa cells together with the TCDD-inducible reporter constructs containing the *CYP1A1* regulatory sequences. An overexpression of NCoR however did not decrease but instead led to a slight increase of the reporter gene activity in the cells. The expected inhibition was observed solely in the case of SMRT that slightly reduced constitutive and TCDD-induced reporter gene activity. A simultaneous expression of NCoR and SMRT shown no further effects and coexpression of HDAC1 with the two corepressors did not alter this situation. Thus, additional factors that are likely involved in the repression of *CYP1A1* gene by HDAC complex remained to be identified.

Taking together, characterisation of an exogenous ligand independent AHR/ARNT complex on DRE in HeLa cells that repress transcription of the *CYP1A1* gene creates a model system enabling investigation of endogenous processes involved in the regulation of AHR function. This study implicates HDAC-mediated repression of *CYP1A1* gene that contributes to the xenobiotic-induced expression in a tissue specific manner. Elucidation of these processes gains an insight into mechanisms leading to deleterious effects of TCDD and related compounds.

2 ZUSAMMENFASSUNG

Cytochrom P450 1A1 Monooxygenase spielt eine wichtige Rolle im Metabolismus von Umweltkontaminanten wie polyzyklischen aromatischen Kohlenwasserstoffen (PAK) oder halogenierten aromatischen Kohlenwasserstoffen (HAK). Oxidation dieser Substanzen liefert Metabolite die mit hydrophilen endogenen Molekülen wie z.B. Glutathion konjugiert werden können. Diese Derivate sind wasserlöslich und können renal oder biliär ausgeschieden werden. CYP1A1 Metabolismus könnte auch schädliche Effekte verursachen, denn er führt zur Entstehung sehr reaktiver Zwischenprodukte, die mit DNA reagieren können und auf diese Weise mutagene Effekte verursachen können. Solche Effekte liegen der Benzo[a]pyren (B[a]P) Mutagenität zugrunde.

Im Normalzustand ist CYP1A1 nicht oder nur sehr gering exprimiert. Viele PAK und HAK, wie z.B. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), können CYP1A1 induzieren. Transkriptionelle Aktivierung des *CYP1A1* Gens wird durch den AHR (engl.: aryl hydrocarbon receptor), einem bHLH (basischer helix loop helix) Transkriptionsfaktor, vermittelt. In seiner nicht DNA bindenden Form verbleibt AHR überwiegend im Cytoplasma. Die Bindung eines Liganden bewirkt die Translokation von AHR in den Zellkern, die Heterodimerisierung mit einem weiteren bHLH Protein, ARNT (aryl hydrocarbon receptor nuclear translocator) und Bindung des AHR/ARNT Heterodimers an das DRE (dioxin responsive element) Motiv auf der DNA. Dieser Prozess führt zu einer transkriptionellen Aktivierung der entsprechenden Gene. TCDD ist der stärkste bekannte Ligand für AHR. Weil TCDD selbst kaum von den aktivierten Enzymen verstoffwechselt wird, führt eine Exposition gegenüber der Substanz zu einer kontinuierlichen AHR-Aktivierung und diversen toxischen Effekten im Organismus.

Um die molekularen Mechanismen der toxischen Effekte von TCDD und ähnlichen Substanzen aufzuklären, wurde im Rahmen dieser Arbeit die AHR-abhängige Regulierung des *CYP1A1* Gens in zwei Zelllinien, HeLa (humanes Zervix Karzinom) und Hepa (murines Hepatom), untersucht.

Analysen der AHR-Aktivierung und deren Konsequenzen für die Expression des *CYP1A1* Gens bestätigten die TCDD-abhängige Formierung des AHR/ARNT-Komplexes an DRE, die zu einer Induktion der *CYP1A1* Transkription in Hepa Zellen führt. In HeLa Zellen jedoch zeigten

diese Analysen, dass die Formierung des AHR/ARNT-Komplexes an der DRE-Sequenz schon in Abwesenheit des jeweiligen exogenen Liganden stattfindet. Außerdem führte die Behandlung mit TCDD zu keiner weiteren Aktivierung von AHR zu seiner DNA-bindenden Form. Obwohl der konstitutive Komplex an DRE in HeLa Zellen existierte, war die Transkription des *CYP1A1* Gens nicht erhöht. Überdies blieb der CYP1A1 Level in HeLa Zellen auch nach Zugabe von TCDD unverändert, was darauf hinweist, dass in diesen Zellen möglicherweise hemmende Mechanismen auf die TCDD-abhängigen Prozesse Einfluss haben. Ähnlich dem nativen humanem *CYP1A1* Gen konnten die Reportergenkonstrukte, die die unterschiedlichen murinen *CYP1A1* regulatorischen Sequenzen beinhalten, nicht in HeLa Zellen durch TCDD aktiviert werden, was eher auf eine Bedeutung von *trans*-aktiven Faktoren in der Wirtszelle, als auf Spezies-spezifische Unterschiede zwischen murinem und humanem *CYP1A1* Genen hinweist.

Die unterschiedliche Regulierung der AHR-vermittelten Transkription des *CYP1A1* Gens in Hepa und HeLa Zellen wurde weiter untersucht, um zwei Aspekte der AHR Funktion aufzuklären: (I) den Mechanismus der Aktivierung von AHR in Abwesenheit von exogenen Ligand und (II) den Faktor, der die Funktion des exogenen Ligand-unabhängigen AHR/ARNT-Komplexes hemmt.

Da in früheren Studien die Transformation von AHR in Abwesenheit eines Liganden und nach Aktivierung von PKA in Hepa Zellen gezeigt wurde, bestand die Vermutung, dass ein PKA-abhängiger Signalweg den endogenen Mechanismus, welcher die konstitutive Aktivierung von AHR in HeLa Zellen auslöst, steuert. Aber weder die Aktivierung von PKA durch Behandlung der Zellen mit Forskolin oder db-cAMP noch die Inhibierung der Kinase durch Behandlung mit H89 führten zu einer Änderung der AHR/ARNT Interaktion oder deren Bindung an DRE. Außerdem zeigten diese Modulatoren der PKA-Aktivität keinen Einfluss auf die CYP1A1-Aktivität sowohl in TCDD behandelten als auch in unbehandelten Zellen. Diese Beobachtungen ergaben somit keine Hinweise auf eine Beteiligung der PKA an der Regulation des *CYP1A1* Gens in HeLa Zellen.

Ausschalten von Genen durch DNA-bindende Transkriptionsfaktoren in Abwesenheit ihrer Liganden wurde für nukleare Rezeptoren beschrieben. Diese Rezeptoren assozieren mit einem Multiprotein Komplex, der auch die Histondeacetylase (HDAC), ein Enzym, welches die Transkription inhibiert, enthält. Deshalb sollte die Funktion von HDAC bei der Regulation des *CYP1A1* Gens untersucht werden. Eine Inhibierung von HDAC durch Behandlung mit Trichostatin A (TSA) oder Natriumbutyrat (NaBu) führte zur Aktivierung der *CYP1A1* Transkription in Anwesenheit aber nicht in der Abwesenheit von TCDD in Hepa und HeLa Zellen.

Western-Blot Analysen der AHR und ARNT Expressionslevel in TSA und NaBu behandelten Zellen zeigten keine Änderungen der Proteinmenge beider Transkriptionsfaktoren. Aufgrund dieser Beobachtungen muss die Hochregulierung des AHR/ARNT responsiven Gens mit Vorgängen an der regulatorischen Sequenz von *CYP1A1* verbunden sein und erfolgt nicht aufgrund einer erhöhten Menge an Transkriptionsfaktoren.
Ähnlich wie das native humane *CYP1A1* Gen wurden auch die Reportergene, die die murine *CYP1A1* Sequenzen beinhalten, von HDAC gehemmt, da die Behandlung mit TSA oder NaBu zu deren Aktivierung in Zellen führte. Das Enhancer Fragment, das drei DREs zusammen mit benachbarten Sequenzen beinhaltet, genügte um die Effekte von HDAC Inhibitoren zu vermitteln. Dies deutet daraufhin, dass die Bindung von AHR/ARNT an DRE wichtig für die Hemmung vom *CYP1A1* Gen sein könnte.

Histondeacetylase kann spezifische Gene inhibieren durch Interaktionen mit so-genannten Corepresoren, die die Transkriptionsfaktoren binden und mit anderen Komponenten des HDAC Komplexes interagieren. Western Blot Analysen der Mitglieder des HDAC Komplexes in HeLa und in Hepa Zellen belegten die Anwesenheit von HDAC1 und den Corepressoren mSin3A (mammalian homolog of yeast Sin3) und SMRT (silencing mediator for retinoid and thyroid receptor) in beiden Zelllinien, während der Corepressor NCoR (nuclear receptor corepressor) ausschließlich in HeLa Zellen exprimiert wird. Ein Fehlen von NCoR könnte für die starke Induzierbarkeit des *CYP1A1* Gens in diesen Zellen verantwortlich sein, im Gegensatz zu HeLa Zellen, wo die Anwesenheit von NCoR die Histondeacetylase abhängige Repression unterstützt. Um diese Hypothese zu überprüfen, wurden Reportergenkonstrukte, die die regulatorischen Sequenzen vom Maus *CYP1A1* beinhalten zusammen mit Expressionskonstrukten für NCoR, HDAC1 oder SMRT in Hepa Zellen transfiziert. Eine Überexpression von NCoR jedoch senkte nicht wie erwartet sondern erhöhte sogar leicht die Reportergenaktivität in Hepa Zellen. Die erwartete Inhibierung der Luziferase-Aktivität wurde ausschließlich mit SMRT gemessen, welches sowohl die konstitutive als auch die TCDD-induzierte Reportergenaktivität schwach senkte. Eine gleichzeitige Expression von NCoR und SMRT zeigte keine weiteren Effekte und ebenfalls nicht bei gleichzeitiger Anwesenheit von HDAC1. Deswegen ist vermutlich ein zusätzlicher Faktor in die Repression des *CYP1A1* Gens involviert.

Die Charakterisierung eines von einem exogenen Liganden unabhängigen AHR/ARNT-Komplexes am DRE in HeLa Zellen, der die Transkription des *CYP1A1* Gens hemmt, schafft ein Model für Studien von endogenen Prozessen, die in die Regulation der AHR Funktion involviert sind. Diese Arbeit zeigt die HDAC-vermitelte Repression des *CYP1A1* Gens auf, die zu der gewebespezifischen, durch Xenobiotika induzierten Expression beiträgt. Die Aufklärung dieser

Prozesse vermittelt eine Einsicht in die Mechanismen der schädlichen Effekte des TCDD's und ähnlicher Substanzen.

3 INTRODUCTION

3.1 Metabolism of xenobiotics

3.1.1 Two step metabolism of foreign compounds

The number of chemical compounds present in the environment either of natural or of anthropogenic origin, such as plant metabolites, mycotoxins, venoms, pharmaceuticals, and the products of industrialization, which daily confront the human organism, may cause toxic or carcinogenic effects, when accumulated in the body. Most of these foreign compounds (xenobiotics) are lipophilic, which is an obstacle to their elimination. Therefore, the body has developed a system of enzymes adept at metabolic processing of the lipophilic xenobiotics to water-soluble products, thus facilitating their elimination and minimizing its exposure to them.

The catalytic process of transforming lipophilic chemicals occurs in two distinct metabolic phases. During phase I either electrophilic or nucleophilic functional groups are generated in the molecule leading to the increased polarity of the compound and furthermore, enabling the conjugation in phase II with endogenous substrates, e.g. glucuronic acid, glutathione or sulphate to form highly hydrophilic molecules, ensuring in this way their elimination**.** The broad and complementary substrate specificity of the xenobiotic metabolizing enzymes (XMEs) of both of these phases serves with the efficient protection against the myriad of heterogeneous compounds that daily penetrate the organism. The majority of these enzymes are localized in the endoplasmic reticulum and the cytosolic fraction of the cell. In mammals they are present in every tissue, but are most abundant in the liver, which consequently functions as the main site of xenobiotic metabolism. Other organs, especially those that are the portals of entry for foreign compounds such as the respiratory and the gastrointestinal tracts, are also involved in the metabolism of foreign compounds.

The majority of phase I reactions are oxidations mediated by cytochrome P-450-dependent monooxygenases (CYPs) (Lu et al. 1980). In eukaryotes CYPs are hemoproteins that

form multimeric complexes in the endoplasmic membrane with the flavoprotein NADPH-P450 reductase. They catalyse reactions such as epoxidation, hydroxylation, desaturation, dealkylation, and a heteroatom oxygenation or replacement by oxygen (reviewed by Guengerich 2001). Human xenobiotic-metabolizing CYPs belong to the families CYP1, CYP2 or CYP3. They are expressed throughout the body; some of them have nearly ubiquitous distribution, some others are present in extrahepatic tissues or are found exclusively in the liver. The broad spectrum of substrates for CYPs consists of polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons (HAHs), aromatic amines, mycotoxins, caffeine, tobacco-specific nitrosoamines, and a broad range of pharmaceutics (Lewis 2000). The members of another family, the flavin containing monooxygenases (FMOs), oxidize molecules containing a sulphur or nitrogen as a heteroatom (Cashman 2000). Xenobiotics are also metabolized by enzymes implicated in the metabolism of endogenous substrates such as the monoamine oxidases (MAOs), which are mainly involved in the process of neurotransmitter inactivation, but can also use a number of xenobiotic aliphatic or aromatic amines as a substrate (Strolin Benedetti and Tipton 1998). Another example of an endogenous substrate metabolizing enzyme, which also plays a role in the xenobiotic metabolism is the prostaglandin H synthase (PGH, also known as cyclooxygenase). This enzyme that catalyzes the initial step in arachidonic acid metabolism is capable of “co-oxidizing” chemicals such as phenolic compounds, aromatic amines and polycyclic aromatic hydrocarbons. In contrast to CYPs, PGH is mainly involved in xenobiotic metabolism in extrahepatic tissues (Vogel 2000).

Many aliphatic, alicyclic and aromatic xenobiotic carbonyl compounds (aldehydes and ketones) are converted through oxidative pathways by alcohol and aldehyde dehydrogenases (ADH and ALDH). A reductive metabolism of these chemicals is also possible. It is carried out by members of the aldo-keto reductase (AKTR), short-chain dehydrogenase/reductase (SDR) and quinone reductase (QR) families (reviewed by Oppermann and Maser 2000). Foreign or by various oxidative processes generated epoxide group containing chemicals are substrates for the soluble (sEH) or microsomal (mEH) epoxide hydrolase (Oesch 1973).

Taking advantage of electrophilic or nucleophilic functional groups already present in the molecule or introduced during phase I metabolism, phase II drug-metabolizing enzymes conjugate xenobiotics using small molecular weight organic donors. Glutathione S-transferases (GSTs) are soluble dimeric enzymes that catalyze the conjugation of the tripeptide glutathione (GSH) with a wide variety of electrophiles (Sheehan et al. 2001). The glutathione conjugate converted to the mercapturic acid in the kidney was one of the earliest recognized xenobiotic metabolite (Chasseaud 1979). Conjugation to glucuronic acid is an important reaction in the

detoxification and elimination of nucleophiles. The uridine diphosphate glucuronosyltransferases (UGTs) catalyse the transfer of UDP-glucuronic acid (UDPGA) to an acceptor with oxygen, nitrogen, sulphur, or carbon atom (King et al. 2000). The sulphotransferases (SULTs) represent a further family of enzymes involved in the elimination of nucleophilic compounds. They catalyze the transfer of sulfuryl groups from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to nucleophilic substrates similar to that of UGTs (Glatt et al. 2001).

3.1.2 Dual role of xenobiotic metabolism

The enzymatic transformation (biotransformation or bioactivation) of xenobiotics is an important process resulting in excretion and detoxification. The biotransformation can be in many cases a 'double edged sword', by the same token enhancing the formation of intermediates more reactive than the parent compound, which can bind to vital cellular macromolecules such as DNA, RNA and proteins (Figure 3-1).

Cytochromes P450 play a key role in the bioactivation reactions because of their unusually broad substrate specificities. An inert compound may be converted by these enzymes to highly reactive metabolites able to form DNA adducts, thus initiating mutagenesis. The mutations that lead to the activation of oncogenes or inactivation of tumor suppressor genes may cause the transformation of a normal into a tumorigenic cell and consequent carcinogenesis (Gelboin 1980; Kozack et al. 2000; Pelkonen and Nebert 1982).

Metabolic activation and subsequent covalent binding to proteins correlates with the toxicity of many drugs. The chemical modification of specific proteins alter their function and may disrupt regulatory pathways leading to cell toxicity (Bessems and Vermeulen 2001; Boelsterli 1993; Pumford and Halmes 1997). Another important consequence of the modification of proteins can be the activation of the immune system. The xenobiotic can initiate an immune response through the covalent binding of its reactive intermediate to proteins, which then serve as hapten, leading to hypersensitivity or autoimmune response (Spracklin et al. 1997).

Although in general conjugation in phase II constitutes a detoxification step, numerous examples are known where the product of the conjugation is still reactive, or even more reactive than the parent compound. The glutathione conjugates have been identified as DNA-reacting species (Chasseaud 1979; Dekant 2001). Examples of bioactivation after conjugation reactions are also known for UDP-glucuronosyl transferase and sulfotransferase metabolism (reviewed by Banoglu 2000; Ritter 2000).

Formation of free radicals is an additional mechanism through which some foreign agents may have deleterious effects. The interaction of these entities with cellular molecules may lead to the formation of secondary radicals derived from proteins, lipids or nucleic acids, and thus initiate a chain reaction leading to cellular damage. Dependent on the nature of the radical species, they may have variable effects, leading to toxic or carcinogenic effects (Aust et al. 1993; Feig et al. 1994; Guyton and Kensler 1993).

A very important practical aspect of the activities of XMEs is their influence on therapeutic drugs efficacies. The metabolic conversion could lead to inactivation of an active drug or activation of a prodrug (Guengerich 1997). Thus, the discovery and development of a new pharmaceutical must involve studies of its metabolism in the organism. Biotransformation is often a reason for disqualification of the prospective drug (Doehmer et al. 1993).

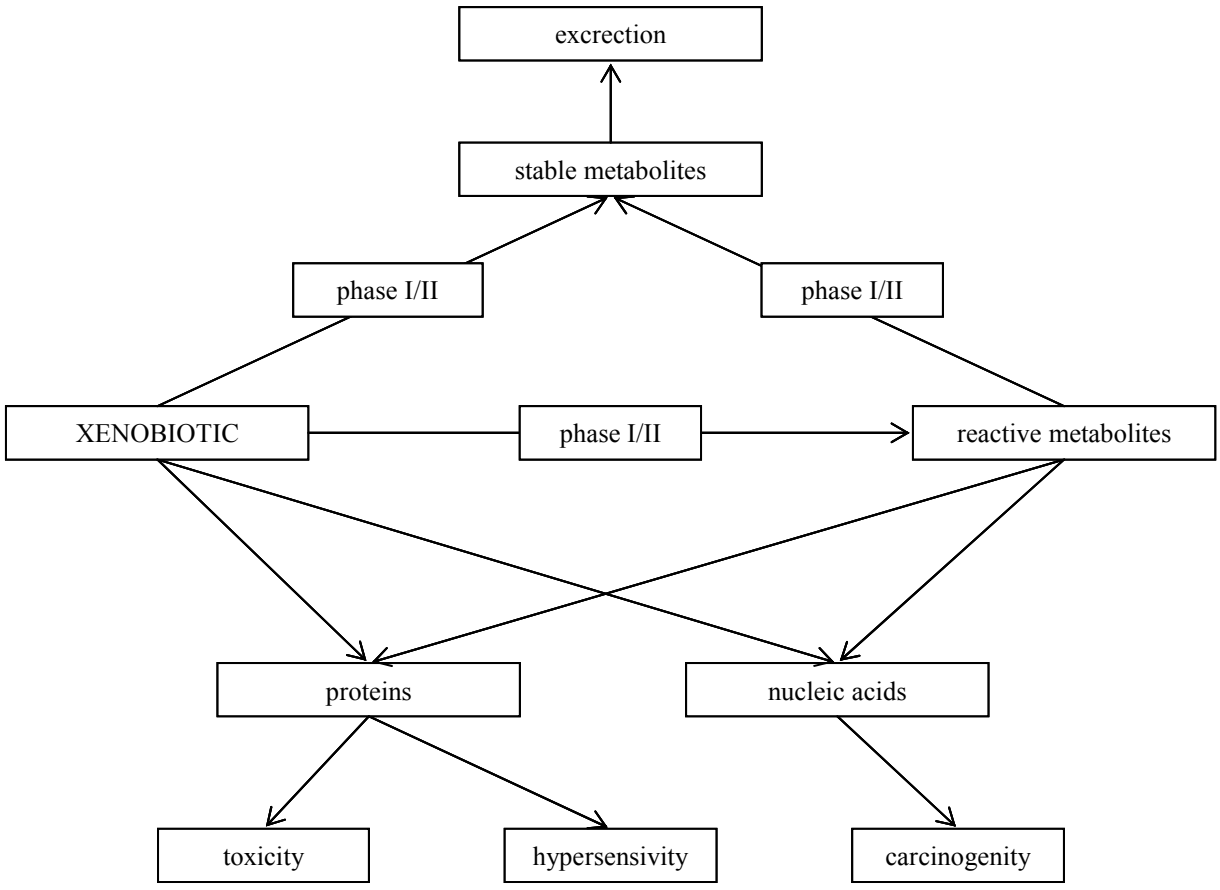


Figure 3-1. Xenobiotic metabolism and bioactivation. Details are described in the text.

3.1.3 Regulation of xenobiotic metabolism

Whether metabolism of a foreign compound results in detoxification and excretion of xenobiotic or in its bioactivation with possible toxic effects, depends on the concerted function of a variety of enzymes involved in the metabolism. The activity of the XMEs must therefore be tightly controlled and there are in fact multiple levels of such a control. The enzymes exhibit tissue-specific expression, and are regulated dependent on the developmental stage or in response to exposure to the foreign chemical.

Most of the regulatory processes take place at the transcriptional level. The tissue- and developmental-specific expression of xenobiotic metabolizing enzymes is regulated by endogenous mechanisms such as methylation of *cis*-acting elements of genes and transcriptional regulation of the genes by tissue enriched transcription factors, hormones or cytokines (Akiyama and Gonzalez 2003; Hines et al. 2001; McCarver and Hines 2002).

An adaptive mechanism leading to effective detoxification is the activation of an enzyme transcription after exposure to a xenobiotic. This activation generally depends on the interaction of the chemical with a xenobiotic receptor, such as specific members of the nuclear receptor family, the “orphan receptors” (receptors whose endogenous ligands are unknown). The most studied members of this family are constitutive androstan receptor (CAR), which mediates the induction of CYP2B by phenobarbital, and pregnane X receptor (PXR), which is involved in the induction of CYP3A4 by dexamethason or rifampicin. The activation of the target genes for these receptors involves dimerization with retinoic X receptor (RXR), a common heterodimerization partner for many orphan nuclear receptors, and binding to nuclear receptor responsive elements, found within the promoter regions of the responsive genes (reviewed by Honkakoski and Negishi 2000; Willson and Kliewer 2002).

Another known xenobiotic-activated transcription factor that regulates expression of XMEs is the aryl hydrocarbon receptor (AHR). Since the transcriptional regulation of the AHR target, the *CYP1A1* gene, is the objective of this study, the functional role of AHR is described in detail in following sections.

Although the increased level of the enzyme activity results mostly from transcriptional activation, posttranscriptional processes like regulation of the mRNA stability also influence the activity of particular enzymes e.g CYP1A1 (Lekas et al. 2000). Postranslational modifications like phosphorylation of enzyme has been shown to regulate the activity of CYP2E1 and CYP2B1 enzymes (Bardag-Gorce et al. 2002; Oesch-Bartlomowicz and Oesch 2002; Oesch-Bartlomowicz et al. 1998; Tindberg 2003).

3.2 AHR and regulation of the *CYP1A1* gene

3.2.1 Role of AHR in the metabolism of xenobiotics

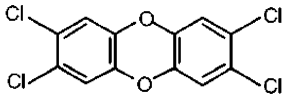
The AHR-mediated transcriptional response to lipophilic compounds was one of the first discovered and the most intensively studied mechanism of xenobiotic-dependent regulation of genes. The understanding of the regulation of many genes involved in metabolism of foreign compounds began with the first seminal observation that administration of polycyclic aromatic hydrocarbons, such as benzantracene, benzo[a]pyrene (B[a]P) or 3-methylcholantrene to rodents led to an induction of arylhydrocarbon hydroxylase activity in these animals (Conney et al. 1957). This observation was supplemented by the identification of CYP1A1, the hemoprotein associated with the induced activity (Leibman et al. 1969; Sladek and Mannering 1966).

The mechanism of CYP1A1 enzyme induction was further elucidated by identification of AHR, the cytosolic receptor capable of binding the PAHs and activating the *CYP1A1* gene (Burbach et al. 1992; Dolwick et al. 1993a; Ema et al. 1992; Knutson and Poland 1982; Whitlock et al. 1989) followed by the identification of the AHR dimerization partner, aryl hydrocarbon receptor nuclear translocator (ARNT), which is necessary for the binding of the activated AHR to DNA (Burbach et al. 1992; Hoffman et al. 1991; Whitelaw M. et al. 1993).

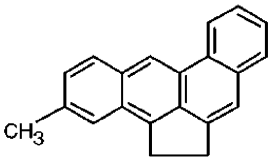
These studies were completed by analysis of the sequences on the *CYP1A1* gene, colled xenobiotic or dioxin responsive elements (XREs, DREs), recognized by the liganded AHR/ARNT heterodimer, needed for the *CYP1A1* activation (Kawajiri et al. 1986). The DRE motif has been found in several other genes coding for xenobiotic metabolizing enzymes, which have been shown to be activated by PAHs in an AHR-dependent manner. Among these genes of the so called ‘AHR gene battery’ (Nebert et al. 2000) are genes coding for XMEs of phase I such as CYP1A2, CYP1B1, an NADPH-quinone reductase (DT diaphorase), an aldehyde dehydrogenase and prostaglandin H synthase, and also phase II XMEs such as GST and UDP-glucuronosyltransferase 1A1 (Emi et al. 1996; Favreau and Pickett 1991; Paulson et al. 1990; Quattrochi et al. 1994; Sutter et al. 1994; Suzuki et al. 1994; Vasiliou et al. 1996; Vogel et al. 2000).

The xenobiotic agonists of AHR are mostly anthropogenic contaminants found in the environment and in food (Denison and Nagy 2003). The PAHs with B[a]P as a representative are found in tobacco smoke and other products of combustion. AHR can also be activated by heterocyclic aromatic amines formed during high-temperature cooking of meat or found in

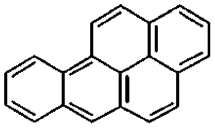
tobacco smoke (Gillner et al. 1989; Kleman et al. 1994) and halogenated aromatic hydrocarbons, among them the most potent known AHR inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, commonly referred to as 'dioxin') (Figure 3-2) (Poland and Glover 1973). TCDD is a by-product of industry working with other chlorinated hydrocarbons such as phenoxyherbicides and chlorophenols and may also be formed by combustion of organic materials including municipal garbage.



2,3,7,8-tetrachlorodibenzo-*p*-dioxin



3-methylcholantrene



benzo[a]pyrene

Figure 3-2. Structure of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and other xenobitotic AHR inducers.

Most of the AHR-induced XMEs are able to metabolize either the same inducing agents or their metabolites, thus being a defense system aimed at elimination of the inducer and its metabolites (Denison and Whitlock 1995). TCDD is an exception since the chlorine atoms prevent this compound from metabolism, thus leading to the persistence of TCDD in the cell and to sustained induction (Dencker 1985; Inouye et al. 2002).

In addition to a protective role of the ligand-dependent AHR-induced activation of the XMEs, there is convincing evidence suggesting that this activation could lead to toxicity. This is best illustrated by the metabolism of PAHs like B[a]P, which carcinogenity depends on the successive metabolism by CYP1A1, epoxide hydrolase and CYP3A4. The metabolic activation of the inert B[a]P ultimately converts this compound to the electrophilic, highly mutagenic diol-epoxide-benzo[a]pyrene. The latter compound as well as other intermediate metabolites can be further detoxified by phase II enzymes such as GSTs and UGTs. At high substrate concentration however, where the detoxification pathways become saturated, induction can increase the production of reactive metabolites beyond the capacity of cellular defense, thereby leading to the accumulation of reactive electrophiles and finally mutagenesis (Brooks et al. 1999; Gelboin 1980; Jerina and Daly 1974; Pelkonen and Nebert 1982).

In addition, the production of reactive oxygen species has been shown to be a consequence of CYP1A1 activation, leading to oxidative stress within the cells (Morel et al.

1999), and subsequent oxidative DNA alterations e.g.8-oxoguanine formation (Park et al. 1996). These observations are in agreement with *in vivo* data showing that TCDD treatment elicits oxidative stress in various mouse tissues (Shertzer et al. 1998) and that the oxidative stress caused by TCDD in mice is controlled by the AH receptor complex (Alsharif et al. 1994).

Other adverse effects linked to the transcriptional activation of XMEs by AHR could result from enhanced metabolism of endogenous substances. Administration of TCDD to rats induced the 2-hydroxylation of 17β-estradiol. The induction of CYP1A1 and CYP1B1 by TCDD in the liver of female rats was suggested to contribute to the carcinogenic effects of not per se genotoxic estradiol by enhancing the formation of reactive estrogen metabolites (Graham et al. 1988; Hayes et al. 1996; Tritscher et al. 1996).

The toxic consequences of elevated CYP1A1 activity are confirmed by epidemiological observations in humans. Exposure to PAHs, particularly among smokers has been shown to result in induction of CYP1A1 and 1A2 (Butler et al. 1992; Pelkonen et al. 1986) and a correlation between high CYP1A1 activity, smoking and lung cancer has also been documented (Kiyohara et al. 1998).

3.2.2 Structure and function of the AHR and ARNT proteins

Two proteins, AHR and ARNT, that are key components of the system controlling the transcription of *CYP1A1* and other genes of the AHR gene battery, belong to an emerging family of bHLH/PAS (basic-helix-loop-helix/PER-ARNT-SIM) transcription factors. Beside response to chemicals, bHLH proteins control other homeostatic responses, such as that to low oxygen tension or light as well as normal development programs (Crews 1998; Dunlap 1998; Wenger 2002). The term PAS stems from the names of the three founding members of the PAS family: PER, a *Drosophila* protein involved in the regulation of circadian rhythm; ARNT, identified as an essential protein for signal transduction by AHR; and another *Drosophila* protein SIM, which was identified through its role in regulation of midline cell lineage (Hoffman et al. 1991; Huang et al. 1993; Nambu et al. 1991).

Members of the bHLH/PAS family share structure and function homology (Figure 3-3). The PAS domain is a region of homology of these three proteins. It typically encompasses 250-300 amino acids and contains a pair of highly conserved 50 amino acid spanning subdomains termed A and B repeats (Hoffman et al. 1991; Jackson et al. 1986; Nambu et al. 1991). The PAS domain functions as a surface for interaction with other PAS proteins and interaction with

cellular chaperones, such as the 90-kDa heat shock protein (Hsp90) (Coumailleau et al. 1995). In the case of AHR, this domain can also function as a ligand-binding region (Dolwick et al. 1993b; Whitelaw M. L. et al. 1993). Most of the PAS proteins contain a basic-helix-loop-helix (bHLH) motif located N-terminally to their PAS domain. The HLH domain is involved in dimerization of two bHLH-PAS proteins, while the basic regions allow specific contact within the major groove of target regulatory elements in DNA (Kadesch 1993; Murre et al. 1994; Reisz-Porszasz et al. 1994). This subdomain is generally 12 to 15 amino acids in length and contains a highly conserved ERXR sequence (where E is glutamic acid, R is arginine, and X is any amino acid). The ERXR motif is found in the basic region of all those bHLH proteins that have been shown to bind to an E-box (CANNTG), a recognition sequence for the bHLH transcription factors (Ellenberger et al. 1994; Murre et al. 1989).

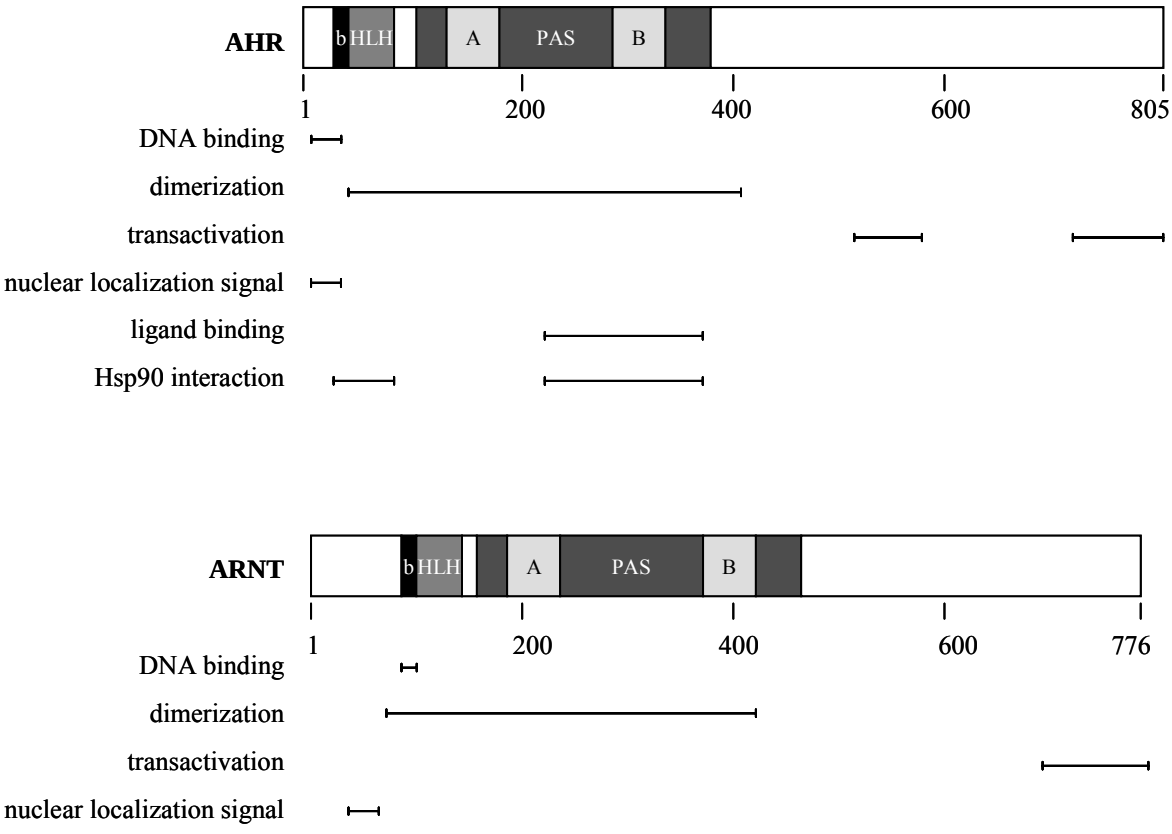


Figure 3-3. Domain structure of AHR and ARNT proteins. Shaded areas indicate the basic (b), helix-loop-helix (HLH), and PER-ARNT-SIM (PAS) domains. The internal repeats within the PAS domain are designated A and B. Numbers mean amino acid positions. Lines indicate regions within which the indicated functions have been mapped. More details are described in the text and reference (Whitlock 1999).

Like other bHLH proteins AHR and ARNT also interact with DNA through their basic regions, whereas their helix and PAS domains are involved in dimerization (Fukunaga and Hankinson 1996; Reisz-Porszasz et al. 1994). In contrast to the classical bHLH transcription factors, which bind the symmetric E-box, the DRE is an asymmetric motif (TNGCGTG), which contains a half of an E-box (underlined) and one nonconsensus site. The four core nucleotides, the CGTG, are critical for the TCDD-inducible protein complex binding to this motif (Denison et al. 1988a, b, 1989; Yao and Denison 1992). The liganded AHR/ARNT heterodimer interacts with this element so that ARNT contacts the GTG trinucleotide identical to the half-site of the E-box element, while AHR binds to the TNGC portion, which does not resembles the E-box (Bacsi and Hankinson 1996; Fukunaga and Hankinson 1996; Shen and Whitlock 1989).

The bHLH-PAS proteins have transcriptionally active domains (TADs) within their C-terminal regions, which however share less homology between particular proteins (Jain et al. 1994). The TAD of human AHR consists of three subdomains: an acidic domain, a glutamine-rich (Q-rich) domain and a proline/serine/threonine-rich domain that are involved in the activation of the AHR responsive genes (Jain et al. 1994; Li et al. 1994; Ma et al. 1995; Rowlands et al. 1996; Sogawa et al. 1995; Whitelaw et al. 1994). The C-terminal 34 amino acids of ARNT can act as a TAD (Sogawa et al. 1995), but the TAD of ARNT is not absolutely required for AHR/ARNT-dependent transactivation of target genes (Ko et al. 1996; Wilson and Safe 1998).

3.2.3 Mechanism of the AHR-mediated transcriptional activation

A model of the AHR-mediated transcriptional response assumes that in unexposed cells AHR resides in the cytoplasm. The cytosolic AHR forms a complex with a dimer of heat shock protein (Hsp90) which represses the intrinsic DNA binding activity and supports its ligand binding conformation (Denis et al. 1988; Perdew 1988; Pongratz et al. 1992). Additional cellular chaperones such as XAP2 (also known as ARA9 or AIP1) and p23 also bind to the AHR-Hsp90 complex and are thought to be required for maintaining its stability (Carver et al. 1998; Kazlauskas et al. 1999; Ma and Whitlock 1997; Meyer and Perdew 1999). The hydrophobic AHR ligand enters the cell by diffusion and binds to the Hsp90-associated AHR (Burbach et al. 1992). Ligand binding causes dissociation of AHR from the cytosolic complex (McGuire et al. 1994) and its conformational change leads to the unmasking of a nuclear localization signal (NLS). This allows its recognition by NLS receptor(s) and consequent nuclear translocation of AHR (Ikuta et al. 1998). Within the nucleus, AHR forms a heterodimer with a nuclear protein

ARNT and generates a DNA-binding transcription factor which recognizes and binds DRE (Probst et al. 1993). Binding of the AHR/ARNT transcription factor to DNA leads to the activation of target genes (Jones et al. 1986; Mason et al. 1994; Matsushita et al. 1993; Reyes et al. 1992).

The ligand-dependent conversion of AHR into its DNA-binding form, observed initially in Hepa (mouse hepatoma, Hepa1c1c7) cells has been defined as "transformation" and is considered to be a critical step for AHR ligand-dependent function (Figure 3-4) (Pollenz et al. 1994; Whitlock 1999).

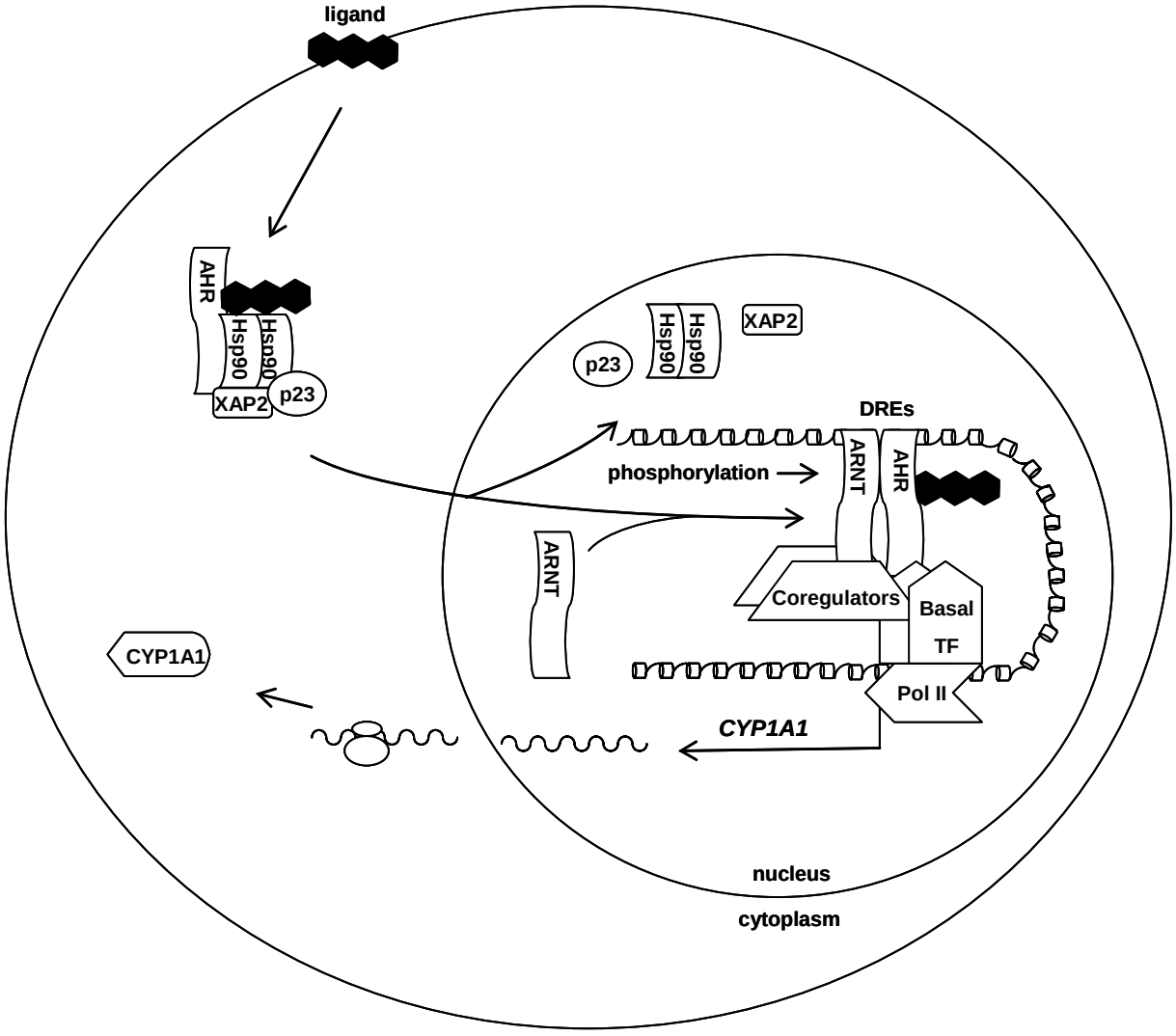


Figure 3-4. Mechanism of the ligand-dependent AHR-mediated transcriptional activation.

3.2.4 *Cis*- and *trans*-elements of the *CYP1A1* transcriptional regulation

The in the previous section described overall model of AHR-mediated transcriptional regulation in response to xenobiotics has further been elucidated in studies aimed at functional analysis of the *CYP1A1* gene and investigation of interactions of AHR and ARNT with other nuclear proteins that contribute to the regulation of *CYP1A1*.

Studies of the *CYP1A1* gene revealed two *cis*-acting elements required for a high level of xenobiotic-inducible gene expression. One distal region which functions as an inducible enhancer and another, proximal to the transcriptional start site, which acts as a promoter (Jones and Whitlock 1990; Jones et al. 1986; Neuhold et al. 1989).

The enhancer region contains several copies of DRE. The nucleotides that flank these motifs, although not involved in primary interaction of AHR/ARNT complex with DNA, contribute to the specificity of the DRE binding (Denison et al. 1988a; Wu and Whitlock 1993; Yao and Denison 1992). In addition to the DRE, a guanosine/cytosine (GC) rich element has been identified in this region, which represents a potential binding site for Sp1 (specific protein 1) like transcription factors (Kadonaga and Tjian 1986). This motif exhibits no intrinsic activity but enhances gene expression if it is linked to a sequence containing DRE elements (Fisher et al. 1990). Footprint analyses of the enhancer region revealed existence of an additional protein binding site, the guanine rich sequence, which has been implicated in the AHR-dependent enhancer function (Watson and Hankinson 1992).

The promoter of the *CYP1A1* gene contains a TATA box located immediately upstream of the transcription start site and a region in its vicinity, named basic transcription element (BTE), which is required for basal as well as inducible gene expression (Jones and Whitlock 1990; Neuhold et al. 1989). The BTE comprises consensus sequences playing a common regulatory role in the basic transcriptional regulation of genes by ubiquitously distributed transcription factors. The CAAT box, which is a nuclear factor 1 (NF-1) binding site and a GC rich box recognized by Sp1 transcription factors have been identified in this region (Jones and Whitlock 1990; Yanagida et al. 1990). Although the promoter does not contain DREs, it is inducible by TCDD and its induction depends on AHR and ARNT, at least in part due to the direct interactions of these proteins with the basal transcription factors (Ko et al. 1997). It was demonstrated that both AHR and ARNT interact with transcription factor (TF) IIB. Interaction of this factor with AHR increases DNA binding ability of the liganded AHR/ARNT (Swanson and Yang 1998). AHR binds also TFIIF and the TATA binding protein, whereas ARNT binds the TFIIF (Rowlands et al. 1996).

Among the proteins associated with *CYP1A1* transcription are also members of the Sp1-like family (Imataka et al. 1992; Kaczynski et al. 2002; Kobayashi et al. 1996; Sogawa et al. 1993; Zhang W. et al. 1998). These common zinc finger transcription factors regulate expression of genes through interactions with the conserved GC-rich DNA elements (Cook et al. 1999; Philipsen and Suske 1999; Turner and Crossley 1999). Binding of Sp1 to its cognate recognition sequence enhances xenobiotic-dependent activation of a reporter construct containing both, DRE and GC motifs. The AHR/ARNT heterodimer directly interacts with Sp1 and this transcription factor enhances the expression of the *CYP1A1* gene synergistically (Kobayashi et al. 1996; Wang et al. 1999).

In contrast to the activation of *CYP1A1*, less is known about mechanisms that silent this gene. A negative regulatory element (NRE) has been identified within the gene (Boucher and Hines 1995; Gonzalez and Nebert 1985; Hines et al. 1988; Sterling et al. 1993). A 21-nucleotide spanning palindrome flanked by conserved GC rich regions is critical for the repressional activity of the NRE, which depends on the specific binding of the nuclear proteins to this motif (Boucher et al. 1995). Another consensus motif within the NRE, the octamer sequence, is also associated with the repressional function of the element. Binding of the transcription factor Oct-1 to this motif suppresses transcription of the *CYP1A1* gene (Bhat et al. 1996; Sterling and Bresnick 1996).

Another mechanism down regulating the expression of the AHR/ARNT responsive genes involves the AHR repressor (AHRR) (Baba et al. 2001; Mimura et al. 1999). AHRR is localized in the nucleus and forms a heterodimer with ARNT. This heterodimer also recognizes the DRE, but functions as a transcriptional repressor by competing with AHR for binding to ARNT (Mimura et al. 1999). AHRR inhibits the AHR-dependent pathway in a feedback regulatory loop, as three DRE sequences in the 5'-flanking region of the AHRR gene enhance expression of the repressor in response to AHR induction (Baba et al. 2001).

3.2.5 Chromatin remodeling in the *CYP1A1* transcriptional regulation

Besides the interactions with transcription factors, AHR/ARNT binding to DREs facilitates the occupancy of the promoter by the common transcriptional machinery through changes in chromatin structure which increase the DNA accessibility in enhancer and promoter regions (Durrin and Whitlock 1989; Ko et al. 1996; Okino and Whitlock 1995). When the gene is silent, the regulatory region of *CYP1A1* is associated with histones and other chromosomal

proteins in a nucleosomal complex (Morgan and Whitlock 1992; Wu and Whitlock 1992). TCDD-induced binding of the AHR/ARNT heterodimer to the enhancer region leads to disruption of the nucleosomal structure in this region, manifested by increased accessibility of DNA. Analogous AHR/ARNT-dependent chromatin alterations are also observed at the *CYP1A1* promoter (Okino and Whitlock 1995). A mechanism responsible for the transmission of an induction signal from enhancer to promoter involves the interaction of AHR and ARNT with nuclear proteins, in particular those with chromatin-modifying activity that have been shown to regulate many genes (reviewed by Kadonaga 1998).

Chromatin assembly, considered to be necessary for the maintenance of the genome, recently proved to play a role in the specific transcriptional regulation. It is a dynamic process modulated by post-transcriptional modifications of the nucleosomal core histones, in particular the acetylation of ε-amino groups of the lysine residues in the conserved N-terminal region of these proteins (Turner et al. 1992). The charge neutralization that occurs upon acetylation of the histone tails weakens the histone-DNA interactions, leading to a decreased condensation of chromatin, which in turn enables the access of transcription factors to the DNA. The link between histone acetylation, chromatin remodelling and gene regulation has been well established (Strahl and Allis 2000; Turner et al. 1992; Wolffe and Pruss 1996; Yuan and Gambee 2001).

The acetylation of the core histones is catalyzed by enzymes referred to as histone acetyltransferases (HATs), while the enzymes that deacetylate histones are called histone deacetylases (HDACs) (Grunstein 1997). The HATs and HDACs exert their promoter specific effects by binding either direct or through proteins referred to as coactivators (for HATs) or corepressors (for HDACs) to the sequence specific transcription factors, which then target the complexes to their recognition motifs on DNA (Xu et al. 1999).

The link between AHR/ARNT transcriptional activity and the mechanisms involved in chromatin remodeling was confirmed in the studies of AHR and ARNT interaction with various coregulator proteins. Among them are members of the steroid receptor coactivator (p160) family as well as p300 and its homologue, the CREB binding protein (CBP), which both posses an intrinsic histone acetyltransferase activity (Ogryzko et al. 1996; Spencer et al. 1997; Xu and Li 2003). The p300/CBP proteins have been implicated in the AHR/ARNT transcriptional regulation of *CYP1A1* via a direct interaction with the ARNT protein (Kobayashi et al. 1997; Nguyen et al. 1999). p160 family members including the nuclear coactivator A-2 (NCoA-2), steroid receptor coactivator-1 (SRC-1) and the estrogen receptor-associated protein 140 (ERAP140) also enhance the TCDD-dependent AHR/ARNT-mediated induction of gene

expression. These three proteins are capable of interacting with AHR as well as ARNT (Beischlag et al. 2002; Kumar and Perdew 1999; Nguyen et al. 1999). Another member of the p160 family, p/CIP and a non-p160 coactivator, the receptor interacting protein 140 (RIP140) are transcriptional coactivators that interact in a ligand dependent manner with AHR (Beischlag et al. 2002; Kumar et al. 1999).

The corepressors, among them are nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT), are known to interact with nuclear receptors in the absence of the receptor ligand (Chen and Evans 1995; Horlein et al. 1995). These proteins connect the nuclear receptors with Sin3 proteins, which in turn assemble with histone deacetylases and other proteins in a multisubunit complex on target genes and repress transcription (Nagy et al. 1997). Interaction of the corepressor SMRT with AHR and modulation of AHR-dependent gene expression by this corepressor has been shown (Nguyen et al. 1999; Rushing and Denison 2002), suggesting the participation of a histone deacetylase-dependent mechanism of *CYP1A1* repression similar to that for nuclear receptors.

Another type of chromatin remodeling factors use the energy derived from ATP hydrolysis to catalyse disruption of the nucleosome by changing the histone octamer position relative to DNA (Vignali et al. 2000). Similar to the HATs and HDACs, the ATP-dependent chromatin remodelling complexes are also targeted to the specific DNA regions via their interaction with DNA binding transcription factors (Ostlund Farrants et al. 1997). The ATP-dependent remodeling complexes are also involved in the regulation of the *CYP1A1* gene. The Brahma/SWI2-related gene 1 (BRG-1), a subunit of a mammalian ATP-dependent chromatin-remodeling complex, modulates the AHR-ARNT-mediated regulation of gene expression probably through interaction of this protein with AHR (Wang and Hankinson 2002).

3.3 Endogenous function of AHR and ARNT

3.3.1 ARNT as a common dimerization partner for bHLH-PAS transcription factors

Significance of the AHR nuclear translocator function for the normal cellular metabolism, growth and development was clearly demonstrated in ARNT deficient mice. These animals are not alive past embryonic day 10.5, they are defective in angiogenesis and show impaired development (Maltepe et al. 1997). Primarily identified as a component of the AHR

transcriptional complex, ARNT proved to act as a dimerization partner for many members of the bHLH–PAS family of transcription factors. Depending on the counterpart, ARNT-containing heterodimers bind diverse core DNA elements (Gu et al. 2000; Swanson et al. 1995). Among the ARNT partners are members of the hypoxia inducible factor (HIF) family, which regulate an adaptive response to oxygen tension (Wang and Semenza 1995), proteins regulating expression of genes involved in development of blood vessels and the tubular system of lung (Ema et al. 1997; Gu et al. 1998) or central nervous system (Ema et al. 1996).

3.3.2 Role of AHR in cell cycle regulation

Exposure of mammals to TCDD leads to a wide range of biochemical and toxic responses including porphyria, liver damage, immunosupression, tumor promotion, teratogenity, wasting syndrome or even death (Poland and Glover 1973; Schmidt et al. 1996). Studies using genetically modified mouse models have revealed that all the examined TCDD effects are mediated by AHR *in vivo* (Fernandez-Salguero et al. 1995). However, not all of the observed effects can be explained by an increased activity of xenobiotic metabolizing enzymes upon exposure to the AHR ligand. Therefore, it is believed that AHR function is also connected with the regulation of important pathways of cell physiology.

Observations in transgenic mice revealed that the AHR deficient animals display smaller livers, reduced fecundity, decreased body weights and defects in their immune systems, suggesting that AHR plays a role in the regulation of normal growth and development (Fernandez-Salguero et al. 1995; Fernandez-Salguero et al. 1996; Lahvis et al. 2000; Mimura et al. 1997; Schmidt et al. 1996). Expression of a mutated AHR constitutively activating transcription, on the other hand, reduces the life span of transgenic mice and induces tumors in the glandular part of the stomach, demonstrating oncogenic potential of AHR and implicating the receptor in regulation of cell proliferation (Andersson et al. 2002). These effects may be, at least in part, due to the AHR-dependent activation of genes involved in cell proliferation and cell cycle regulation like those coding for IL-1β , PAI-2, p27 or jun B (Hoffer et al. 1996; Kolluri et al. 1999; Son and Rozman 2002; Vogel et al. 1997). Most of these genes were found to contain DREs in their regulatory regions indicating a direct upregulation by liganded receptor.

AHR may also affect the cell cycle through its interaction with retinoblastoma (Rb) protein. This interaction represses E2F-dependent transcription and cell cycle progression regulated by Rb (Ge and Elferink 1998; Puga et al. 2000).

3.3.3 Regulation of the AHR function by endogenous mechanisms

A xenobiotic-independent role of AHR is further supported by studies indicating activation of the AHR/ARNT complex in the absence of exogenous ligand (Chang and Puga 1998; Singh et al. 1996). A high level of nuclear AHR that forms a complex with ARNT without exposure to xenobiotic has been shown in HeLa cells (Singh et al. 1996). This observation suggests involvement of an endogenous mechanism or ligand in the activation[1] of AHR under physiological conditions in these cells. Several endogenous compounds have been identified that can bind to AHR and activate AHR-dependent gene expression. For instance: prostaglandins, tryptophan and its indole-containing metabolites, bilirubin, 7-ketocholesterol and arachidonic acid metabolites have been shown to be agonists for AHR (Chen et al. 1995; Miller 1997; Savouret et al. 2001; Schaldach et al. 1999; Seidel et al. 2001; Sinal and Bend 1997; Wei et al. 1999) (Figure 3-5). Compared to TCDD, the numerous endogenous compounds show a relatively weak affinity to AHR and are rapidly degraded by the induced detoxification enzymes. The function of endogenous ligands is regulated in a feedback mechanism, in which an increased transcription of gene coding for corresponding enzyme leads to increased metabolism, inactivation of the ligand and therefore consequent decrease in transcriptional activity. Such a phenomenon has been described for the *CYP1A1* gene, where overexpression of the enzyme leads to the repression of a reporter gene driven by the *CYP1A1* gene promoter (Jorgensen and Autrup 1996; RayChaudhuri et al. 1990). This negative autoregulation is abolished by ellipticine (an inhibitor of CYP1A1) (Morel et al. 1999).

Regulation of the AHR function by endogenous signalling cascades has also been described, straightening the hypothesis of xenobiotic-independent regulation of the receptor. Both AHR and ARNT are phosphoproteins (Mahon and Gasiewicz 1995; Minsavage et al. 2003) and their regulation by phosphorylation has been postulated. Protein kinase C (PKC) dependent phosphorylation is required for ligand-dependent transcriptional activation of the AHR regulated genes (Berghard et al. 1993). This phosphorylation influences the ligand-dependent formation of the specific DNA binding complex (Carrier et al. 1992; Minsavage et al. 2003; Park et al. 2000)

[1] In context of this study, "AHR activation" refers to the process of the AHR nuclear translocation, formation of the complex containing ARNT and binding of this complex to DRE. This term encompasses both, the transformation of AHR upon exogenous ligand binding and formation of the nuclear AHR complex induced by endogenous, yet unknown, mechanisms. Appropriate, the nuclear AHR complex on DRE will be termed "activated AHR". This however will not describe its transcriptional activity but solely the nuclear localization in a DNA bound protein complex, in contrast to the latent cytosolic AHR form.

and is also implicated in the regulation of the AHR nuclear transport (Ikuta et al. 2004). On the other hand, the ligand activated release of the AHR from the cytosolic complex with Hsp90 is regulated by tyrosine kinase activity, which influences the AHR-mediated transcriptional regulation (Gradin et al. 1994).

The serine specific cAMP-dependent protein kinase A (PKA) is also implicated in the regulation of the AHR pathway. It has been observed that administration of the N^6,$O^{2'}$-dibutyryl cyclic adenosine-3',5'-monophosphate (db-cAMP), an analog of cyclic AMP, reduces the tumor promoting effects of 7,12-dimethylbenz[a]antracene in mice (Cho-Chung et al. 1983). The carcinogenity of this compound depends on the metabolism by CYP1A1 (Wislocki et al. 1980). A cAMP-modulated activation of the metabolism by CYP1A1 has been observed in cultured fetal human adrenocortical cells. Treatment of these cells with the hormone adrenocorticotropin (ACTH) and other compounds which stimulate the production of the endogenous cAMP (forskolin and cholera toxin) stimulates the metabolism of the classical CYP1A1 substrate benzo[a]pyrene (Hornsby et al. 1985). The synergistic action of β-naphthoflavone and db-cAMP in *CYP1A1* gene activation has been shown in IEC-18 cells (Zhang et al. 1997). The specific serine/threonine phosphatases influence the activity of the DRE driven reporter construct. Inhibition of these phosphatases leads to an increase in reporter gene expression (Li and Dougherty 1997).

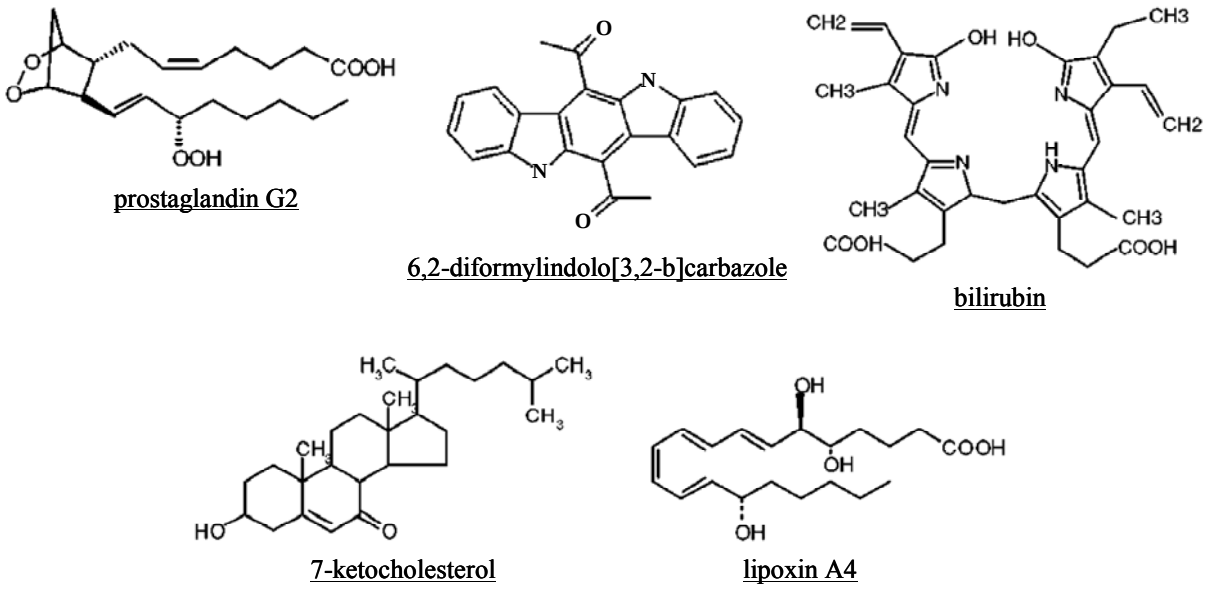


Figure 3-5. Structures of naturally occuring AHR inducers.

The above presented studies implicate that a cAMP-dependent pathway may be involved in the regulation of the xenobiotic metabolism by CYP1A1. The molecular mechanisms underlying this regulation remain hitherto unclear.

Performed in our laboratory initial studies of the PKA-dependent regulation of the AHR transformation have contributed to the understanding of PKA role in the regulation of the *CYP1A1* gene. These studies revealed that treatment of Hepa cells with the activators of PKA, db-cAMP or forskolin, initiates events leading to the translocation of the AHR to the nucleus, similar to the effect caused by treatment of these cells with TCDD. They have also shown that an increased level of cAMP in these cells triggers the binding of a specific nuclear protein complex to DRE even in the absence of TCDD. The effect of PKA-dependent activation of the AHR and ARNT binding to DRE concerning transcriptional regulation of the target genes was examined in reporter gene assays. These assays revealed that cAMP was able to decrease the TCDD-dependent activity of the construct containing the regulatory sequences of *CYP1A1* gene fused to the luciferase gene. These observations have inspired further study of the *CYP1A1* transcriptional regulation by the PKA-dependent phosphorylation.

4 AIMS OF THE STUDY

To better understand and predict the toxicity of xenobiotics like TCDD and other related chemicals able to activate the AHR-dependent gene transcription, processes involved in the control of the *CYP1A1* gene transcription were investigated. This study focused on the AHR-dependent regulation of the *CYP1A1* gene in two cell lines in which the constitutive cellular distribution of AHR and formation of AHR/ARNT heterodimer in the absence of TCDD differ noticeably. These cells were used as a model system facilitating study of the following aspects:

(I) Molecular mechanisms involved in the activation of AHR in the absence of exogenous ligand. In this view, regulation of the AHR/ARNT heterodimer formation, binding of this heterodimer to its responsive element on DNA and regulation of the *CYP1A1* transcription after modulation of PKA activity in Hepa and HeLa cells were investigated as the influence of PKA on the AHR cellular distribution and formation of protein complex on DRE has been observed in Hepa cells.

(II) Identification of factors that repress function of the exogenous ligand-independent AHR/ARNT complex bound to DRE in HeLa cells. In this respect, a participation of histone deacetylase was supposed, through the analogy to the function of unliganded nuclear receptors.

5 MATERIALS

Antibodies

Anti-Actinin: Santa Cruz Biotechnology, Heidelberg, Germany
Anti-AHR: Biomol, Hamburg, Germany
Anti-ARNT 1: Santa Cruz Biotechnology, Heidelberg, Germany
AntiHDAC1: Sigma-Aldrich, Taufkirchen, Germany
Anti-NCoR: Upstate biotechnology, distributed by Biomol, Hamburg, Germany
Anti-mSin3A: Santa Cruz Biotechnology, Heidelberg, Germany
Anti-SMRT: Dianova, Hamburg, Germany
Anti-rabbit IgG, coupled with alkaline phosphatase: Calbiochem, San Diego, USA

Cell culture media

FCS: PAA Labolatories, Cölbe, Germany
MEM alfa and Dulbecco's modifiziertes Eagle Medium (DMEM) mit Natriumpyruvat, 4500 mg/l Glucose und Glutamax-I was purcased from Gibco BRL, Karlsruhe, Germany
Tripsin/EDTA: Gibco BRL, Karlsruhe, Germany

Chemicals

ATP: Sigma-Aldrich, Taufkirchen, Germany
BCIP: Sigma-Aldrich, Taufkirchen, Germany
Bradford reagent: Bio-Rad; München, Germany
Coenzym A: Aplichem, Darmstadt, Germany
Colentrazine: Aplichem, Darmstadt, Germany
Db-cAMP: Sigma-Aldrich, Taufkirchen, Germany
D-Luciferin: Aplichem, Darmstadt, Germany
DMSO: Sigma-Aldrich, Taufkirchen, Germany

7-ethoxyresorufin: Sigma-Aldrich, Taufkirchen, Germany
Forskolin Sigma-Aldrich, Taufkirchen, Germany
H89 Sigma-Aldrich, Taufkirchen, Germany
MTT: Sigma-Aldrich, Taufkirchen, Germany
NaBu: Calbiochem, San Diego, USA
NBT: Sigma-Aldrich, Taufkirchen, Germany
Passive Lysis Buffer 5x: Promega, Manheim, Germany
Poly [d(I-C)]: Roche, Mannheim, Germany
PolyFect Transfection Reagent: Qiagen, Hilden, Germany
Protease Inhibitor Cocktail: Sigma-Aldrich, Taufkirchen, Germany
Quick Hybridization Solution: Stratagene, La Jolla, Canada
Resorufin: Sigma-Aldrich, Taufkirchen, Germany
Sonicated salmon sperm DNA: Stratagene, La Jolla, Canada
Streptavidin-Sepharose: Amersham Pharmacia, Freiburg, Germany
TSA: Sigma-Aldrich, Taufkirchen, Germany

The common chemicals used for buffers preparation were purchased from Sigma-Aldrich, Taufkirchen, Germany or ROTH, Karlsruhe, Germany

Disposable materials

Cryovials,tubes, pipets, cell culture dishes, flasks and cryovials: Greiner, Frickenhausen, Germany

Enzymes

T4 polynucleotide kinase (supplied with reaction buffer): Invitrogen, Karlsruhe, Germany
StuI (supplied with reaction buffer): MBI Fermentas, St. Leon-Rot, Germany

Equipment

Fluoreszenz-Spektralphotometer F2000: Hitachi, Berks, UK
MicroLumatPlus Luminometer: Berthold, Bad Wildbad, Germany

Phosphoimager STORM: Amersham Pharmacia, Freiburg, Germany
Spektralphotometer MPS-2000: Shimadzu, Kyoto, Japan
Scintilation counter Packard Tri-Carb A 300 CD, Canaberra packard,, Frankfurt a.M., Germany

Kits

HexaLabel™ DNA labeling Kit: MBI Fermentas, St. Leon-Rot, Germany
RNeasy Midi Kit: Qiagen, Hilden, Germany
QIAquick Nucleotide Removal Kit: Qiagen, Hilden, Germany

Materials

Hybond N+: Amersham Pharmacia, Freiburg, Germany
NICK Column: Amersham Pharmacia, Freiburg, Germany
PVDV membrane: Millipore, Schwalbach, Germany

Oligonucleotides

DRE: 5'-GATCCGGAGTTGCGTGAGAAGAGCCA
MDRE: 5'-GATCCGGAGTTATTGTAGAAGAGCCA
DRE and MDRE and their 5'-biotin labeled counterparts were synthesized by MWG Biotech AG, Ebersberg bei München, Deutchland

Radiochemicals

[γ-^{32}P]-ATP: Hartmann Analytic, Braunschweig, Germany
[α^{32}P]-dCTP: Hartmann Analytic, Braunschweig, Germany

6 EXPERIMENTAL PROCEDURES

6.1 Cell culture

Cell cultures are created by removing cells from an organism and placing them at 37 °C in a medium with nutrients and atmosphere containing CO_2. Tumor cells lack normal control of cell growth, therefore cells derived from tumors are able to grow indefinitely giving the possibility to establish cell lines. The cells growing in culture are very useful for studying the physiology of the cell in tightly controlled environmental conditions that may be altered in order to analyse response of the cells to a particular factor. Furthermore, cells can be manipulated genetically to support functional studies of genes and their products.

To preserve cells growing in culture for a long time from bacterial or fungal contaminations, basic principles of sterile technique have to be kept. Thus, all cell culture manipulations were carried out in a laminar flow hood. Plasticware including pipets, culture flasks, dishes and cryovials were purchased as sterile disposable materials. Other materials like glassware, pipette tips, Pasteur-pipettes were sterilized by autoclaving. Buffers and media were either obtained as ready to use sterile ware or sterilised by autoclaving or filtration. The cells were cultured as described in table 6-1 in an incubator at 37 °C and 5% CO_2 atmosphere. All liquids intended to contact cells were warmed up to 37 °C before use.

Table 6-1. Cell culture conditions. Cell lines used in this study were cultured as follows:

Cell line	Cell type	Growth conditions
Hepa1c1c7	mouse hepatoma	90% MEM alfa + 10% FCS, monolayer culture
HeLa	human cervix carcinoma	90% Dulbecco's + 10% FCS, monolayer culture
HepG2	human hepatoma	90% Dulbecco's + 10% FCS, monolayer culture

6.1.1 Inoculation of a new culture

Cells growing in a monolayer were allowed to proliferate to a semi-confluent (80-90 %) state. The exponentially growing cells were detached from the dish surface using trypsin and EDTA. Trypsin is a protolytic enzyme, which disrupts matrix and attachment proteins, while EDTA is a divalent ion chelating agent, which supports the release of the cells from the surface.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- Trypsin/EDTA: 0.5 g/l trypsin, 0.2 g/l EDTA in PBS (received as a ready to use sterile solution).

Prior to splitting, cells were washed with PBS, rinsed with trypsin/EDTA solution and incubated for 5 minutes at 37 °C in order to detach them from the surface of the dish. The released cells were resuspended in a fresh growth medium and diluted either 1:3 to 1:5 for a further culture or according to the requirement of the following experiment.

6.1.2 Cryopreservation of cells

Aliquots of each cell line were stored at temperatures from -135^0 to -175^0 C in liquid nitrogen. To prevent intracellular ice crystal formation during freezing, the cells were stored in medium containing DMSO as a cryoprotectant.

Solutions:

- Freezing medium: the appropriate culture medium, containing 10 % FCS and 10 % DMSO,
- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- Trypsin/EDTA: 0.5 g/l trypsin, 0.2 g/l EDTA in PBS.

Exponentially growing cells collected after trypsin treatment (as described in section 6.1.1) and centrifugation (500 x g, 5 minutes, 4 °C) were resuspended in freezing medium in a concentration of 5 x 10^6 cells/ml. The cell suspension was transferred to cryovial and frozen in a stepwise manner until the aliquots reached the temperature of -80 °C, then the cells were transported into the liquid nitrogen chamber.

When needed, an aliquot of the cells was removed from the liquid nitrogen, warmed up to 37 °C

in as short as possible time and immediately transferred to the culture dish containing a fresh culture medium. After the cells had attached to the dish surface (from 4 hours to over night, depending on the cell type), the culture medium was changed to a fresh portion and the cells were cultured under usual conditions for several days prior to using them in appropriate experiments.

6.1.3 MTT test

MTT tests are used for the estimation of cell viability in various conditions. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrasolium bromide; thiazolyl blue), which yields a yellow water solution, is converted to a purple formazan by dehydrogenase enzymes. Only active mitochondrial dehydrogenases of living cells causes this conversion, thus the concentration of the formazan gives information about the viability of cells and allows estimation of toxic effects in cell culture.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- MTT solution: 5 mg/ml MTT diluted in PBS
- Isopropanol

5 x 10^4 cells/ml medium/well were seeded in a 24 well plate and cultured for 24 hours. The cells were then treated with a chemical used in various concentrations for the course of time intended in the final experiment and a control group of the cells was incubated in the presence of solvent. After treatment, the cells were washed with PBS and incubated with 0.5 ml medium/well containing MTT solution (0.5 ml MTT solution/10 ml medium) for 3-4 hours, until the purple crystals became visible. The MTT-medium was washed out with PBS and 500 µl of isopropanol was added to each well followed by incubation on a shaker (150 rpm) for 30 minutes at room temperature. The absorbance of the formazan containing isopropanol solution was measured at λ=570 nm. Cytotoxicity of a chemical was established by comparison of absorbance of the solution raised from chemically treated cells with that from control cells, the later assumed as 100 % viable.

6.1.4 Transient transfection

The aim of transient transfection is a delivery of foreign DNA into eukaryotic cells, but without its integration into the chromosome. In consequence, many copies of the gene of interest are present in the cell nucleus, leading to a high level of expressed protein within 24 to 96 hours after introduction of DNA, depending on the plasmid used. For this study, the transfection was carried out using a circular, supercoiled plasmid DNA and PolyFect Transfection Reagent, according to the manufacturer handbook. When a treatment of the transfected cells was required, it was initiated 24 hours after transfection and carried out as described in a legend of the figure illustrating the experiment.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- Serum free medium: medium appropriate for the used cell type not supplemented with FCS,
- PolyFect Transfection Reagent,
- TE buffer: 10 mM Tris-HCl ph 8.0, 1 mM EDTA.

5 x 10^3 cells/100 µl medium/well were seeded in a 96 well plate and cultured for 24 hours. 200 ng DNA and 0.5 µl PolyFect reagent diluted in serum free medium, 30 and 20 µl respectively, were used for a single-well transfection which was then carried out according to the manufacturer protocol. For cotransfection experiments, DNA constructs were used in amounts described in the figure legends and adjusted with an appropriate amount of pCDNA3.1 plasmid to maintain the same 200 ng DNA/well. All transfections were carried out in six to eight replications.

6.2 Protein-protein and protein-DNA interactions *in vivo*

6.2.1 Preparation of nuclear proteins

A rapid method for preparation of nuclear proteins from cells bases on the procedure described by Andrews (Andrews and Faller 1991). Basic principles of this method are hypotonic lysis of the cells, isolation of nuclei by centrifugation and subsequent high salt extraction of the

nuclear proteins. To prevent degradation of isolated proteins, all steps were performed on ice, all buffers were cold and buffers A and C were supplemented with protease inhibitors.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- 10 % NP-40 in H_2O,
- Buffer A: 10 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.01 v/v Protease Inhibitor Cocktail,
- Buffer C: 20 mM Hepes-KOH pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 0.01 v/v Protease Inhibitor Cocktail (DTT and protease inhibitors were added just before use),
- Buffer D: 20 mM Hepes pH 7.9, 20% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT (PMSF and DTT were added just before use).

Cells were seeded in 145 mm dishes, cultured until they achieved 80-90 % of confluence and treated with chemicals as described in the figure legends. After treatment, cells were washed with PBS, scraped into a 15 ml tube and pelleted by centrifugation (500 x g, 5 min, 4 °C). Cell pellet was resuspended in 1.6 ml of cold buffer A by vortexing and 60 µl of 10 % NP-40 was added to the suspension. Cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were centrifuged (1000 g, 5 minutes, 4 °C) and supernatant fractions were discarded. Nuclei were washed twice with 1 ml buffer A, resuspended in 200 µl of cold buffer C and incubated on ice for 20 minutes for a high-salt extraction. Cellular debris was then removed by centrifugation (maximal speed, 10 minutes, 4 °C). The supernatant fraction was divided into 100 µl aliquots, rapidly frozen in liquid nitrogen and stored at -80 °C until use. Optionally, if the extracts were intended to be used in electromobility shift assays, protein extracts were subjected to dialysis against 50 x sample volume of buffer D for 1 hour in order to reduce the salt concentration. Afterward, aliquots were prepared and stored as described above.

6.2.2 Preparation of cellular proteins

Isolation of cellular proteins was carried out essentially as described (Heinzel et al. 1997). The procedure bases on the observation that detergent molecules destroy cell membranes enabling extraction of cellular proteins. Similar to the procedure for nuclear extract preparation, all steps are prepared at 4 °C and lysis buffer is supplemented with protease inhibitors.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- NETN buffer: 20 mM Tris pH 8.0, 0.1 % NP-40, 100 mM NaCl, 1 mM EDTA, 10 % glycerine, 1 mM DTT, 0.5 mM PMSF, 0.01 v/v Protease Inhibitor Cocktail (DTT and protease inhibitors were added just before use).

Cells were cultured, treated and collected as described in section 6.2.1. Cell pellet was resuspended in 1 ml of cold buffer NETN by vortexing, incubated for 10 minutes on ice and then centrifuged (maximal speed, 10 minutes, 4 °C). The supernatant fraction containing cellular proteins was divided into 200 µl aliquots that were rapidly frozen in liquid nitrogen and stored at -80 °C until use.

6.2.3 Estimation of protein concentration

Estimations of a protein concentration in nuclear or cellular protein extracts were carried out according to the Bradford method. This method bases on the observation that a red acidic solution of Coomassie Brilliant Blue yields a blue solution when the dye binds to proteins. The absorbance at λ=595 nm correlates with the protein concentration.

Solutions:

- BSA standard: 100 µg/ml BSA in H_2O,
- 5 x Bradford Dye Reagent .

1-10 µl of protein extract were mixed with H_2O to give 800 µl protein solution. Several dilutions containing 2 to 20 µg of BSA in 800 µl H_2O were also prepared prior every measurement. 200 µl of 5 x Bradford Dye Reagent was added to each sample and 5 minutes later the absorbance at λ=595 was measured. The concentrations of proteins in each sample were calculated using the spectrophotometer software.

6.2.4 Immuncoprecipitation of protein complexes

In protein extracts prepared from the cells under nondenaturating conditions many of the protein-protein associations that exist in intact cells are maintained. This fact enables the

identification of physiologically relevant protein-protein interactions by the means of immuncoprecipitation assays. In the assays, a particular protein is bound by a specific antibody, and other proteins stably associated with the epitop carrying protein *in vivo* may also stay in this complex. The antibody-protein complexes are precipitated using agarose beads coupled with protein A or protein G. Both, staphylococcal protein A and streptococcal protein G, have high but different and thus complementary affinities for the Fc region of mammalian antibodies which enable an efficient isolation of the immune complexes. Coprecipitation of a protein of known identity may be subsequently detected by Western blot.

Solutions:

- Extracts of nuclear proteins: described in section 6.2.1,
- Buffer C: 20 mM Hepes-KOH pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT (added just before use),
- 2 x Laemmli buffer: 100 mM Tris-HCl pH 6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 200 mM DTT (added just before use).

An appropriate antibody (1 µg) was added to the sample containing 1000 µl of nuclear extract (about 2.5 mg protein) that was then incubated on ice for 2 hours. Afterwards 10 µl of Protein A/G+ Agarose was added and the sample was incubated for the next 1 hour on a rotating platform at 4 °C. The agarose bound complexes were precipitated by centrifugation (maximal speed, 5 minutes, 4 °C) and washed three times with 800 µl of cold buffer C. The precipitated protein complexes were resolved in 30 µl of 1 x Laemmli buffer (one volume of 2 x Laemmli buffer + one volume H_2O), boiled for 5 minutes and analysed by Western blot.

6.2.5 Electromobility shift assay

Electromobility shift assays (EMSAs) rely on the fact that during electrophoresis under nondenaturating conditions, DNA fragment bound to protein complexes has a reduced mobility compared to protein-free DNA. The DNA fragment is typically radiolabeled with [^{32}P], which enables visulization of its position in an acrylamide gel. Binding of a DNA probe to proteins is carried out in the presence of poly-[d(I-C)], a competitor which reduces the non-specific interactions. Essentially, assays were carried out as described (Denison et al. 1988b).

Solutions:

- nuclear extracts prepared as described in section 6.2.1. subjected to dialysis against buffer D,

- buffer D: 20 mM Hepes pH 7.9, 20 % glycerol, 80 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT (PMSF and DTT were added just before use),
- poly [d(I-C)]: 250 ng/µl in HEDG buffer (25 mM Hepes, 1 mM EDTA, 10 % glycerol, pH 7.5)
- [^{32}P]-DRE probe prepared as described in section 6.4.1,
- loading buffer: 25 % w/v Ficoll 400, 0.25 % w/v bromophenol blue,
- 10 x TAE: 400 mM Tris base, 200 mM sodium acetate, 10 mM EDTA,
- 30 % acrylamide mix: 1 % N,N'-methylenebisacrylamide, 29 % acrylamide,
- APS: 10 % ammonium persulfate in H_2O,
- TEMED
- running buffer: 1 x TAE

Nuclear extracts containing 15 µg proteins were mixed with buffer D to bring the final volume to 21 µl. The protein samples were preincubated with 2 µl of poly [d(I-C)] at room temperature for 15 minutes, afterward 2 µl of a [^{32}P]-DRE probe (~ 100 000 cpm/µl, described in section 6.4.1) was added and samples were incubated at room temperature for further 15 minutes. After incubation, 2.8 µl of loading buffer was added to each sample and 20 µl of the reaction mixture was loaded onto a 4 % non-denaturating polyacrylamide gel, prepared as follows. Using values given in table 6-2, solutions for the gel were mixed and immediately poured into the gap between glass plates, then a Teflon comb was inserted into the gel which then polymerized. After polymerization of the gel, a preelectrophoresis was carried out at 4 °C and 80 V for 1 hour prior to loading of the protein-DNA samples. Electrophoretical separation of protein-DNA complexes was carried out at the same conditions until the bromophenol blue dye reached the bottom of the gel.

Table 6-2. Solutions for preparing 4 % non-denaturating gels.

Solutions	Volume (ml)
H_2O	38.2
30 % acrylamide mix	6.5
10 x TAE	5
10 % APS	0.35
TEMED	0.0175

After the electrophoresis was finished, the gel plates were pried apart, the gel was transferred to a piece of Whatman 3 MM blotting paper and dried on a gel dryer. The dried gel was then exposed on a phosphor imager to visualize protein-DNA complexes.

6.2.6 Supershift assay

A particular protein bound to DNA can be identified using a specific antibody that binds a protein member of the complex observed in EMSA, leading to an increase of its molecular weight observed as a "supershift". To perform supershift assays, 1µl of antibody was added to the reaction mixture before formation of the complex with a DNA probe. Protein extracts containing antibody were incubated at room temperature for 15 minutes. After incubation poly-[d(I-C)] and the [^{32}P]-probe were added to the samples and the remaining procedure was carried out as described in the previous section.

6.2.7 Avidin-biotin complex on DNA

"Avidin-biotin complex on DNA" (ABCD) assays utilize a similar principle, as described for the immuncoprecipitation method. The difference between the two methods is that the protein complexes are precipitated from the nuclear extracts based on their specific binding to oligonucleotide sequences instead of antibodies. Thus the ABCD assay is an excellent tool for the analysis of protein-DNA interactions. In this assay, the biotin-labeled double stranded oligonucleotide is incubated with protein extracts to form a complex with the proteins that recognizes its sequence. Sepharose beads conjugated to streptavidin that binds the biotin entity with high affinity, are used for the precipitation of the biotin-DNA-protein complexes. The DNA-bound proteins may be subsequently detected by Western blot.

Solutions:

- Extracts of nuclear proteins: see section 6.2.1,
- 10 x hybridization buffer: 0.2 M Tris pH 7.4, 0.5 M NaCl,
- ssDNA: salmon sperm DNA 10 mg/ml,
- Buffer C: 20 mM Hepes-KOH pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT (added fresh).

- Buffer B: 20 mM Hepes-KOH pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT (added fresh),
- 2 x Laemmli buffer: 100 mM Tris-HCl pH 6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 200 mM DTT (added fresh).

For hybridization of the biotinylated oligonucleotides, a 100 µl mixture containing 50 µg of the 5' biotin-labeled oligonucleotide, 50 µg of the 5' biotin-labeled complementary strand and 1 x hybridization buffer, was boiled for 5 minutes and allowed to chill slowly to room temperature. 2 µl of the double stranded biotinylated oligonucleotide and 1µl of ss DNA were added to 100 µl of nuclear extract (containing about 250 µg protein). The samples were incubated for 5 minutes at 30 °C and then for 1 hour on ice. During the time of the incubation, an aliquot of 500 µl streptavidin-sepharose beads was washed twice with 800 µl buffer B and resuspended in 500 µl of fresh buffer B. 50 µl of the beads suspension was added to the protein-DNA containing samples and incubated for the following 1 hour on a rotated platform at 4 °C. After incubation, the protein-DNA complexes were precipitated by centrifugation (maximal speed, 5 minutes, 4 °C) and washed three times with 800 µl of cold buffer C. Then the precipitated material was resolved in 30 µl of 1 x Laemmli buffer (one volume of 2 x Laemmli buffer + one volume of H_2O), boiled for 5 minutes and analysed by Western blot.

6.2.8 Western blot

Western blot analysis of proteins consists of three steps: electrophoretical separation of the proteins on a polyacrylamide gel, transfer to a solid support and detection of the protein by a specific antibody. In this study all these steps were carried out according to standard procedures.

6.2.8.1 SDS-polyacrylamide gel electrophoresis

SDS-PAGE is carried out under denaturating conditions that minimize aggregation of proteins and ensure dissociation of protein complexes into their individual subunits. The gradients of pH and acrylamide density of the stacking and resolving gels enable concentration of the proteins in the sample into a very small volume and increase the resolution of gels. Concentration of acrylamide in the resolving gel depends on the molecular weight of the protein to be detected (Table 6-3).

Solutions:

- 2 x Laemmli buffer: 100 mM Tris-HCl pH 6.8, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 200 mM DTT (added fresh),
- 30 % acrylamide mix: 1 % N,N'-methylenebisacrylamide, 29 % acrylamide,
- SDS: 10 % sodium dodecyl sulphate in H_2O,
- APS: 10 % ammonium persulfate in H_2O,
- TEMED,
- 1.5 mM Tris-HCl pH 8.8,
- 1.0 mM Tris-HCl pH 6.8,
- Tris-glycine electrophoresis buffer: 25 mM Tris-base pH 8.3, 250 mM glycine, 0.1 % SDS.

Using the values given in table 6-3-A, solutions for the resolving gel were mixed and immediately poured into the gap between glass plates; the surface of the gel was carefully covered with water. After complete polymerization of the gel (~ 30 minutes), solutions for the stacking gel (Table 6-3-B) were mixed and poured over the resolving gel. A Teflon comb was inserted into this gel, which then polymerized.

Table 6-3. Solutions for preparing gels for SDS-PAGE. A Composition of resolving gels. **B.** Composition of a 5 % stacking gel.

A.	Molecular weight of the protein	200- 300 KD	80 – 100 KD	40 – 70 KD
	Acrylamide concentration	6 %	8 %	10 %
Solutions Volume (ml)	H_2O	21.2	18.5	15.9
	30 % acrylamide mix	8.0	10.7	13.3
	1.5 M Tris pH 8.8	10.0	10.0	10.0
	10 % SDS	0.4	0.4	0.4
	10 % APS	0.4	0.4	0.4
	TEMED	0.032	0.024	0.016

B.

Solutions	Volume (ml)
H_2O	4.1
30 % acrylamide mix	1.0
1.0 M Tris pH 6.8	0.75
10 % SDS	0.06
10 % APS	0.06
TEMED	0.006

Protein samples, prepared by adding the equal volume of 2 x Laemmli buffer to the protein extract and boiling for 5 minutes, were loaded onto a gel in the presence of a molecular weight marker. In the case of immuncoprecipitation or ABCD assays, care was taken to avoid loading of the beads, which were present in the samples. The electrophoresis was performed at 60 V until the dye reached the resolving gel and then at 120 V until it reached the bottom of the gel.

6.2.8.2 Semi-dry transfer of proteins to a solid support

For the forward analyses, the proteins separated in the polyacrylamide gel are transferred from the gel to a PVDV membrane, where they immobilize. The semi-dry transfer is achieved by placing the gel directly onto the membrane and keeping the “sandwich” in an electric field, which causes a migration of the proteins from the gel towards the membrane, where they bind covalently.

Solutions:

- Methanol,
- Transfer buffer: 25 mM Tris-base pH 8.3, 250 mM glycine, 0.1 % SDS, 20 % methanol.

The PVDV membrane was activated for 1 minute in methanol and rinsed for 2 minutes in water. The gel and the methanol-activated membrane were equilibrated in transfer buffer for 15 minutes prior to transfer. The following ”sandwich” was constructed on the anode surface of the blotter: three pieces of Whatman 3 MM wetted in transfer buffer/membrane/polyacrylamide gel/three pieces of the Whatman 3 MM wetted in transfer buffer. The “sandwich” was covered with the cathode plate and the transfer was started with the applied current depending on the size of the gel (0.65 mA/1 cm^2). The transfer was carried out for 3 hours.

6.2.8.3 Immunodetection

After the protein transfer, remaining binding sites on the membrane are blocked to eliminate any unspecific reaction of the antibodies with the membrane. The target protein is than identified by exposure of the membrane to the primary antibody that specifically binds to the epitop harbouring protein, followed by incubation of the membrane with secondary antibody coupled to alkaline phosphatase (AP). The enzyme catalyzes the conversion of NBT and BCIP to a blue purple insoluble dye, which precipitates at sites of the alkaline phosphatase activity, colouring the membrane purple-blue.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- Blocking solution: 5 % non fat dried milk in PBS,
- Antibody solution: antibodies in concentrations as follows: α-AHR 1 : 1000, α-ARNT 1 : 500, α-actinin 1 : 1000, α-HDAC1 1: 2000, α-NCoR 1 : 500, α-mSin3A 1:1000, α-SMRT 1: 2000, AP-conjugated α-rabbit IgG 1 : 2000, were diluted in blocking solution,
- Washing buffer: 0.05 % tween 20 in PBS,
- AP buffer: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$,
- NBT solution: 4-nitro blue tetrazolium chloride 50 mg/ml in 70 % dimethyl-formamide,
- BCIP solution: 5-bromo-4-chloro-3-indolyl phosphate 50 mg/ml BCIP in 100 % dimethylformamide,
- AP reaction buffer: 10 ml of AP buffer containing 66 µl of NBT solution and 33 µl of BCIP solution,
- Quenching solution: 2 mM EDTA in PBS

After transfer the membranes were incubated in blocking solution for 1 hour at room temperature or over night at 4 °C, followed by incubation in the primary antibody solution for 2 hours at room temperature or over night at 4 °C. Afterwards, the antibody was washed out three times with washing buffer for 30 minutes, the membranes were covered with the secondary antibody solution and incubated for 1 hour at room temperature. The antibody was washed out in the same way as the primary antibody, and after a short equilibration in AP buffer, the membranes were incubated with AP reaction buffer. When the bands became visible, the reaction was stopped by rinsing of the membrane with quenching solution.

6.3 Analysis of the *CYP1A1* gene expression

6.3.1 Ethoxyresorufin-*o*-deethylase assay

In the ethoxyresorufin-*o*-deethylase (EROD) assay, CYP1A1 utilizes the substrate 7-ethoxyresorufin and oxidizes the α-C-H to α-C-OH entities. The product is a hemiacetal that spontaneously dissociates to give resorufin, which can be quantified by fluorescence spectroscopy. The presence of dicumarol during the reaction protects the produced resorufin from forward conversion by the cytosolic DT-diaphorase activity. EROD assays were performed directly in intact cell cultures based on the procedure described by Petrulis (Petrulis and Bunce 1999).

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- 7-Ethoxyresorufin solution: 500 µM in ethanol,
- Resorufin solution: 1 µM resorufin sodium salt in ethanol,
- Dicumarol solution: 600 µM in 100 mM Tris-HCl pH 8.0,
- Methanol,
- PLB:1 volume 5 X Passive Lysis Buffer (Promega) in 4 volumes H_2O.

A range of solutions containing 1 to 50 pmol/ml resorufin sodium salt was prepared by dilution of resorufin solution in 500 µl medium followed by addition 500 µl methanol. Samples were vortexed and centrifuged (maximal speed, 5 min) and then the fluorescence of the samples was quantified by fluorescence spectroscopy with excitation at λ=550 nm and emission at λ=585 nm. The standard equation of the concentration-fluorescence dependence, linear in this concentration range, was subsequently used for estimation of the resorufin concentration in the cell culture medium carried out as described below.

5×10^4 cells/ml medium/well were plated in 24 well plates and treated with chemicals as described in figure legends. After treatment, the cells were washed with PBS. 650 µl medium containing 1.25 µM 7-ethoxyresorufin and 1.5 µM dicumarol was added to each well and the cells were incubated with the CYP1A1 substrate for 1 hour. Afterwards the reaction was stopped by mixing 500 µl of the incubation medium with 500 µl methanol. Samples were centrifuged (maximal speed, 5 minutes) and a fluorescence of the samples was measured as described for the standard resorufin solutions.

Remaining cells were washed with PBS, covered with 50 µl of PLB per well and lysed with gentle rocking on an orbital shaker for 15 min at room temperature. The cell lysates were subsequentially used for a determination of protein amounts as described in section 6.2.3. An average mean of EROD activity received in 4 to 6 repetitions was expressed as picomoles of resorufin formed per milligram of protein per 1 minute of incubation time.

6.3.2 Northern blot analysis of the *CYP1A1* mRNA

Northern blot analysis is a common method to monitor gene expression by quantification of the specific mRNA transcribed from the gene of interest. The analysis of mRNA is performed in several steps: isolation of the total RNA from the cells, its electrophoretic separation in a denaturing gel followed by transfer to a nylon membrane. mRNA may be identified by hybridization to a specific [^{32}P]-labeled DNA probe and visualization using a phosphor imager system. Aseptic conditions should be kept during the preparation in order to avoid a damage of isolated RNA by very stable and active RNases. Thus, the ware used for the assays was either disposable, sterile and RNase free or decontaminated by treatment with 0.1 mM NaOH, 1 mM EDTA followed by washing in RNase free water.

6.3.2.1 Isolation of total RNA

Total RNA was isolated from the cells using RNeasy Midi Kit (Qiagen) according to the manufacturers protocol. The protocol utilizes cell lysis in the presence of highly denaturating guanidine isothiocyanate which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol present in the lysates provides appropriate binding conditions enabling isolation of the total RNA on a column.

RNA was isolated from cells that grown to ~80 % confluence on 145 mm culture dishes and treated as described in figure legends. The amount of RNA in each sample was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (concentration of RNA in the sample = 40 x A_{260} x dilution factor).

6.3.2.2 Formaldehyde agarose gel electrophoresis

Samples of RNA were denatured by treatment with formamide and separated by electrophoresis through agarose gels containing formaldehyde.

Solutions:

- 10 x FA gel buffer: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA,
- 1 x FA gel running buffer: 100 ml 10 x FA gel buffer, 20 ml 37 % (12.3 M) formaldehyde, 880 ml RNase-free water,
- 1.2 % FA gel: 1.2 g agarose, 10 ml 10 x FA gel buffer, RNase-free water to 100 ml,
- 5 x Loading buffer: 16 µl saturated aqueous bromophenol blue solution, 80 µl 500 mM EDTA, 720 µl 37 % (12.3M) formaldehyde, 2 ml 100 % glycerol, 3084 µl formamide, 4 ml 10 x FA gel buffer, RNase-free water to 10 ml,

All components of 1.2 % FA gel were mixed and resolved by boiling in a microwave oven. After cooling to 65 °C, 1.8 ml of 37 % (12,3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution were added to the gel. The gel was mixed, poured onto a gel support and allowed to polymerize. Prior to running the gel was equilibrated in 1 x FA gel running buffer at 60 V for at least 30 minutes. 25 µl samples containing 10 µg of total RNA and 5 µl of 5 x loading buffer in RNAse free water were denaturated by heating at 65 °C for 5 minutes and rapidly chilling in ice water. The samples were loaded onto the gel and subjected to electrophoresis at 60 V for 2 hours. After completion of the electrophoresis, integrity and size distribution of isolated RNA was tested by ethidium bromide staining and visualization in UV light. Afterwards the RNA was transferred to nylon membranes.

6.3.2.3 Transfer and fixation of RNA to membranes

Transfer of RNA fractionated by agarose gel electrophoresis to the positively charged nylon membranes was carried out in neutral pH. RNA was then covalently linked to the membranes at hight temperatures.

Solution:

- 20 x SSC: 3 M NaCl, 0.3 M sodium citrate ($Na_3C_6H_5O_7$**.**H_2O), pH 7.0,

The gel and a piece of Hybond + membrane were rinsed in RNase-free water and soaked for 5 minutes in 20 x SSC transfer buffer. Then the capillary transfer was carried out over night in 20 x SSC buffer using the Turbobloter Rapid Downward Transfer System according to the

manufacturers protocol. After transfer, the membranes were air dried and baked for 2 hours at 80 °C.

6.3.2.4 Hybridization

RNA samples immobilized on the membranes were subsequently hybridized with specific [^{32}P]-labeled DNA probes using QuickHyb hybridization solution. Hybridization was carried out in rolled bottles in a hybridization oven.

Solutions

- QuickHyb solution (Stratagene)
- [^{32}P]-radiolabeled DNA probe prepared as described in section 6.4.2,
- ssDNA: salmon sperm DNA 1 mg/ml
- 20 x SSC: 3 M NaCl, 0.3 M sodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$), pH 7.0,
- SDS: 10 % sodium dodecyl sulphate in H_2O

Membranes were rinsed in deionized water for 15 minutes. To decrease non specific binding of the probe, the membranes were prehybridized in Quick Hyb solution (33 µl of the solution per 1 cm^2 of the blot) at 68 °C for 15 minutes prior to addition of the radiolabeled probe. During the time of prehybridization, the probe was prepared as follows: the double-stranded probe (3 x 10^7 cpm/ml of hybridization solution) was mixed with 100 µl of ssDNA, boiled for 5 minutes and rapidly chilled in ice water. 1 ml of the prehybridization solution was removed from the blot-containing bottle, mixed with the denaturated probe and placed back to the bottle. The incubation of the blot at 68 °C was then continued for the next 2 hours. After hybridization the unspecific bound probe was washed out by incubation of the blot at room temperature in two changes of 2 x SSC/0,1 % SDS buffer for 15 minutes followed by incubation at 60 °C in 0,1 x SSC/0,1 % SDS for 30 minutes. After washes, the membranes were wrapped in a plastic bag and visualized using a phosphor imager.

6.3.3 Dual luciferase reporter gene assay

A reporter gene assay is a tool used to determine how *cis*-acting sequences, such as promoters and enhancers, control eukaryotic gene expression. For this purpose, the *cis*-acting sequence from the gene of interest is linked to the coding sequence of an unrelated reporter gene and the construct is introduced into the cells by transfection (described in section 6.1.4) where its

activity can be measured under various conditions. The advantages of luciferase reporter genes are first, the absence of this enzyme in mammalian cells which reduces background activity and, on the other hand, the possibility of quick, easy and sensitive measurement of the reporter activity using a luminometer.

The "dual" reporter gene system uses the transfection of two individual reporter enzymes within a single experiment. The experimental construct: firefly (*Photinus pyralis*) luciferase coupled to the enhancer element of the *CYP1A1* gene was cotransfected with the control *Renilla* (*Renilla reniforms*) luciferase coupled to SV40 early promoter/enhancer region, providing strong, constitutive expression of the reporter activity in a variety of cell types. The cotransfection of the two reporter genes aids in normalizing the activity of the experimental construct to the activity of the internal control and minimizes variability caused by differences in the transformation efficiency.

Solutions:

- PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH adjusted to 7.4,
- PLB: 1 volume 5 X Passive Lysis Buffer (Promega) in 4 volumes H_2O,
- 2 x Luciferase buffer: 60 mM Tricine pH 7.8, 0.2 mM EDTA, 30 mM $MgSO_4$, 20 mM DTT (added just before use),
- D-Luciferin: 25 mM in 25 mM NaOH,
- Coenzym A: 27 µM in H_2O,
- ATP: 100 mM in H_2O,
- Luciferase substrate buffer: 5 ml of 2 x Luciferase buffer, 188 µl D-Luciferin, 100 µl Coenzym A, 53 µl ATP, filled with H_2O up to 10 ml,
- *Renilla* buffer: 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $CaCl_2$,
- Colentrazine: 90 µM in ethanol,
- *Renilla* substrat buffer: 10 µl Colentrazine in 10 ml Renilla buffer,

Cells were seeded in 96 well plates, transfected as described in section 6.1.4 and treated as described in the figure legends. After treatment, the cells were washed with PBS and lysed in 50 µl PLB by gentle rocking on an orbital shaker at room temperature for 15 minutes. The assays for firefly and *Renilla* luciferase activities were performed sequentially using aliquots of 20 µl of cell lysate for each measurement. For this purpose 2 x 20 µl of each lysate were transferred to 96 well plates. Using an auto-injector, 100 µl of Luciferase substrate buffer was injected to the first aliquot and the measurement was performed with a 2 seconds premeasurement delay followed by a 10 seconds measurement period for each assay. *Renilla* luciferase activity was estimated in the

second aliquot, using *Renilla* substrate buffer and the same parameters as for the firefly luciferase assay. The average means of reporter enzyme activity obtained from six to eight wells per treatment was then expressed as relative firefly to *Renilla* luciferase activity units (RLU).

6.4 Radiolabeling of DNA probes

6.4.1 Labeling of DNA-probes using T4 polynucleotide kinase

T4 polynucleotide kinase catalyzes the transfer of γ-phosphate residues from [γ-^{32}P]-ATP to the 5'-hydroxyl termini of single stranded or 5'-protruded double stranded DNA fragments. Probes labeled using this method are suitable for electromobility shift assays.

Solutions:

- TE buffer:
- oligonucleotides: 100 ng/µl in TE buffer,
- [γ^{32}P]-ATP with specific activity of 3000 Ci/mmol,
- 10 x T4 kinase buffer: supplied with the enzyme,
- T4 kinase (30u/µl).

For hybridization of the oligonucleotides, 100 µl mixture containing 500 ng of the sense oligonucleotide and 500 ng of the antisense strand in TE buffer was boiled for 5 min and allowed to chill slowly to room temperature. The double stranded oligonucleotide was subsequentially subjected to the phosphorylation reaction in 50 ml mix containing: 25 µl double stranded DNA (250 ng), 5 µl [γ^{32}P]-ATP (50 µCi), 5 µl 10 x T4 kinase buffer, 18 u T4 kinase. Reaction was carried out at 37 °C for 30 minutes. Radioactive oligonucleotides were then purified from the enzymatic reaction mixture using QIAquick Nucleotide Removal Kit (Qiagen) according to the protocol. The activity of the probe was estimated by measurement in a scintillation counter, the activity of the probes obtained by this method was usually ~100 000 cpm/µl.

6.4.2 Labeling of DNA fragments by extension of random oligonucleotides

Short (6-8 base pairs) oligonucleotides can serve as primers for the initiation of DNA synthesis on single-stranded templates by a DNA polymerase. Using heterogeneous

oligonucleotides of random sequences that form hybrids at many positions of any DNA strand and a mix of four deoxyribonucleotides containing one [α^{32}P]-dNTP as precursors, it is possible to generate a radiolabeled complementary strand with an activity enabling specific detection e.g. RNA in Northern blot analyses. CYP1A1 and GAPDH probes were labelled using HexaLabel^{TM} DNA Labeling Kit (Fermentas).

Solutions:

- DNA templates: (I) a 450 bp Stu I restriction fragment of pBluescript SK+/- cloning vector containing CYP1A1 cDNA (kindly provided by M. Lohman) generated by standard methods of molecular cloning and (II) a PCR amplified GAPDH cDNA (kindly provided by M. Christman).
- Kit components containing: hexanucleotide mix in 5 x reaction buffer, deoxynucleotide mix (minus dCTP) and Klenow fragment (exo$^-$) of Polymerase I (5 u/µ).
- [α^{32}P]-dCTP with specific activity of 3000 Ci/mmol

The labeling procedure was performed as described in the manufacturers protocol using 100 ng template DNA and 50 µCi [α^{32}P]-dCTP in every reaction. The labelled DNA was subsequently purified using prepacked columns containing Sephadex G-50 NICK (Pharmacia Biotech) according to the supplied protocol. Activity of the probe was estimated by measurement in a scintillation counter, the probes obtained by this method usually had an activitxy of ~100 000 – 300 000 cpm/µl.

7 RESULTS

7.1 Optimization of experimental conditions

7.1.1 Chemical treatment of cells

Two cell lines HeLa and Hepa were used for studying the following phenomena: (I) protein kinase A dependent phosphorylation as an endogenous mechanism that control AHR function (II) participation of histone deacetylase in the regulation of *CYP1A1* transcription.

Experiments aimed in the explanation of the PKA and HDAC roles in AHR function were performed in cells treated with activators or inhibitors of these enzymes. To avoid a false interpretation of the effects caused by the toxic doses of the chemicals, the non-toxic concentration of each compound was established using the MTT test. For this purpose, cells were cultured under their usual conditions and treated with various amounts of a particular compound for the period of time intended to be used in the ultimate experiment. The concentration of the chemical, which did not cause a decrease in viability of the cells greater then 5 %, as compared to untreated cells, was assumed to be non cytotoxic and maintained in the subsequent experiments.

The toxic effect of TCDD was not analysed because this compound was conventionally used in our laboratory and no toxic effects were observed even when it was used in the concentration of 10 nM.

7.1.1.1 Viability of cells treated with inhibitors of the HDAC activity

Trichostatin A (TSA), originally used as a fungistatic antibiotic, is a potent and specific inhibitor of the histone deacetylase activity. This compound acts as a competitive inhibitor, which competes with a substrate in binding to the active centre of enzyme. It induces accumulation of highly acetylated histones *in vivo* and is able to inhibit the activity of histone

deacetylase when used in the nanomolar range (Yoshida et al. 1990). Sodium butyrate (NaBu), short fatty acid produced in humans by bacterial fermentation in the colon (Cummings and Stephen 1980), is a non competitive inhibitor, but the mechanism of its action is not well understood. In milimolar concentrations NaBu inhibits HDAC and has pleiotropic effects on the cell physiology (Davie 2003; Riggs et al. 1977).

The analysis of the viability of cells in the presence of TSA revealed that this inhibitor did not exhibit any significant toxic effects on HeLa cells up to 160 nM. Hepa cells proved to be more sensitive and have shown a decreased viability when exposed to 32 nM TSA. The toxic effects of NaBu was observed in HeLa cells exposed to 4 mM and in Hepa cells exposed to 1.8 mM NaBu (Table 7-1).

Table 7-1. Viability of cells treated with inhibitors of histone deacetylase. Cells were incubated for 48 hours in the presence of NaBu or TSA in concentrations as indicated in the tables, followed by the estimation of their viability using MTT test.

HeLa		Hepa	
TSA (nM)	% of viability ± standard deviation	TSA (nM)	% of viability ± standard deviation
0	100 ± 2.7	0	100 ± 1.3
40	102 ± 5.1	8	98 ± 2.6
80	97 ± 4.7	16	99 ± 8.6
120	98 ± 4.8	24	96 ± 3.8
160	95 ± 3.2	32	87 ± 3.6
200	77 ± 6.7	40	76 ± 6.0

HeLa		Hepa	
NaBu (mM)	% of viability ± standard deviation	NaBu (mM)	% of viability ± standard deviation
0	100 ± 4.7	0	100 ± 1.3
1	98 ± 1.1	0.6	100 ± 2.6
2	99 ± 5.4	1.2	99 ± 4.6
3	97 ± 3.9	1.8	92 ± 3.8
4	94 ± 6.2	2.4	86 ± 3.6
5	86 ± 10.7	3	74 ± 14.8

7.1.1.2 Viability of cells treated with modulators of the PKA activity

Protein kinase A is activated in the cell by the low molecular weight second messenger, cyclic AMP. An analogue of endogenous cAMP, N^6,$O^{2'}$-dibutyryl cyclic adenosine-3',5'-monophosphate (db-cAMP) binds to PKA and activates the PKA dependent processes in cultured cells when applied to the culture medium in a concentrations range from 0.1 to 1 mM

Table 7-2. Viability of cells treated with modulators of protein kinase A activity. Cells were incubated for 48 hours in the presence of db-cAMP, forskolin (Fsk.) or H89 in concentrations as indicated in the tables, followed by the estimation of their viability using the MTT test.

	HeLa	Hepa
db-cAMP (μM)	% of viability ± standard deviation	% of viability ± standard deviation
0	100 ± 3.0	100 ± 7.0
100	96.3 ± 3.8	96.3 ± 5.5
200	88.8 ± 3.3	98.3 ± 3.7
300	87,0 ± 8.4	94. 0 ± 9.0
400	78.9 ± 6.8	76.4 ± 7.3
500	75.4 ± 2.2	81.1 ± 2.8

	HeLa	Hepa
Fsk. (μM)	% of viability ± standard deviation	% of viability ± standard deviation
0	100 ± 3.1	100 ± 3.4
5	99.7 ± 2.3	99.8 ± 5.0
10	99.3 ± 5.6	97.8 ± 3.7
15	96.7 ± 1.6	96.4 ± 2.9
20	76.2 ± 3.2	96,6 ± 4.6
25	77.7 ± 2.8	97.4 ± 2.2

	HeLa	Hepa
H89 (μM)	% of viability ± standard deviation	% of viability ± standard deviation
0	100 ± 3.0	100 ± 1.9
5	96.1 ± 1.5	97.7 ± 4.7
10	93.5 ± 0.9	97.9 ± 3.4
15	89.8 ± 1.0	95.7 ± 4.0
20	83.2 ± 8.2	88.4 ± 2.9
25	45.2 ± 9.4	82.3 ± 6.2

(Insel et al. 1975). Activation of PKA can also be achieved by an elevation of the endogenous cAMP level. Forskolin, a diterpene isolated from *Coleus Forscoli*, activates the cAMP dependent protein kinase by stimulation of adenylate cyclase, an enzyme responsible for the formation of the cAMP in the cell. This compound shows effects when applied to the cells at micromolar concentrations (Metzger and Lindner 1981). The potent and selective PKA inhibitor *N*-[2-(*p*-bromocynamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) inhibits activity of the PKA at micromolar concentrations (Chijiwa et al. 1990).

The MTT test performed in the cells treated with these compounds revealed that db-cAMP decreased the viability of HeLa and Hepa cells at concentrations of 200 and 300 μM, respectively. Forskolin had no toxic effects on the viability of HeLa cells up to 15 μM, while Hepa cells were not sensitive to toxic effects of forskolin up to 25 μM. H89 decreased cell viability at 10 μM in HeLa and at 15 μM in Hepa cells.

7.1.2 Preparation of protein extracts

Contribution of protein-protein and protein-DNA interactions to the regulation of the AHR function was analysed using immuncoprecipitation and ABCD (avidin-biotin complex on DNA) assays respectively. The experiments were performed using native nuclear proteins isolated from the cells according to the method described by Andrews (Andrews and Faller 1991). This method utilizes hypotonic lysis of the cells followed by a high salt extraction of the nuclear proteins. Among other methods of protein isolation from the cells, also extraction of cellular proteins in the presence of detergent (Heinzel et al. 1997), isolation of the nuclear proteins using MENG buffer (Chen and Perdew 1994) and the large-scale nuclear protein isolation (Dignam et al. 1983) were tested. Preparation of the cellular protein extracts in the presence of 0.1 % detergent, although rapid and efficient, yielded in AHR and ARNT proteins, that binding to DRE was week and accompanied by a high background of unspecific bound proteins. All three methods of nuclear protein isolation yilded proteins of comparable quality. An advantage of Andrew's method was the much higher efficiency of the extraction compared to the methods described either by Chen or Dignam. Extraction of the nuclear proteins from a 145 mm dish at about 90 % cell confluence resulted in 100-200 μg protein, dependent on the cell line used, while the other two procedures delivered 30-50 μg protein from a single dish. In addition to using of the biological material sparingly, saving time was also an advantage of this method. The time required for the complete procedure was approximately 1 hour, in contrast to the other

methods that took about 2.5 hours. In consequence, the Andrews method was chosen to isolate the nuclear proteins used in the experiments of this study.

Western blot analyses of proteins isolated using this method revealed an about 2-fold higher amount of the nuclear ARNT in HeLa in comparison to Hepa cells, as estimated by visual examination of the intensity of apriopriate bands. The AHR protein was not detectable in the extracts of nuclear proteins from the control, but was observed as a very intensive band in the TCDD treated Hepa cells, due to the nuclear translocation of AHR after ligand binding. In contrast, AHR was present in the nucleus even of untreated HeLa cells (Figure 7-1).

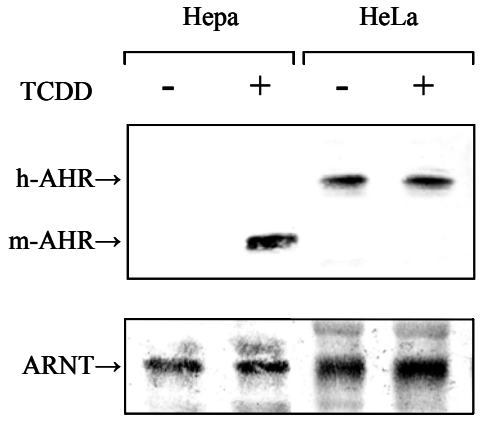


Figure 7-1. Western blot analysis of the AHR and ARNT proteins in nuclear extracts. Extracts of nuclear proteins were prepared from Hepa or HeLa cells treated with 2 nM TCDD or with the appropriate amount of DMSO (control group) for 1 hour. Samples containing 50 µg protein were resolved in 12 % SDS-PAGE, blotted onto PVDV membranes and probed with α-AhR or α-ARNT antibodies. h-AHR: human AHR (110 kDa), m-AHR: mouse AHR (97 kDa), ARNT has a molecular weight of 87 kDa.

Lack of AHR in control extracts and the presence of this protein in the nucleus of TCDD treated Hepa cells confirmed that the extracts of nuclear proteins were not contaminated with cytosolic proteins and contained only the AHR, which was tightly associated with the nuclear matrix, due to its activation by TCDD binding. The relative amount of AHR and ARNT in the two cell lines corresponded with published studies (Singh et al. 1996).

7.1.3 Interaction of the AHR complex with DRE

The ability of isolated nuclear proteins to specifically bind DNA was tested using the electromobility shift assay (EMSA). The high-salt extraction of the nuclear proteins delivered extracts containing 420 mM NaCl. In order to ensure a 80 mM concentration of salt, which is required for the optimal binding of the AHR/ARNT heterodimer to the [^{32}P]-labeled probe carrying the sequence of the dioxin responsive element (DRE), the extracts were dialysed against

buffer containing the appropriate amount of KCl prior to the generation of the protein-DNA complexes. Incubation of the nuclear proteins prepared from TCDD-exposed Hepa cells resulted in the formation of a protein-DNA complex which shifted [^{32}P]-DRE. The shift was not observed in the control cells. In HeLa cells the [^{32}P]-DRE probe was shifted in both, control and TCDD treated cells, indicating the exogenous ligand-independent formation of a protein complex on DRE in these cells (Figure 7-2 A). The supershift of the complex in the presence of antibodies against AHR or ARNT but not in the presence of preimmune serum, confirmed that the TCDD-dependent protein complex on DRE observed in Hepa cells consisted of AHR and ARNT proteins (Figure 7-2 B).

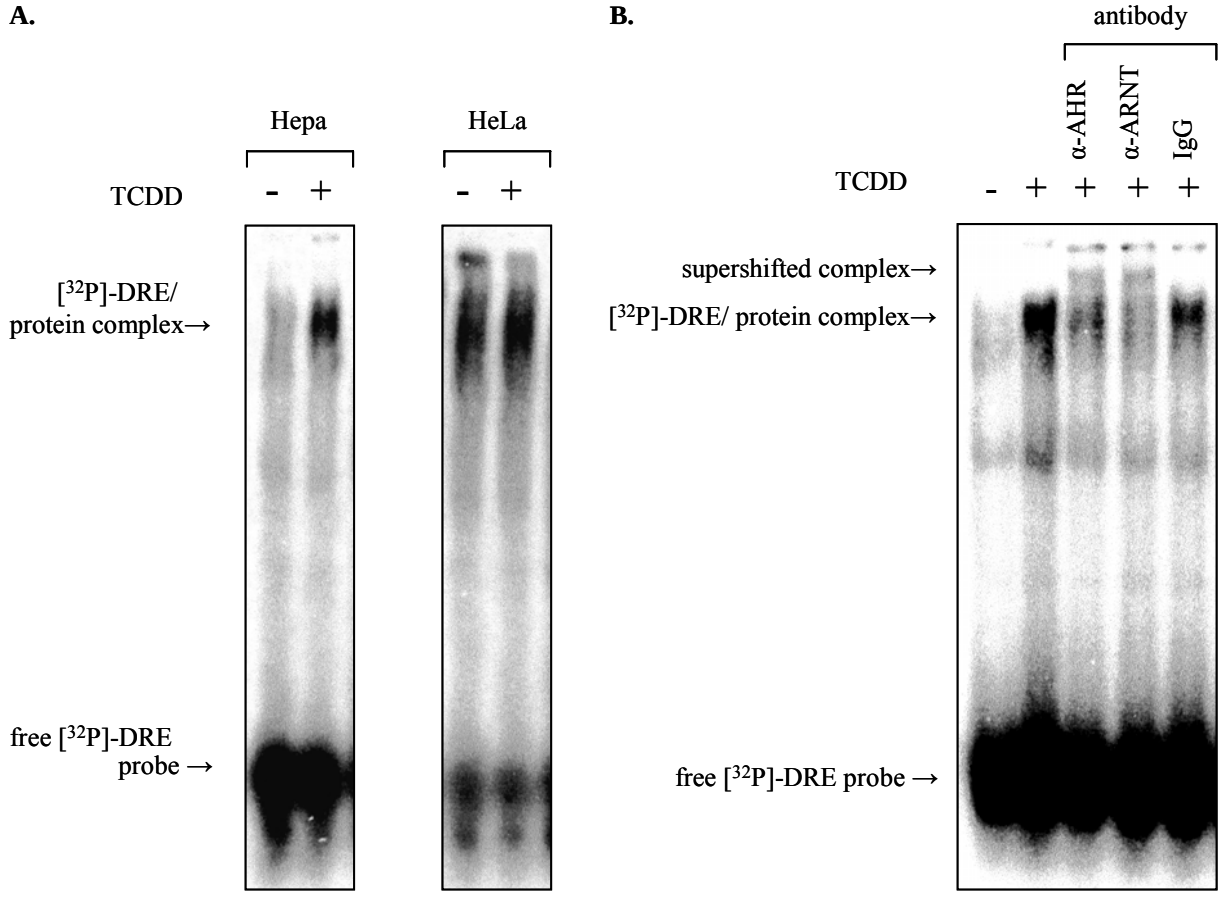


Figure 7-2. EMSA analysis of the AHR/ARNT complex formation on DRE. A. Hepa and HeLa cells were treated with 2 nM TCDD or the appropriate amount of DMSO (control) for 1 hour prior to the isolation of nuclear proteins. Samples containing 15 µg protein were incubated with the [^{32}P]-labeled DRE probe and resolved in nondenaturating polyacrylamide electrophoresis. **B.** To determine the specificity of the [^{32}P]-DRE bound proteins, the extracts from TCDD-treated Hepa cells were incubated with α-AHR, α-ARNT antibodies or preimmune serum (IgG) prior to incubation with the DRE probe.

7.1.4 Effect of salt concentration on the binding of ARNT to DRE

The specific protein-DNA interactions were subsequently analysed in "Avidin-biotin complex on DNA" (ABCD) assays. The assay which based on the method described by Korzus (1998), has been modified in order to utilize native proteins isolated from the cells, instead of the recombinant proteins. Since salt concentration maintained in the binding buffer is a critical factor influencing the specificity of protein-DNA interactions, the optimal concentration of NaCl for AHR complex binding to DRE was established. To this aim, nuclear proteins isolated from TCDD treated Hepa or untreated HeLa cells were incubated with biotinylated DRE in binding buffer that contained various amounts of NaCl. To analyse intensity of the binding, the amounts of ARNT which is a component of AHR complex, in the material bound to the biotinylated oligonucleotides were analysed in Western blots. As shown in figure 7-3, ARNT bound specifically to the wild type of the dioxin responsive element (DRE), but not to its variant, which harboured an extensively mutated core fragment (GCGTG→ ATTGT) (M-DRE) in the presence of 420 mM NaCl. Lower (210 and 105 mM) salt concentration in binding buffer led to a significant increase of unspecific binding to an oligonucleotide deprived of the DRE core sequence. The conditions maintained in the first buffer proved to be optimal and were consequently used in further studies.

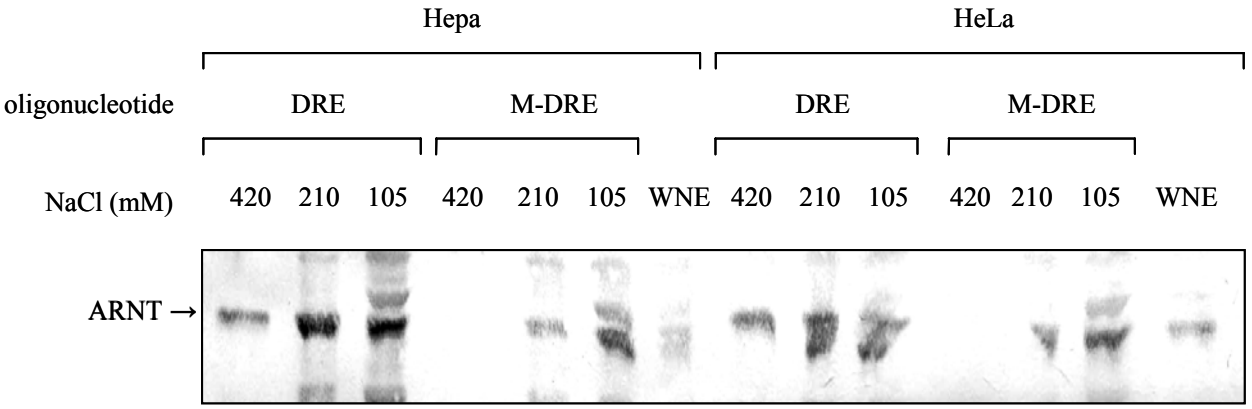


Figure 7-3. Effect of the salt concentration on specific binding of ARNT to biotinylated-DRE. Nuclear proteins from Hepa cells grown in the presence of 2 nM TCDD for 1 hour or untreated HeLa cells were extracted in the presence of 420 mM NaCl. The protein extracts were then adjusted with NaCl-free buffer to obtain the final salt concentration as indicated and subsequently incubated with biotinylated DRE or M-DRE oligonucleotides. DNA-bound protein complexes were precipitated using streptavidin-agarose. After extensive washing, proteins interacting with biotinylated oligonucleotides were recovered from the streptavidin agarose by boiling in SDS-sample buffer, resolved by SDS-PAGE and analysed by Western blot. WNE: whole nuclear extract.

7.2 Constitutive and TCDD-dependent activation of AHR

7.2.1 Formation of the AHR/ARNT heterodimer in the presence and in the absence of TCDD

In the first attempt to characterize the different mechanisms of the AHR activation in Hepa and HeLa cells, the TCDD-dependent interaction of the AHR with its dimerization partner ARNT was examined in these two cell lines by immuncoprecipitation. Figure 7-4 shows that treatment of Hepa cells with 2 nM TCDD for 1 hour was sufficient to cause a pronounced dimerization of AHR with ARNT, which was not observed in the cells treated with DMSO (vehicle). For the comparison of the TCDD effects in the two cell lines, the same conditions were maintained in HeLa cell cultures. In contrast to Hepa, in HeLa cells the ARNT protein was detected in the protein complex precipitated with α-AHR antibodies even in untreated cells. Presence of TCDD in the cell culture medium prior to the protein isolation and immunprecipitation assays did not significantly influence the AHR/ARNT interaction as the similar amount of ARNT was detected in α-AHR protein complexes precipitated from both, control and TCDD treated cells.

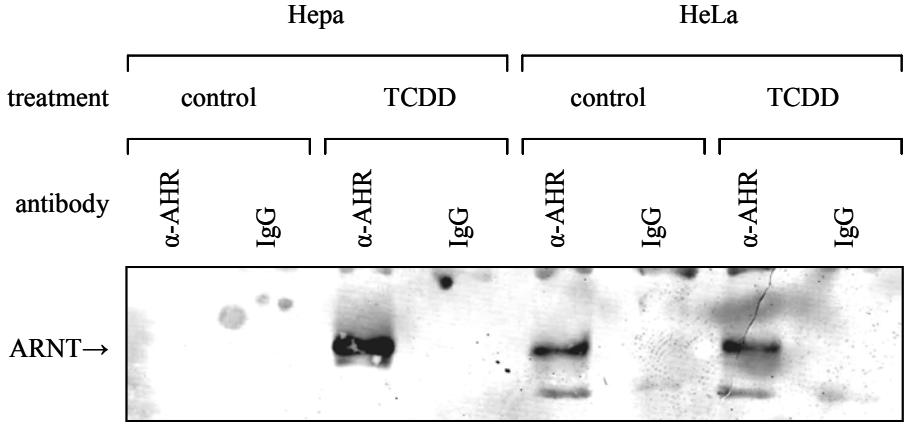


Figure 7-4. Constitutive and TCDD-induced heterodimerization of AHR and ARNT in Hepa and HeLa cells. Nuclear proteins were prepared from Hepa and HeLa cells incubated in the presence of 2 nM TCDD or the appropriate amount of vehicle (control) for 1 hour. Proteins were precipitated with antibodies against AHR or preimmune serum (IgG). The antibody-bound protein complexes were resolved by SDS-PAGE, transferred to PVDV membranes and immunodetected using antibodies against ARNT.

7.2.2 AHR and ARNT binding to the DRE in the presence and in the absence of TCDD

To analyse the subsequent step of the ligand-dependent AHR activation, namely the binding of the AHR/ARNT complex to its responsive element on DNA, ABCD assays were performed. Results presented in figure 7-5 reveal that both proteins AHR and ARNT isolated from Hepa cells treated with TCDD, but not those isolated from untreated cells, bound to the biotinylated DRE. Mutation of the core sequence abolished binding of the TCDD-dependent complex, thus confirming its specificity. As expected considering the exogenous ligand-independent dimerization of AHR and ARNT in HeLa cells, both proteins were found to be able to bind DRE independently of TCDD. The binding of AHR and ARNT to DRE observed in HeLa cells was not significantly increased after treatment of the cells with TCDD.

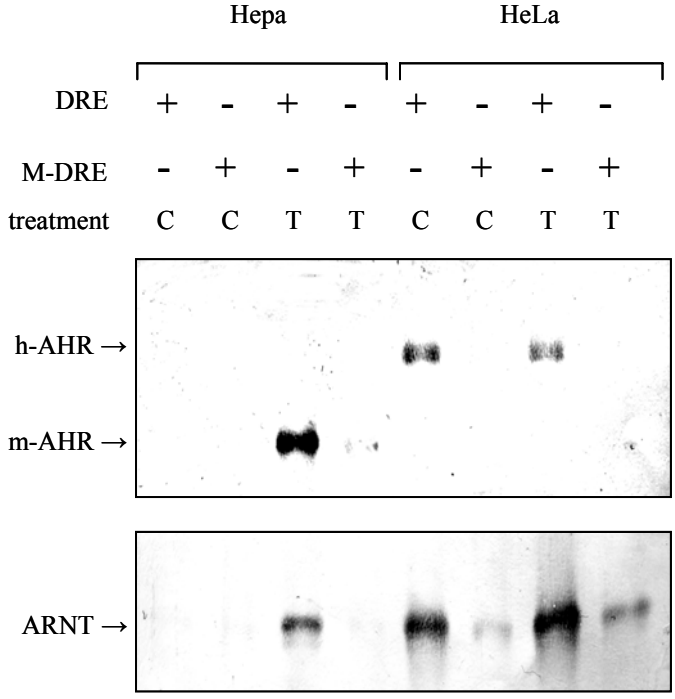


Figure 7-5. Constitutive and TCDD-induced binding of the AHR/ARNT heterodimer to biotinylated DRE. The DRE and M-DRE biotin-labeled oligonucleotides were incubated with nuclear extracts prepared from Hepa or HeLa cells growing in the presence of DMSO (C) or 2 nM TCDD (T) for 1 hour. The DNA bound protein complexes were precipitated using streptavidin-agarose. After extensive washing, the proteins interacting with biotinylated oligonucleotides were recovered from the streptavidin agarose by boiling in SDS-sample buffer and analysed in Western blots.

7.2.3 Regulation of the CYP1A1 activity in the presence and in the absence of TCDD

A physiological consequence of the ligand-dependent activation of the AHR dimerization with ARNT and binding to DRE is a transcriptional activation of responsive genes. Thus, it was interesting to ascertain whether the TCDD-independent binding of AHR and ARNT to DRE in

HeLa cells leads to the transcriptional activation of *CYP1A1* gene similar to the TCDD-dependent induction of the *CYP1A1* transcription in Hepa cells. The activation of the *CYP1A1* gene expression was followed by the analysis of the CYP1A1 monooxygenase activity in the cells by EROD assay (Burke and Mayer 1974). As illustrated in figure 7-6, Hepa cells expressed constitutively a low level of EROD activity. The exposure of the cells to TCDD led to a 20-fold increase in EROD activity compared to cells treated with vehicle alone. Surprisingly, in HeLa cells no EROD activity was detectable, even if the cells were treated with TCDD.

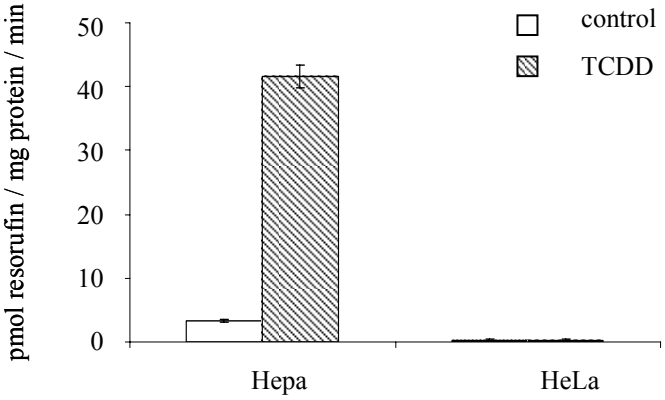


Figure 7-6. EROD activity in Hepa and HeLa cells in the absence and presence of TCDD. The cells were treated with 2 nM TCDD or with DMSO (control) for 16 hours followed by the measurement of EROD activity in cell culture. Each result represents an average of four experiments.

Taken together, the data of immuncoprecipitation, ABCD and EROD assays confirmed that the mechanism of AHR activation varied in the two studied cell lines. In Hepa cells, AHR was activated to a form that binds to ARNT and forms a complex on the DRE only after exposure of the cells to TCDD. In HeLa cells the AHR/ARNT heterodimer is bound to DRE in the absence of any exogenous ligand. This suggests the presence of a putative endogenous factor, which activates the transformation of AHR in these cells. However the AHR/ARNT heterodimer constitutively present on DRE in HeLa cells, unlike the heterodimer activated by TCDD in Hepa, does not induce the transcription of *CYP1A1* gene. The observation that TCDD failed to activate the transcription of *CYP1A1* in HeLa cells led to the assumption that a factor with a repressor activity controls the function of this complex.

7.3 Effect of PKA on the AHR function

7.3.1 Formation of the AHR/ARNT heterodimer upon activation of PKA

Studies in our labolatory have shown that the nuclear translocation of AHR and formation of the high molecular protein complex on DRE in Hepa cells could be induced in the absence of exogenous AHR ligand, namely by the activation of PKA. These studies together with the above presented results led to the question whether the constitutive activation of AHR in HeLa cells resulted from PKA-dependent phosphorylation. To answer this question, the influence of the cAMP stimulated phosphorylation on the interaction between AHR and ARNT was analysed in immuncoprecipitation experiments.

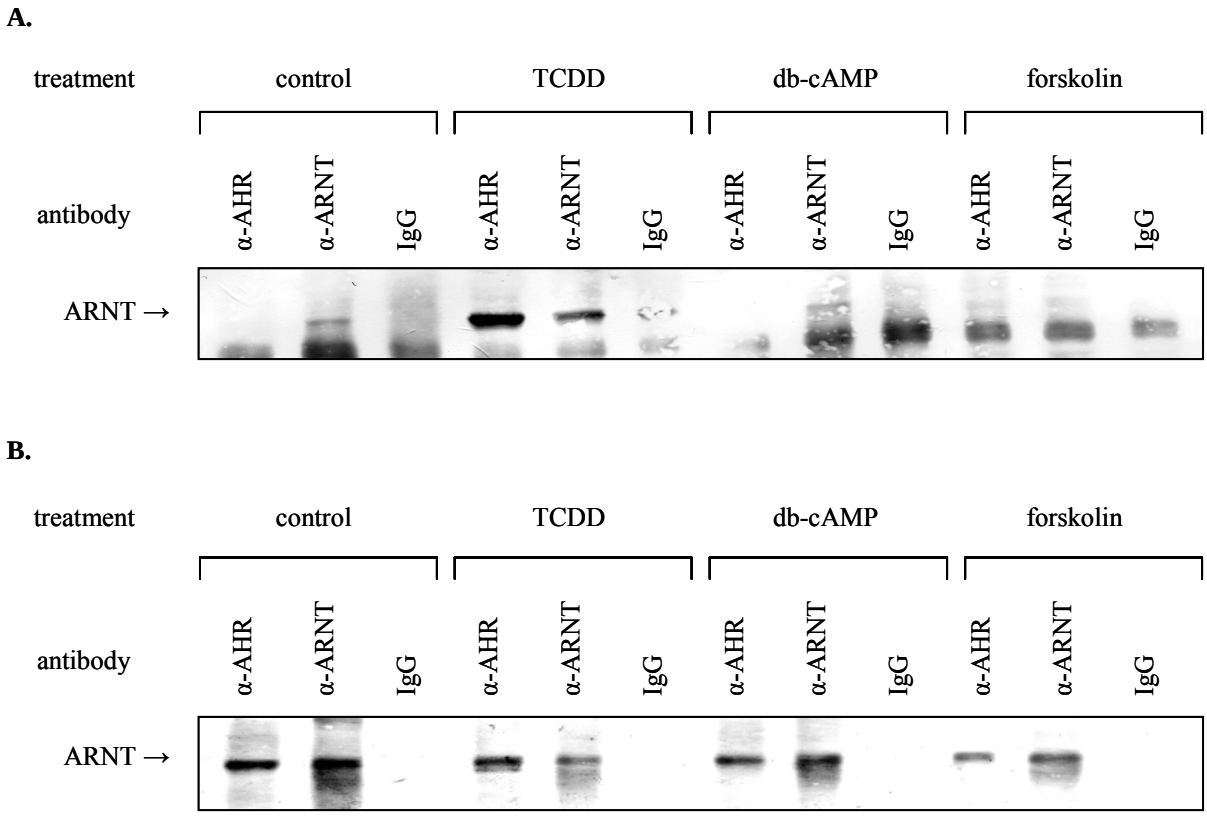


Figure 7-7. Interaction of AHR with ARNT in the presence of activators of PKA. Nuclear proteins isolated from Hepa **(A)** and HeLa **(B)** cells incubated with 2 nM TCDD for 1 hour, with the PKA activator db-cAMP (2 mM) or forskolin (10µM) for 15 minutes, or the appropriate amount of DMSO (control), were precipitated with antibodies against AHR, ARNT or preimmune serum. The antibody-bound protein complexes were denaturated by boiling in SDS-sample buffer, resolved in SDS-PAGE and analysed using Western blot.

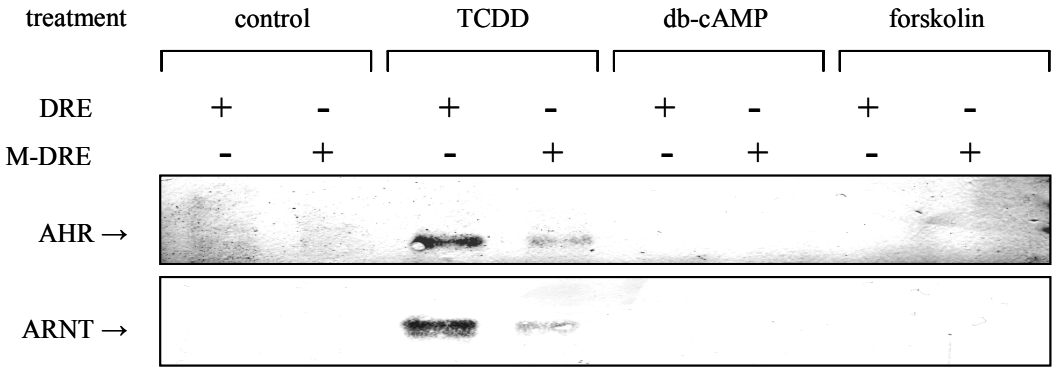
Presence of ARNT in the α-AHR-bound protein complexes precipitated from the TCDD-treated cells confirmed the ligand-dependent interaction between AHR and ARNT in Hepa cells. Treatment of these cells with db-cAMP or forskolin however did not alter this interaction as compared to the solvent treated (control) cells (Figure 7-7- A). In HeLa cells the AHR/ARNT heterodimer was detected in the control as well as in TCDD, db-cAMP or forskolin treated cells. A slight variations in the amount of ARNT precipitated with α-AHR from particular extract were not reproducible and thus probably resulted from the differences in the quality of isolated proteins rather than were a result of specific modulation of the AHR interaction wih ARNT (Figure 7-7 B). Since PKA modulators did not cause any significant alterations in the amount of the AHR/ARNT heterodimer formed as compared to the control cells, the involvement of PKA in this process was not established.

7.3.2 AHR and ARNT binding to DRE upon activation of PKA

Subsequent experiments aimed in the analysis of the AHR/ARNT complex binding to DRE upon stimulation of the PKA activity in Hepa and HeLa cells.

ABCD assays were performed using nuclear proteins isolated from the cells treated with TCDD or PKA activators. Figure 7-8 A shows that in Hepa cells only TCDD but not db-cAMP or forskolin treatment resulted in AHR and ARNT binding to DRE. In HeLa cells both proteins were found to be bound to the biotinylated DRE irrespective whether the nuclear extracts were isolated from the control TCDD, db-cAMP or forskolin treated cells. Similar to the AHR binding to ARNT presented in previous chapter, also binding of the AHR and ARNT proteins to DRE varied slightly in particular experiments. However, no reproducibility of these variantions was observed, therefore they were not considered to be significant (Figure 7-8-B).

A.



B.

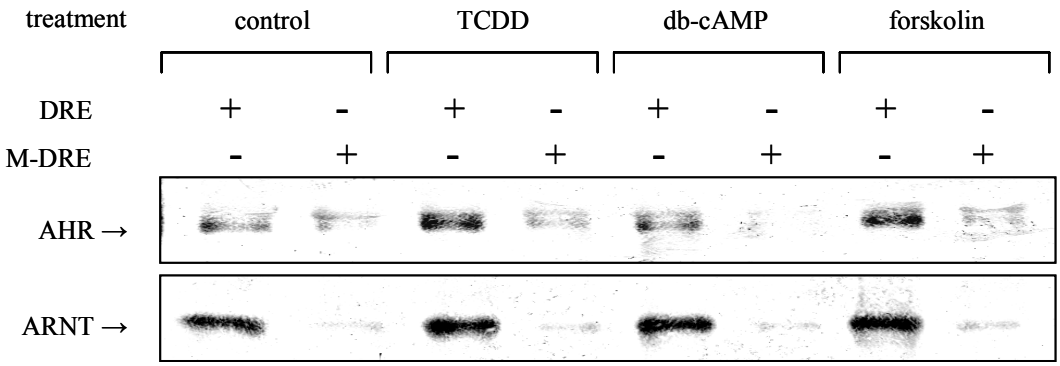


Figure 7-8. Formation of the AHR/ARNT complex on DRE in the presence of the PKA activators. Nuclear proteins isolated from Hepa (A) and HeLa (B) cells treated with TCDD (2 nM) for 1 hour, db-cAMP (2mM) or forskolin (15 μM) for 15 minutes or with DMSO (control) for 1 hour, were incubated with biotinylated oligonucleotides representing DRE (wild type) or M-DRE (DRE with extensively mutated core motif). Biotinylated-DNA-bound protein complexes were precipitated using streptavidin-agarose. After extensive washing the proteins were recovered from the streptavidin agarose by boiling in SDS-sample buffer, resolved by SDS-PAGE and analysed in Western blot.

7.3.3 Regulation of the CYP1A1 activity upon activation of PKA

Effect of the PKA-dependent phosphorylation on the AHR function appeared to be distinct from the TCDD-dependent activation of the receptor. In addition, the function of the cAMP-dependent complex in the transcriptional regulation is not similar to the well-known pathway of the TCDD-dependent activation of *CYP1A* gene. Reporter gene analyses have

revealed that the PKA-phosphorylation-dependent complex on DRE does not activate reporter gene containing 5' regulatory sequence of the mouse *CYP1A1* regulatory region (Oesch-Bartlomowicz et al., manuscript submitted)

To study the effects of PKA-dependent phosphorylation on the transcription of AHR responsive genes, the influence of the PKA activation or inhibition on the regulation of the native CYP1A1 gene in Hepa and HeLa cells was analysed by EROD assays.

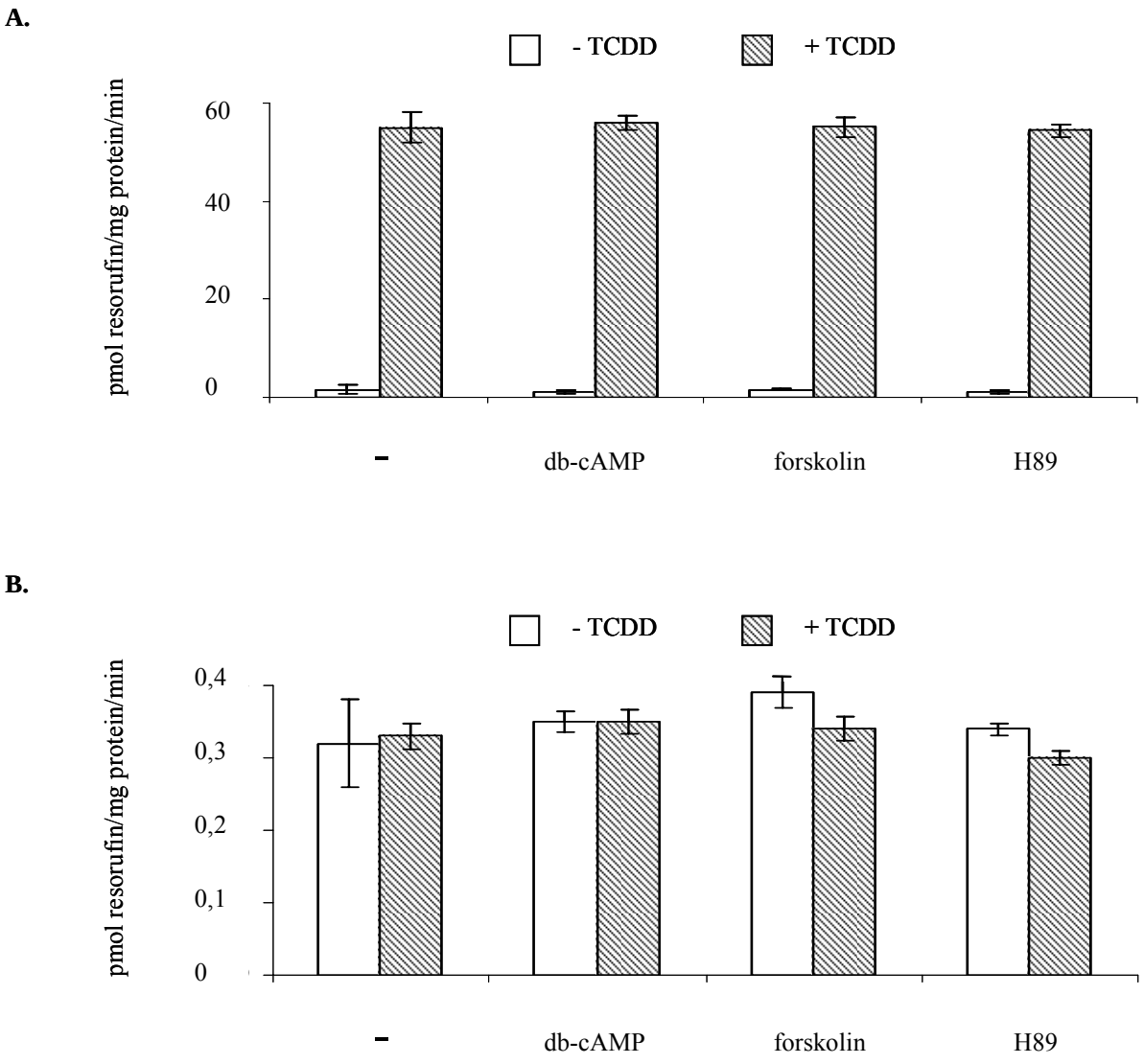


Figure 7-9. Effect of PKA on the constitutive and TCDD-dependent EROD activity. Hepa **(A)** and HeLa **(B)** cells were treated with db-cAMP (100 µM), forskolin (5 µM) or H89 (1 µM) in the presence or in the absence of 2 nM TCDD that was added to the cells after 24 hours of pretreatment with the PKA modulators and maintained in cell culture medium for further 24 hours. After treatment the EROD activity was measured in the cell cultures. Each value represents the average of six experiments.

Figure 7-9 A demonstrates that the activation of PKA by treatment the cells with forskolin or db-cAMP or inhibition by treatment with H89 had no effect on the constitutive as well as TCDD-induced EROD activity in Hepa cells. Also in HeLa cells the modulators of the PKA activity did not influence the EROD activity in either presence or absence of TCDD (Figure 7-9 B).

These results confirmed the observations on the regulation of the *CYP1A1* driven reporter gene by PKA and support the hypothesis of the unique character of the PKA-dependent protein complex on DRE. Lack of an effect of the PKA activation on the AHR/ARNT dimerization and binding of the AHR/ARNT dimer to DRE did not permit conclusion concerning involvement of PKA in these processes.

7.4 Role of HDAC in the regulation of the *CYP1A* gene

7.4.1 Members of the HDAC complex

Nuclear receptors are transcription factors that bind in the absence of their ligands to regulatory regions of their responsive genes and suppress transcription. The repressional character of an unliganded nuclear receptor depends on its interaction with a multiprotein complex containing histone deacetylase. Lack of transcriptional activity of AHR that is constitutively bound to DRE in HeLa cells evoked a question whether histone deacetylase might also be involved in the observed repression of *CYP1A1* gene and thus contributes to the distinct activity of the receptor in Hepa and HeLa cells.

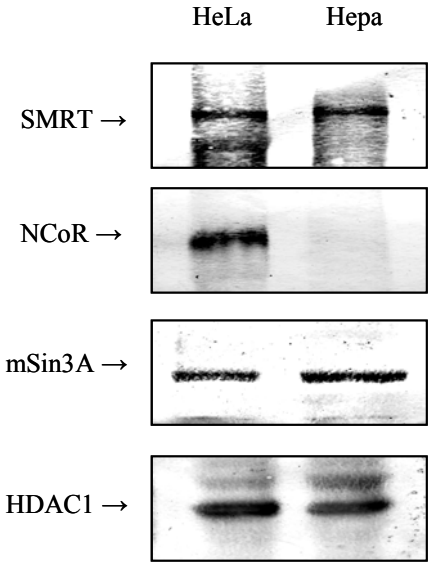


Figure 7-10. Western blot analysis of the SMRT, NCoR, Sin3A and HDAC1 proteins. Samples containing 50 µg nuclear proteins isolated from Hepa or HeLa cells were resolved by SDS-PAGE (6 % acrylamide gel for SMRT and NCoR, 10 % for mSin3A and 12 % acryamide gel for HDAC1). After electrophoresis the proteins were blotted to PVDV membranes and immunodetected.

To answer this question, presence of the members of histone deacetylase complex namely corepressors NCoR, SMRT and mSin3A as well as histone deacetylase 1 was analysed in Hepa and HeLa cells by the means of Western blot. The analyses showed that SMRT, mSin3A and HDAC1 are expressed at similar levels in Hepa and HeLa cells. NCoR was however present in nuclei of HeLa but not detected in Hepa cells (Figure 7-10). Thus, NCoR might be involved in the repression of *CYP1A1* in HeLa cells. On the other hand, high inducibility of Hepa cells could be explained by the absence of this corepressor in the cells.

7.4.2 Effect of HDAC inhibitors on the TCDD-dependent induction of the CYP1A1 activity

In further studies a role of the histone deacetylase activity in the repression of the *CYP1A1* gene was analysed by EROD assays in cells treated with the inhibitors of HDAC, NaBu and TSA. Figure 7-11 shows that the presence of NaBu in the culture medium had no detectable effect on the basal EROD activity in Hepa cells. Addition of TCDD to the cells that were pretreated with NaBu led to a modest additional activation of EROD as compared to the cells treated with TCDD alone. Similar results were obtained with the specific HDAC inhibitor TSA, which also did not affect the basal, but slightly increased the TCDD-dependent EROD activity in the cells. A significant restoration of EROD inducibility by TCDD was observed in HeLa cells. A synergistic action of TCDD and an inhibitor of histone deacetylase was dose dependent and reached a significant higher EROD activity in HeLa cells treated with TCDD in the presence of 3 mM NaBu compared to the cells treated only with TCDD or NaBu. Similar, the presence of TSA in the culture medium prior to the addition of TCDD restored the inducibility of *CYP1A1*, although to a lesser degree than NaBu did.

These observations strongly supported the hypothesis that histone deacetylase contributes to the repression of the *CYP1A1* gene in HeLa cells.

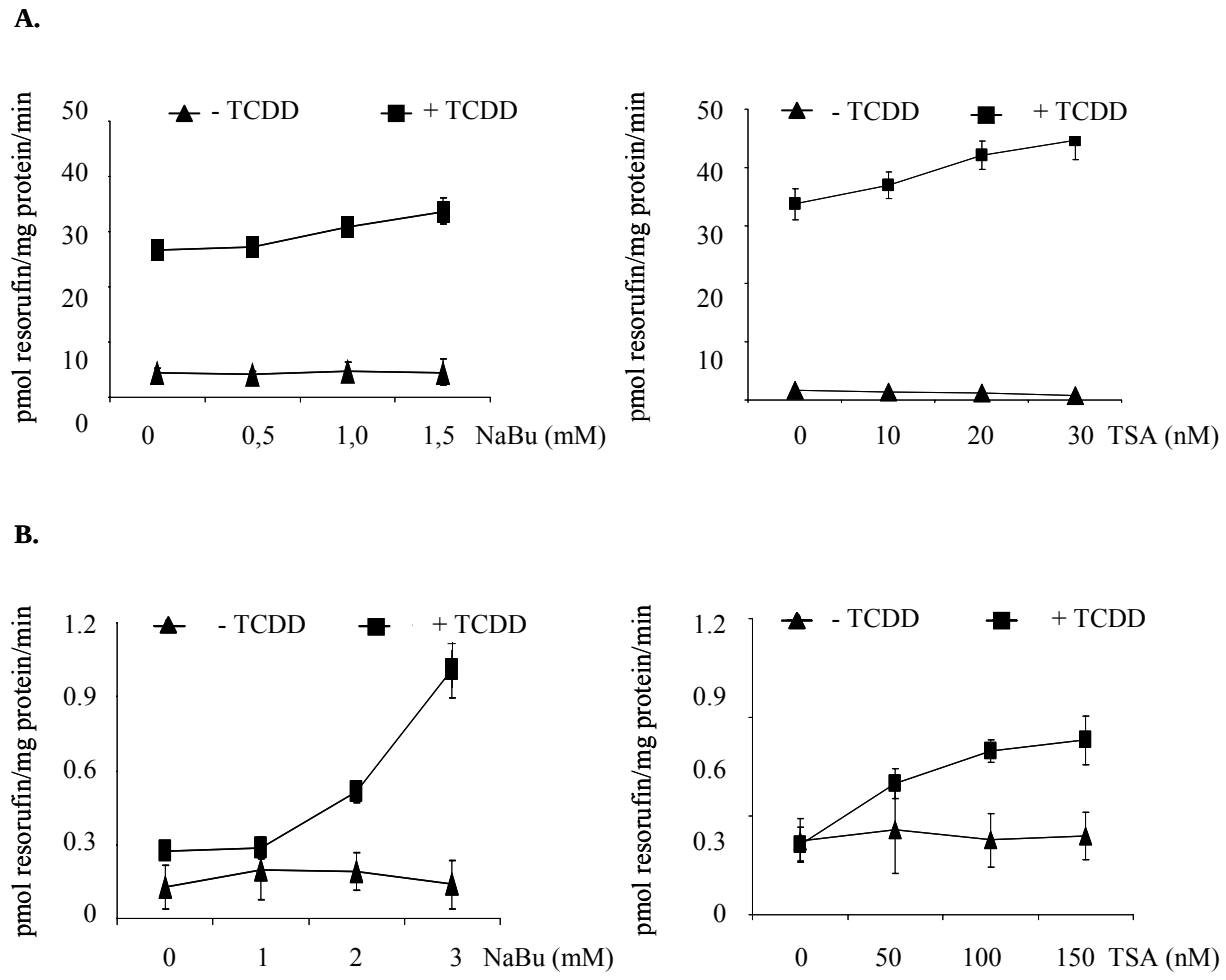


Figure 7-11. Effects of NaBu and TSA on the constitutive and TCDD-inducible EROD activity in the cells. Hepa **(A)** and HeLa **(B)** cells were treated with NaBu or TSA in concentrations as indicated for 12 hours followed by addition of 2 nM TCDD or solvent (DMSO) and further incubation of the cells for 15 hours. Each value on the diagrams represents the mean of six experiments.

7.4.3 Effect of HDAC inhibitors on the TCDD-dependent expression of the *CYP1A1* mRNA

To confirm that the induction of the EROD activity in Hepa and HeLa cells (resulting from the increased acetylation) reflects the alterations in the *CYP1A1* gene activity, the analysis of *CYP1A1* mRNA expression was performed by Northern blot assays. The analysis of the total RNA isolated from the cells treated with TCDD alone or in the presence of HDAC inhibitors, revealed a similar response of the cells as observed in EROD assays.

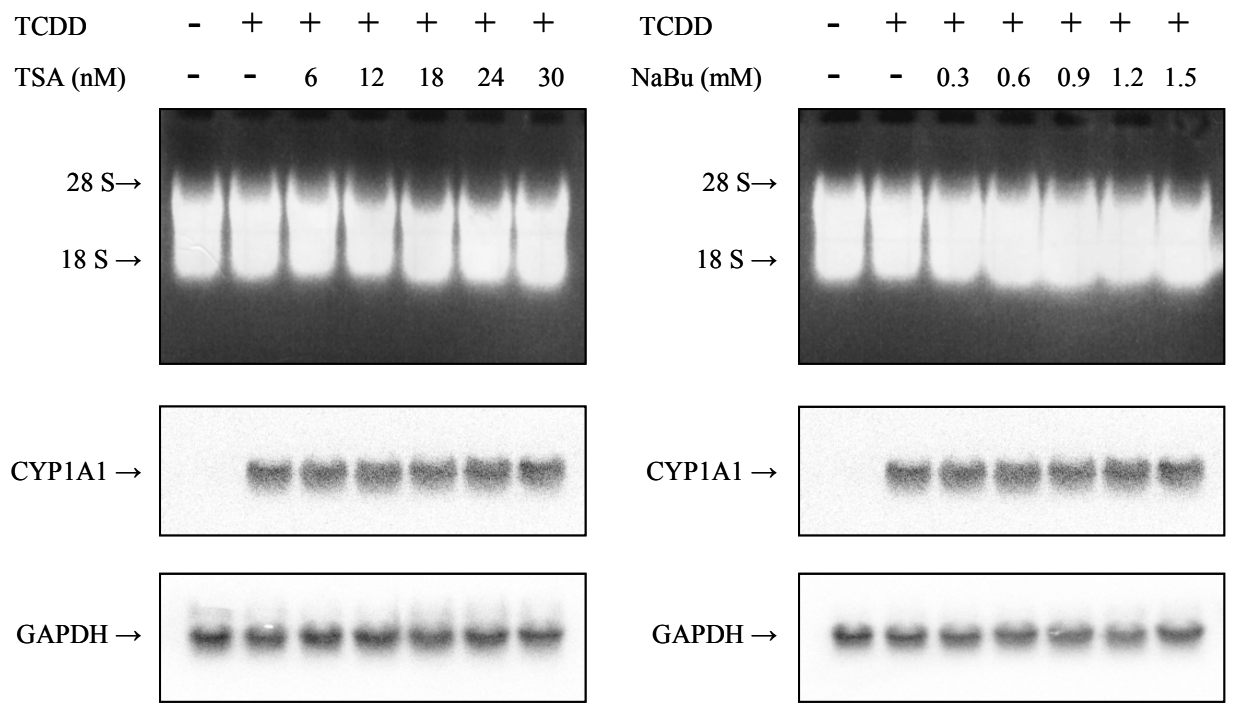


Figure 7-12. Effects of TSA and NaBu on the constitutive and TCDD-inducible *CYP1A1* mRNA level in Hepa cells. Cells were treated with NaBu or TSA in various concentrations for 12 hours followed by the addition of 2 nM TCDD and incubation was continued with the HDAC inhibitor and TCDD for another 15 hours. Total RNA isolated from the cells was fractionated in formaldehyde agarose gels in the presence of ethidium bromide and visualized in UV light for the control of integrity of the isolated RNA in the samples (28 S and 18 S RNA). The RNA was transferred to Hybond+ nylon membranes, probed with a radiolabeled *CYP1A1* specific probe and visualized by phosphorimaging. As a loading control, a second blot was performed under the same conditions and hybridized to a GAPDH specific probe.

Treatment of Hepa cells with TCDD led to a significant increase of *CYP1A1* mRNA level in these cells. Because the amount of mRNA in uninduced cells was under the limit of detection, the quantitative analysis of the induction was not performed. The intensity of the *CYP1A1* mRNA bands (relative to the amount of GAPDH mRNA) in samples isolated from the cells grown in the presence of TCDD alone or together with histone deacetylase inhibitor, revealed a slight additional induction of the *CYP1A1* mRNA expression in the latter group (Figure 7-12).
No increase of the *CYP1A1* mRNA level was observed in HeLa cells treated with TCDD alone, supporting the above described inability of these cells to respond to TCDD. However, treatment of these cells with NaBu followed by addition of TCDD to the culture medium led to an increase

of *CYP1A1* mRNA level in a NaBu dose dependent manner. Such a dose-dependent derepression of TCDD-induced *CYP1A1* mRNA expression was also observed in the cells pretreated with TSA prior to addition of TCDD. Similar to the observations of the regulation of the EROD activity, the effect of TSA was weaker than that of NaBu (Figure 7-13).

In both tested cell lines EROD assay as well as *CYP1A1* mRNA analysis confirmed that the TCDD response was potentiated by an increase of acetylation state. Repression of this gene in HeLa cells could, at least in part, be overcome by an inhibition of deacetylase activity, thus proving an involvement of HDAC in the regulation of the *CYP1A1* gene. Because the derepression was not complete, it is probable that additional factors contribute to the observed silencing of the gene.

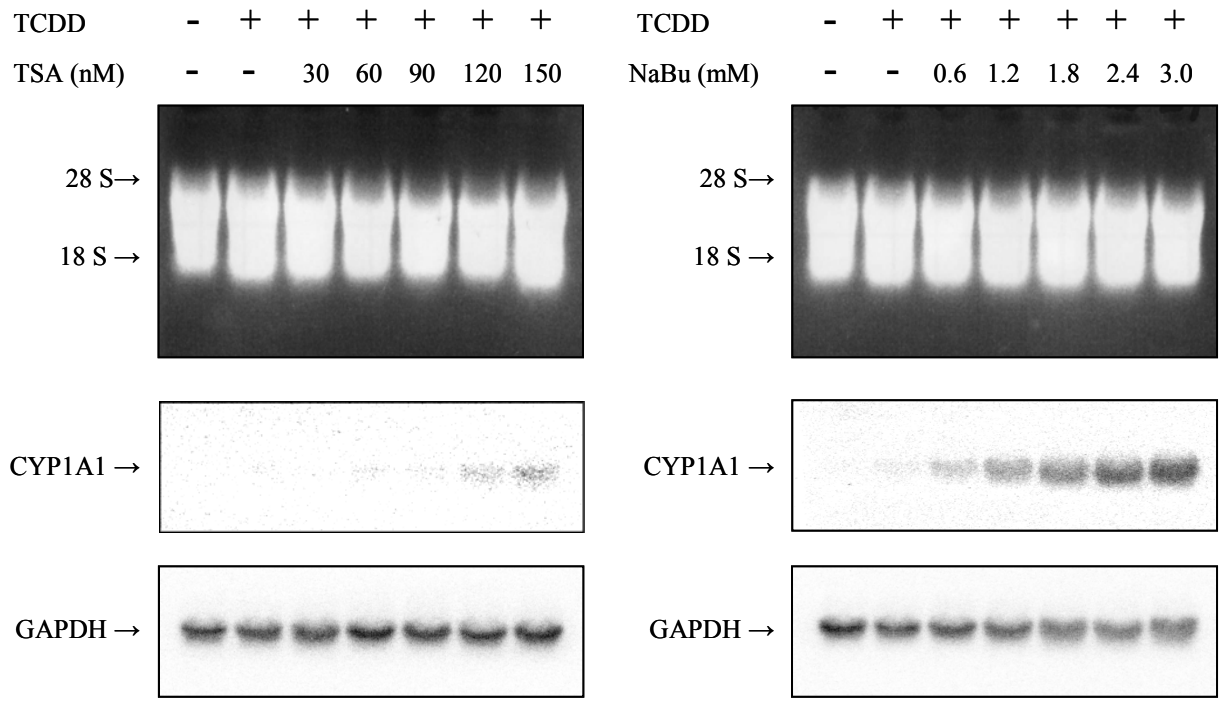


Figure 7-13. Effects of TSA and NaBu on the constitutive and TCDD-inducible *CYP1A1* mRNA level in HeLa cells. Cells were treated with NaBu or TSA in various concentrations for 12 hours followed by the addition of 2 nM TCDD and incubation was continued with the HDAC inhibitor and TCDD for another 15 hours. Total RNA isolated from the cells was fractionated in formaldehyde agarose gels in the presence of ethidium bromide and visualized in UV light for the control of integrity of the isolated RNA in the samples (28 S and 18 S RNA). The RNA was transferred to Hybond+ nylon membranes, probed with a radiolabeled *CYP1A1* specific probe and visualized by phosphorimaging. As a loading control, a second blot was performed under the same conditions and hybridized to a GAPDH specific probe.

7.4.4 Expression of the AHR and ARNT proteins in cells treated with HDAC inhibitors

Expression of a variety of genes has been reported to be influenced by histone deacetylase, among them the gene coding for AHR. The expression of AHR can be activated in the presence of HDAC inhibitors (Garrison et al. 2000). Since an increased amount of transcription factor might influence expression of its responsive gene, it has been tested whether the increased activation of *CYP1A1* gene in the presence of NaBu or TSA was due to this effect. For this purpose, the expression of AHR and ARNT proteins in Hepa and HeLa cells treated with TCDD or HDAC inhibitors was analysed by Western blot. The conditions that caused an activation of the *CYP1A1* gene did not led to a detectable increase in the amount of either the AHR or ARNT proteins (Figure 7-14). According to these observations, the alterations in the

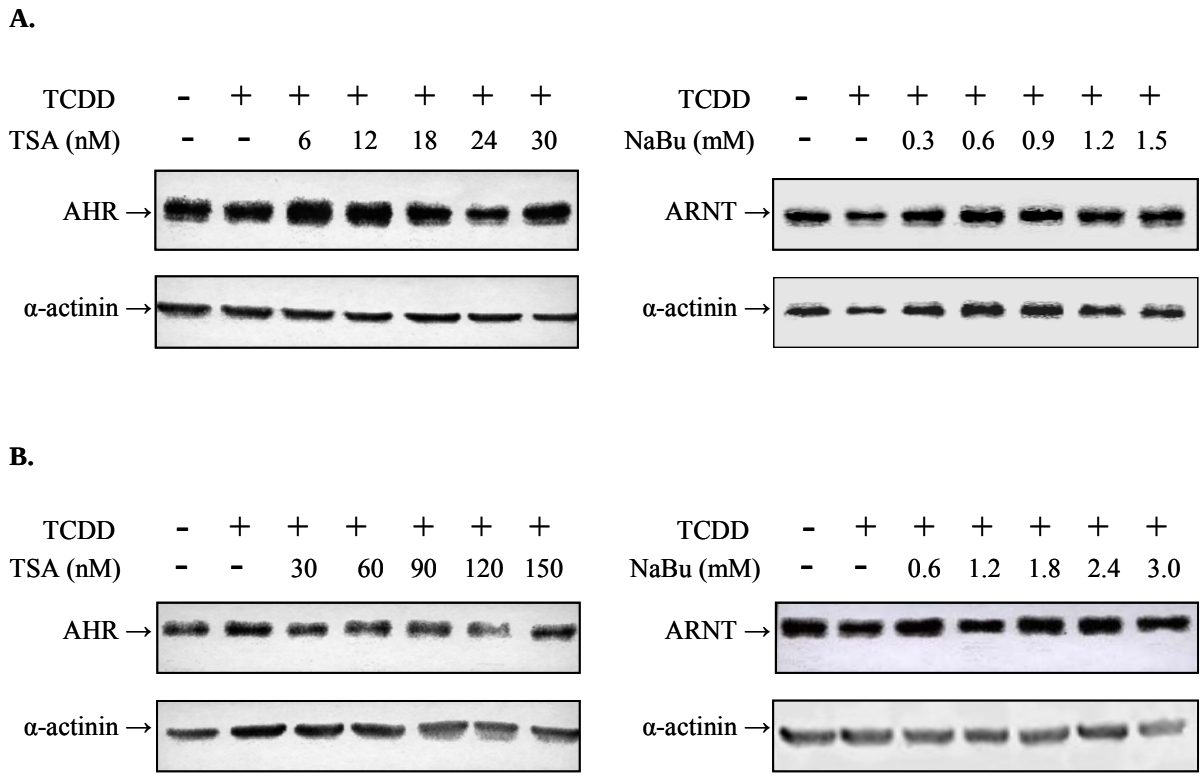


Figure 7-14. Western blot analysis of the AHR and ARNT in the presence of histone deacetylase inhibitors and TCDD. Hepa **(A)** or HeLa **(B)** cells were treated with HDAC inhibitor in concentration as described in the figure for 12 hours followed by addition of 2 nM TCDD and further incubation of the cells in the presence of both chemicals for 15 hours. Samples containing 50 µg of cellular proteins were resolved in SDS-Page and analysed by Western blot.

CYP1A1 gene expression were probably associated with the events occurring on the *CYP1A1* promoter region and were not consequences of the altered intracellular concentrations of the two regulatory proteins.

7.5 Mechanism of HDAC mediated regulation of the *CYP1A* gene

7.5.1 TCDD-dependent regulation of the mouse *CYP1A1* regulatory sequences in murine and human cell lines

Role of the mouse *CYP1A1* regulatory sequences in the transcriptional repression of this gene by histone deacetylase was further studied in reporter gene assays. Two constructs expressing the firefly luciferase gene adjacent to the different murine *CYP1A1* 5' regulatory regions were used in this study. The p1646P1Luc3 containing the murine *CYP1A1* sequences from -1646 to +57 and the pAhRDtkLuc3 containing the sequences from -1100 to -896 (cloned into the pGL3 basic vector) were gratefully obtained from A. Puga (Chang and Puga 1998). Figure 7-15 represents a schematic illustration of these regulatory regions.

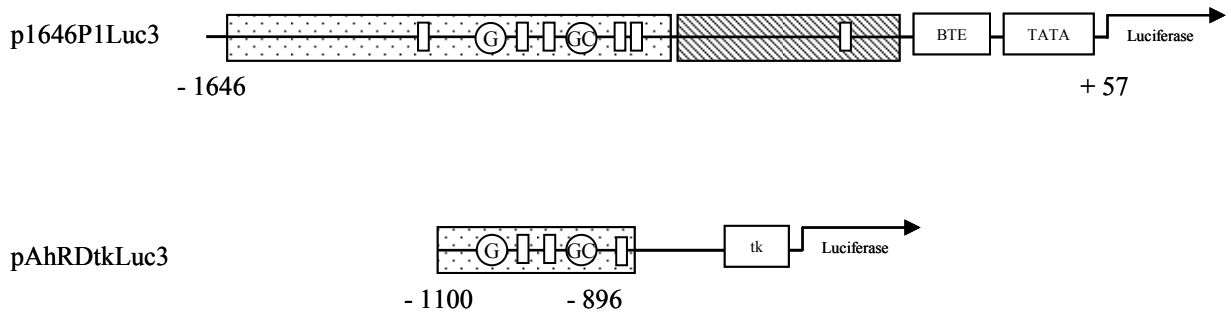


Figure 7-15. Schematic representation of the luciferase gene fused to the regulatory sequences of the murine *CYP1A1*. Numbers at the bottom indicate the distance from the transcription start site in the native *CYP1A1* gene. TATA: promoter containing TATAAA motif, BTE: basic transcription element containing a guanine-cytosine-rich box, tk: herpex simplex virus type1 thymidine kinase minimum promoter (deprived of SP-1 binding site), (G):a guanine-rich sequence, (GC): GC-rich motif, open boxes represent DRE motifs, hatched box depicts the position of the negative regulatory element while the dotted box depict the enhancer region of the gene. Arrows indicate the reporter gene transcriptional start site. Further details of the *CYP1A1* gene structure and function are described in the text.

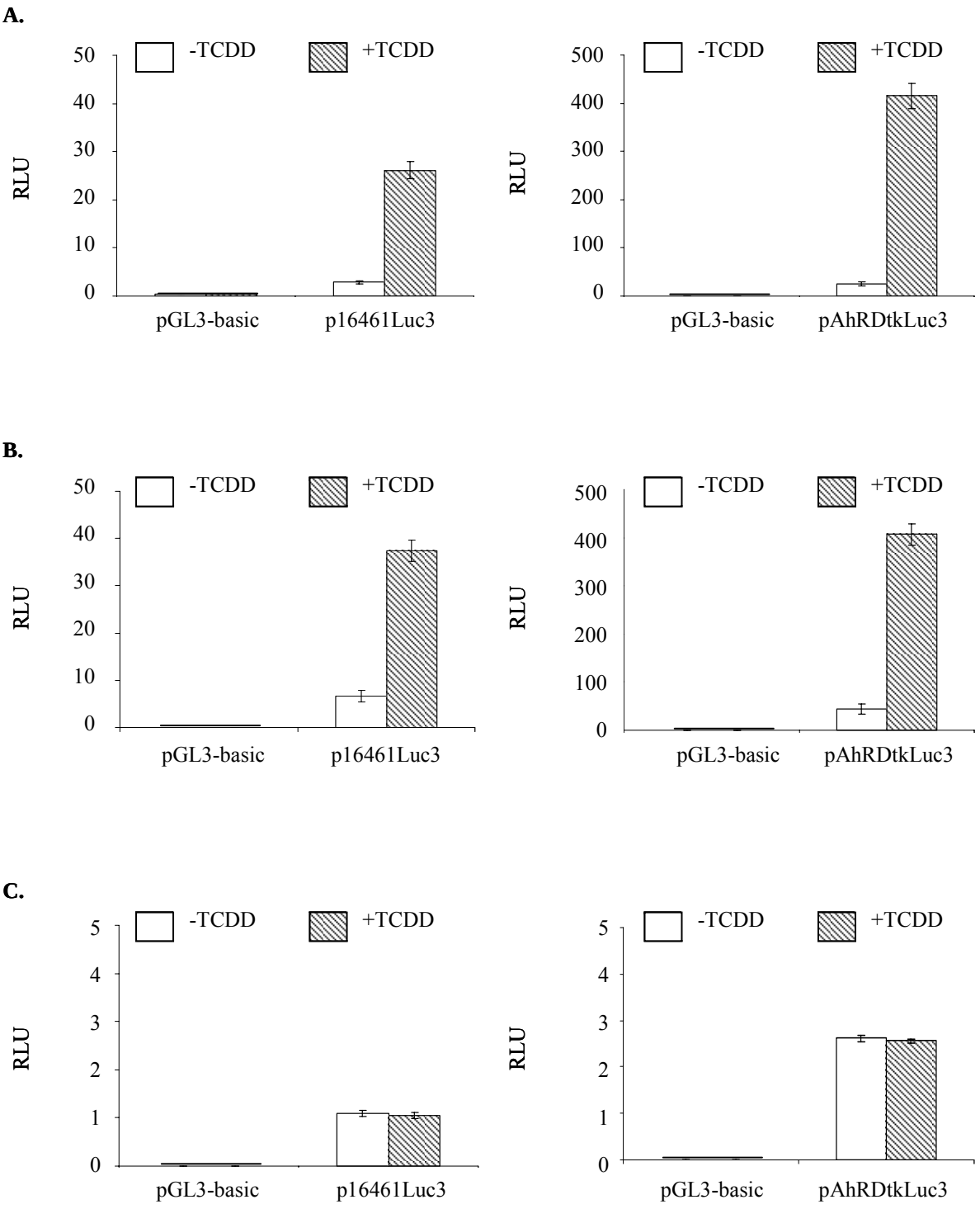


Figure 7-16. Luciferase reporter activity driven by the mouse *CYP1A1* regulatory regions in mouse and human cells. Hepa **(A)**, HepG2 **(B)** and HeLa **(C)** cells were transiently transfected with pGL3basic, p1646P1Luc3 or pAhRDtkLuc3 constructs (200 ng DNA/well). The pRLSV40 plasmid expressing a *Renilla* luciferase gene under control of the SV40 early enhancer/promoter region was included in every transformation mixture (2.5 ng DNA/well) as an internal control of transfection efficiency. 24 hours after transfection, the cells were treated with 2 nM TCDD for 24 hours prior to the measurement of the reporter activities. RLU represents an average of firefly luciferase normalized to *Renilla* luciferase activity in six wells.

A pGL3-basic vector without *CYP1A1* sequences was used in the transfection experiments as a control of the specificity of the observed effects.

In order to examine inducibility of reporter gene controlled by murine *CYP1A1* regulatory sequences in mouse and human cells, luciferase activity was analysed in Hepa (a murine cell line), HeLa and HepG2 (two human cell lines) transfected with the pGL3-basic, p1646P1Luc3 or pAhRDtkLuc3 constructs and treated with TCDD or solvent.

Figure 7-16 shows no reporter activity in the cells transiently transfected with the control pGL3-basic vector. A low level of constitutive reporter activity was observed in the cells transfected with p1646P1Luc3. This activity was about 10-fold increased in the presence of TCDD in both Hepa (A) and HepG2 (B) cells, but was not affected in HeLa (C) cells. The second reporter construct pAhRDtkLuc3 responded with much higher basal as well as TCDD-induced luciferase activity and was inducible in a TCDD-dependent manner in Hepa and HepG2 but not in HeLa cells.

The murine *CYP1A1* regulatory regions spanning the sequences from -1646 to +57 or from -1100 to -896 fused to a reporter gene were activated by TCDD in a similar, tissue specific, way in murine (Hepa) and human (HepG2) hepatoma cell lines. The regulation of the reporter gene mimicked the well known regulation of the native *CYP1A1* gene in these cells. Lack of the reporter gene induction in HeLa cells transfected with either p1646P1Luc3 or pAhRDtkluc3 is in a good agreement with the observation that these cells were non-responsive to TCDD regarding the activation of the native *CYP1A1* gene.

7.5.2 Contribution of the enhancer region of *CYP1A1* to the HDAC-mediated repression of this gene

In subsequent experiments, the function of the *CYP1A1* regulatory regions in the presence of HDAC inhibitors in HeLa cells was studied. Figure 7-17 shows no significant alterations in the luciferase activity in the cells transfected with p1646P1Luc3 and treated with TCDD. Also treatment of these cells with NaBu alone did not increase the reporter activity, while treatment of the cells with NaBu followed by addition of TCDD resulted in a synergistic action of these two compounds leading to a significantly increased reporter activity, an effect, which was already observed in the case of the native *CYP1A1* gene in these cells. Interestingly, unlike the native *CYP1A1* gene, which responded weakly to TSA and only in the presence of TCDD, the activity of the reporter construct was increased about 10-fold upon TSA treatment even in the absence of TCDD and no further significant activation was observed after addition of TCDD.

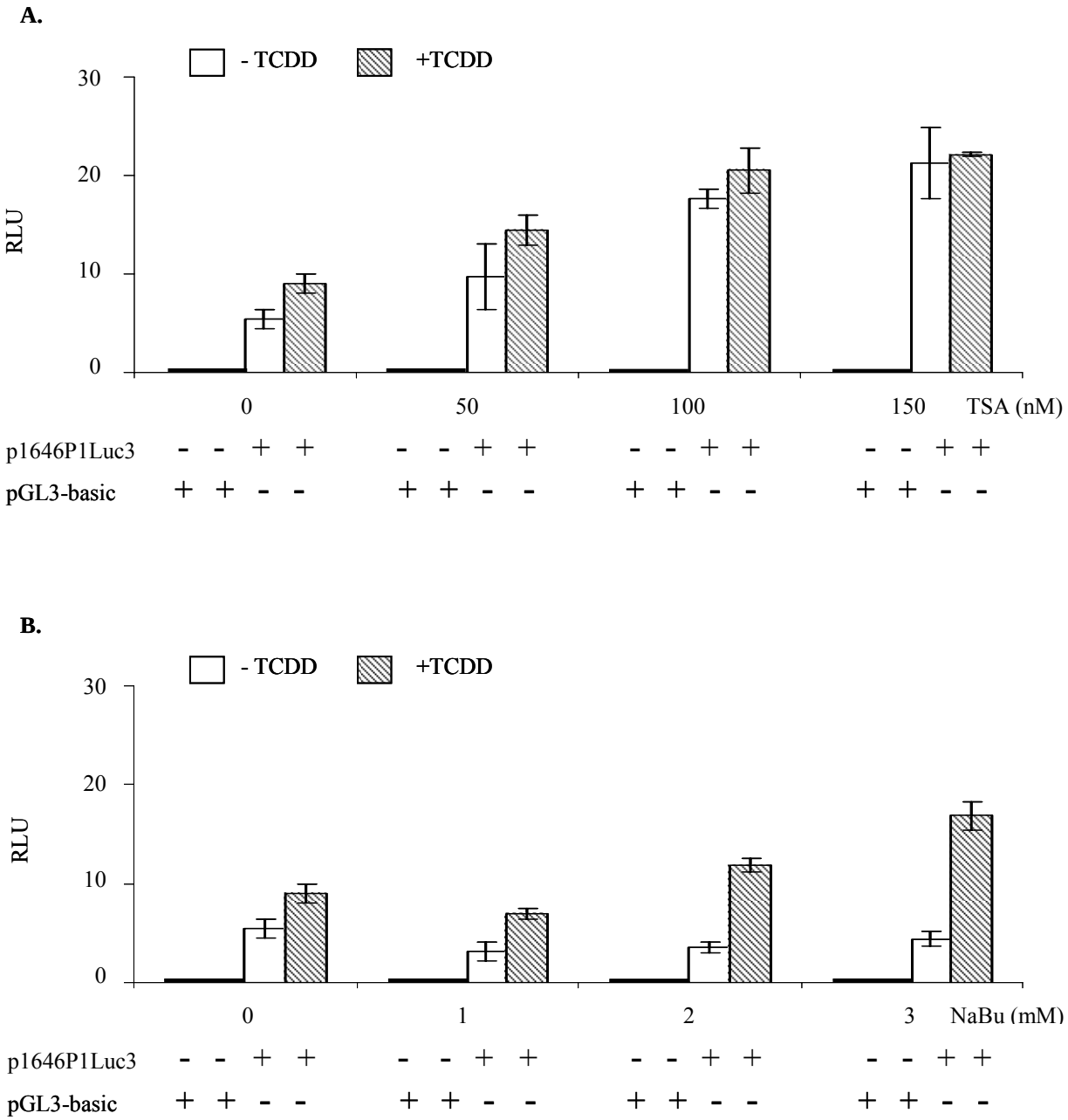


Figure 7-17. Activity of the p1646P1Luc3 reporter construct in HeLa cells treated with TCDD and with HDAC inhibitors TSA (A) and NaBu (B). Cells were transfected with p1646P1Luc3 or pGL3-basic constructs in the presence of pRLSV40 control plasmid. 24 hours after transfection, the cells were treated with TSA or NaBu in concentrations indicated in the figure for 24 hours followed by treatment of the cells with HDAC inhibitor together with 2 nM TCDD for further 24 hours prior to the measurement of reporter activity. RLU represents a mean of firefly normalized luciferase to *Renilla* luciferase activity in six wells.

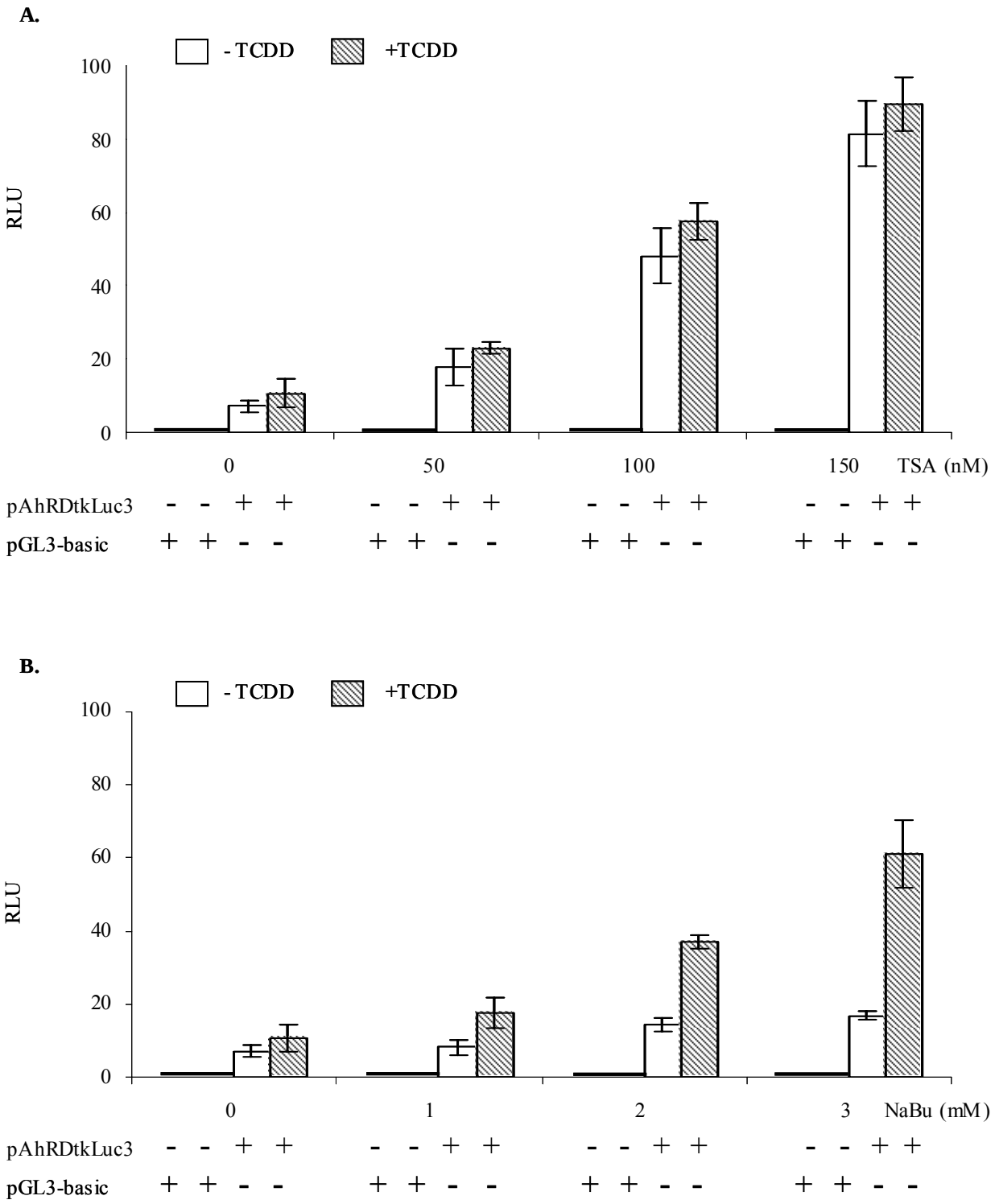


Figure 7-18. Activity of the pAhRDtkLuc3 reporter construct in HeLa cells treated with TCDD and with HDAC inhibitors TSA (A) and NaBu (B). Cells were transfected with pAhRDtkLuc3 or pGL3-basic constructs in the presence of pRLSV40 control plasmid. 24 hours after transfection, the cells were treated with TSA or NaBu in concentration indicated in the figure for 24 hours followed by treatment of the cells with HDAC inhibitor and 2 nM TCDD for further 24 hours prior to the measurement of reporter activity. RLU represents a mean of firefly luciferase normalized to *Renilla* luciferase activity in six wells.

The same pattern of the *CYP1A1* driven reporter gene activation was observed using the reporter plasmid containing the shorter version of the *CYP1A1* regulatory region, the pAhRDtkLuc3 construct. In this case NaBu also had no effect when applied to the transfected cells alone, but activated luciferase in the presence of TCDD. Activation of the reporter gene by TSA in the absence of TCDD was still observed regardless deletions of the *CYP1A1* promoter and proximal sequences in pAhRDtkLuc3 compare to p1646P1Luc3.

NaBu and TSA activated the two reporter genes p1646P1Luc3 and pAhRDtkLuc3 in a similar manner. This led to the conclusion that inhibitors of histone deacetylase may regulate *CYP1A1* gene via DRE motifs and/or surrounding sequences. This means the interaction of the HDAC complex with the AHR/ARNT heterodimer or proteins bound to the G- or GC-reach regions in the enhancer of *CYP1A1* gene could contribute to this effect. Interaction with other regulatory sequences present in p1646P1Luc3 but deleted in the pAhRDtkLuc3 construct, like the negative regulatory element, basic transcription element or *CYP1A1* promoter likely do not play a role in the observed effects of NaBu and TSA.

Although the different action of TSA regarding activation of the native human *CYP1A1* and the murine *CYP1A1* driven reporter genes was confirmed in a repeated experiment, but it should be mentioned that further verification of the reproducibility of this observation is required.

7.5.3 AHR and ARNT interaction with the HDAC complex

Because the above described effects of NaBu and TSA on the *CYP1A1* driven reporter construct may be mediated by the AHR/ARNT responsive elements, AHR and ARNT proteins were expected to interact with the histone deacetylase complex. The putative interactions of AHR and ARNT with the members of the HDAC complex were analysed in immuncoprecipitation assays. The examined members of the HDAC complex were, however not detectable in the protein complexes precipitated using α-AHR or α-ARNT antibodies when analysed by Western blot (data not shown). Thus, the putative physical interaction of AHR or ARNT with the members of the histone deacetylase complex required further verification.

In following studies the involvement of the particular members of the complex in the regulation of the *CYP1A1* gene was analysed in coexpression experiments.

7.5.4 Effect of the HDAC complex's individual members (HDAC1, SMRT and NCoR) overexpression on the regulation of the *CYP1A1* driven reporter gene

To study the role of particular members of the HDAC complex in the regulation of the *CYP1A1* gene, the effects of the HDAC1, SMRT or NCoR proteins expressed in Hepa cells on the activity of the coexpressed reporter gene under control of the *CYP1A1* regulatory region was analysed. The pCMX expression vectors coding for FLAG-tagged proteins were kindly provided by R. Evans (pCMX-HDAC1-FLAG and pCMX-SMRT-FLAG, described by Nagy et al. (1997)), and T. Heinzel (pCMX-NCoR-FLAG, described by Zamir (1996)).

As already shown in previous experiments, the control pGL3-basic construct did not show a detectable reporter activity either in the presence or in the absence of TCDD. To simplify interpretation of the presented diagrams, results obtained with the control plasmid are not included in the following figures.

Figure 7-19 shows that both the p1646PLuc3 (A) and pAhRDtkLuc3 (B) constructs expressed a constitutive level of the reporter activity in Hepa cells. This activity was not significantly affected by the presence of the control pCMX plasmid expressing the FLAG peptide. Unexpectedly, in the presence of HDAC1-FLAG-tagged protein the p1646P1Luc3 reporter gene expressed increased level of the luciferase activity. Coexpression of the SMRT-FLAG slightly suppressed the reporter activity in the cells transfected either with p1646PLuc3 or pAhRDtkLuc3 when 50 ng of pCMX-SMRT-FLAG DNA per well was applied to the cells. Similar to the constitutive activity also the TCDD-dependent transcriptional activity of the p1646P1Luc3 reporter gene was increased in the presence of the HDAC1-FLAG expression plasmid, whereas SMRT-FLAG coexpressed with any of the two reporter constructs was able to modestly decrease their activity (Figure 7-20). Coexpression of the NCoR-FLAG protein with any of the two reporter genes had no significant effect on the constitutive (Figure 7-19) as well as TCDD-induced (Figure 7-20) reporter enzyme activity.

The negligible modulation of the reporter activities did not permit any explicit conclusion regarding an involvement of HDAC1, NCoR or SMRT alone proteins in the repression of the *CYP1A1* gene.

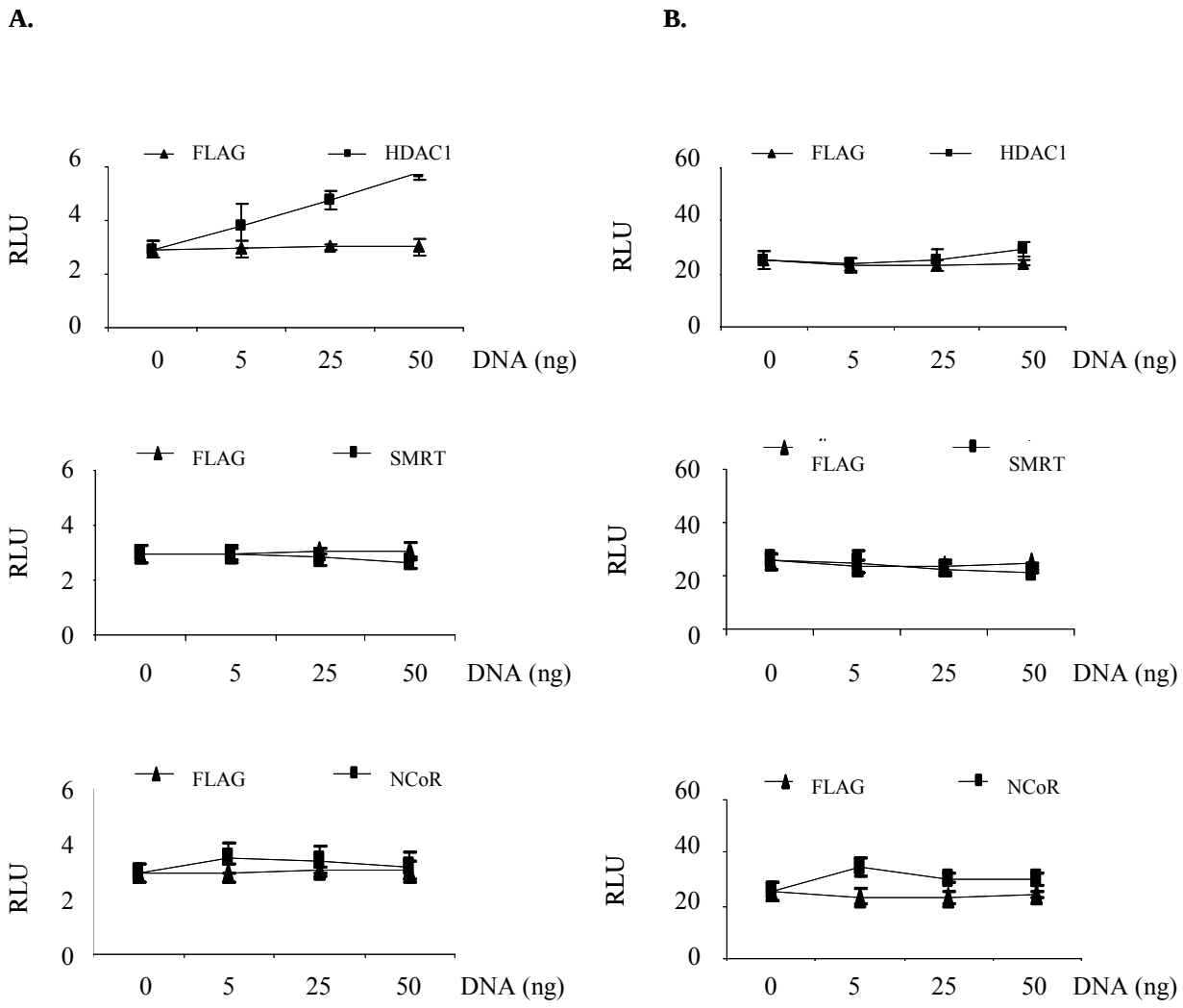


Figure 7-19. Effects of members of histone deacetylase complex on the constitutive activity of the *CYP1A1* driven reporter genes. Hepa cells were cotransfected with p1646P1Luc3 **(A)** or pAhRDtkLuc3 **(B)** in the presence of the pCMX expression plasmid coding for HDAC1-, SMRT-, or NCoR-FLAG fusion proteins. Transfection mix applied per well contained 20 ng of the *CYP1A1* derived construct, 5 ng pRLSV40, and pCMX coding for the appropriate member of the HDAC complex in the amounts indicated in the figure. pcDNA3.1 plasmid was added to the transfection mixtures to maintain an equal amount of 200 ng DNA per well. The pCMX vector expressing the FLAG peptide was used as a negative control. 40 hours after transfection, firefly luciferase was measured in the cells. RLU represents the firefly luciferase normalised to the *Renilla* luciferase activity expressed by the pRLSV40. Values represent the mean of results from four wells.

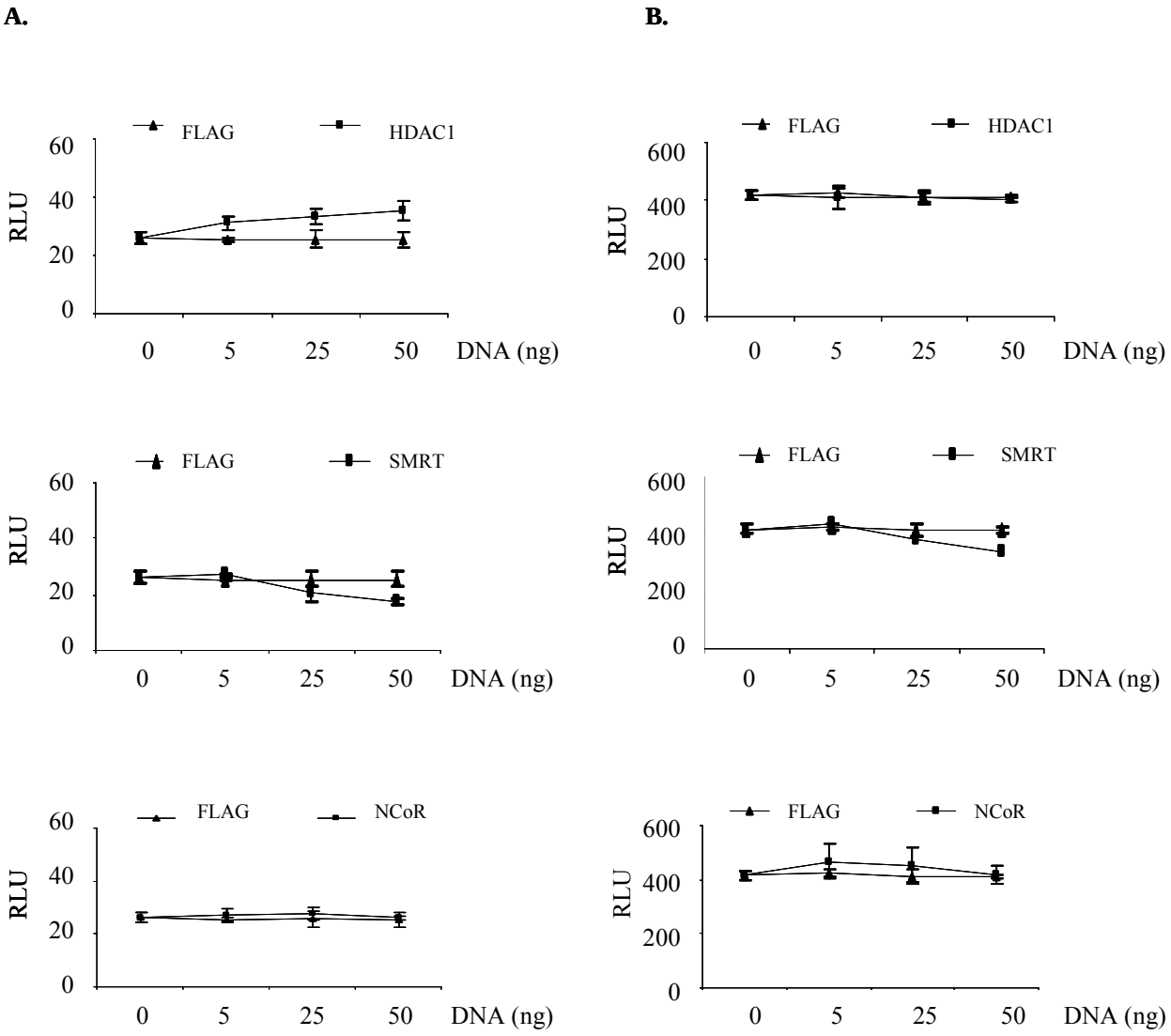


Figure 7-20. Effects of members of histone deacetylase complex on the TCDD-inducible activity of the *CYP1A1* driven reporter genes. Hepa cells were cotransfected with p1646P1Luc3 **(A)** or pAhRDtkLuc3 **(B)** in the presence of the pCMX expression plasmid coding for HDAC1-, SMRT-, or NCoR-FLAG fusion proteins. Transfection mix applied per well contained 20 ng of the *CYP1A1* derived construct, 5 ng pRLSV40, and pCMX coding for the appropriate member of the HDAC complex in the amounts indicated in the figure. pcDNA3.1 plasmid was added to the transfection mixtures to maintain an equal amount of 200 ng DNA per well. The pCMX vector expressing the FLAG peptide was used as a negative control. 24 hours after transfection, the cells were treated with 2 nM TCDD for 16 hours followed by measurement of the reporter enzyme activity. RLU represents the firefly luciferase normalised to the *Renilla* luciferase activity expressed by the pRLSV40. Values represent the mean of results from four wells.

7.5.5 Effect of HDAC1, SMRT and NCoR combined overexpression on the regulation of the *CYP1A1* driven reporter gene

In most cases histone deacetylases are targeted to the specific responsive elements on DNA by corepressors, proteins that bind transcription factors but have no deacetylase activity themselves and thus are not able to down regulate transcription. Therefore the effects of the overexpression of the particular members of the HDAC complex might not resemble the putative action of the multiprotein HDAC complex on the gene in the native situation.

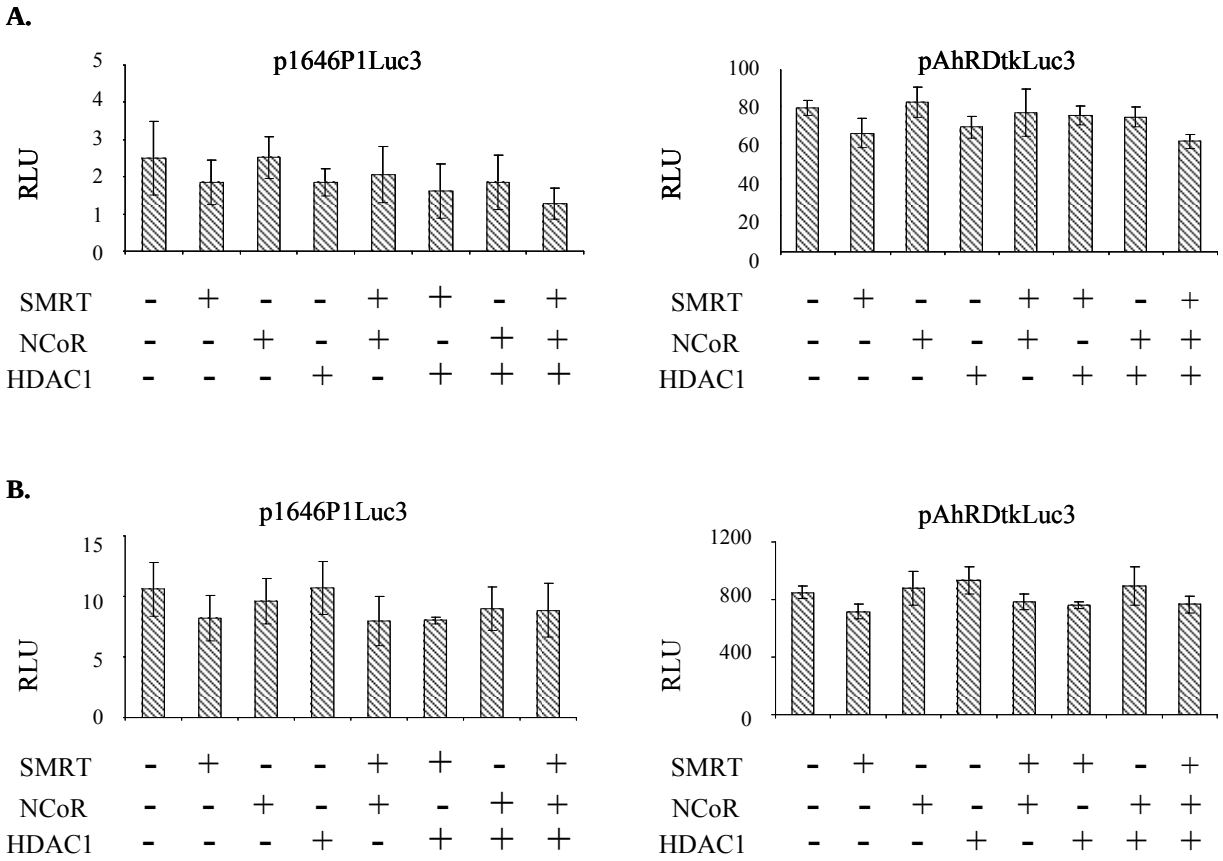


Figure 7-21. Effects of SMRT, NCoR and HDAC1 coexpression on the TCDD-inducible activity of the *CYP1A1* driven reporter genes. Hepa cells were cotransfected with 20 ng of p1646P1Luc3 or pAhRDtkLuc3 in the presence of 5 ng of each of the pCMX expression plasmids coding for HDAC1-, SMRT-, or NCoR-FLAG fusion proteins combined as indicated in the diagram. Transfection mix applied per well contained also 5 ng of pRLSV40 and pcDNA3.1 plasmid added to the transfection mixtures to maintain an equal amount of 200 ng DNA per well. **(A)** Firefly luciferase normalized to *Renilla* luciferase activity measured 30 hours after transfection, RLU represents the mean of the activity in four wells. **(B)** 2 nM TCDD was added to the cell culture medium 24 hours after transfection and the cells were incubated with the compound for the next 16 hours prior to the measurement of the reporter activity in the cells. RLU represents the mean of the activity in four wells.

To analyse the complex function of the multisubunit-repressor that likely leads to the silencing of the *CYP1A1* gene, the influence of the pCMX vectors coding for the SMRT, NCoR and HDAC1 proteins cotransfected in various combinations with one of the *CYP1A1* driven reporter gene on the constitutive and TCDD-induced luciferase activity in Hepa cells was assayed. As illustrated in figure 7-21-A, the constitutive activities of both p1646P1Luc3 and pAhRDtkLuc3 reporter constructs, modestly decreased in the presence of SMRT, were not further reduced after coexpression with either HDAC1-FLAG or NCoR-FLAG proteins. Combined overexpression of SMRT-FLAG, HDAC1-FLAG and NCoR-FLAG did not straighten the repressional effect of SMRT. Similar to the constitutive activity, also the TCDD-induced luciferase activities of the two reporter constructs were not affected by the presence of NCoR-FLAG, HDAC1-FLAG or both of these proteins coexpressed with SMRT (Figure 7-21-B).
Thus, these results did not confirm the cooperated action of the two corepressors and histone deacetylase in the control of the murine *CYP1A1* regulatory sequences. This suggests an involvement of an additional factor that probably is required for the repression of this gene.

7.5.6 Role of PKA in the NaBu mediated TCDD-dependent increase of *CYP1A1* expression

TSA is a competitive inhibitor of HDAC that binds to the active site of the enzyme (Finnin et al. 1999). The mechanism of action of NaBu is more complex. NaBu is a non competitive inhibitor of HDAC, having also pleiotropic effects on cell physiology. The additional (to the histone deaceylase inhibition) effects of NaBu could also be involved in the regulation of the *CYP1A1* gene. In this view, the most interesting pathways affected by NaBu would be the transcriptional regulation mediated by a serine/threonine phosphatase (Cuisset et al. 1997, 1998). Because previous studies from our labolatory revealed that PKA modulates AHR in Hepa cells, it was interesting to investigate whether the NaBu-dependent modulation of the *CYP1A1* gene in HeLa and Hepa cells was mediated by PKA. In order to find out whether the PKA-dependent protein phosphorylation may play a role in the NaBu mediated increase of the *CYP1A1* gene transcription, the effect of modulators of PKA activity, namely db-cAMP, forskolin and H89 on the NaBu-stimulated activation of the constitutive and TCDD-induced EROD was analysed.
As shown in figure 7-22, the PKA activators db-cAMP and forskolin as well as the PKA inhibitor H89 had no effect on the EROD activity in both cell lines (Hepa and HeLa) either in the presence or absence of TCDD. Alterations in the PKA-dependent serine phosphorylation in the

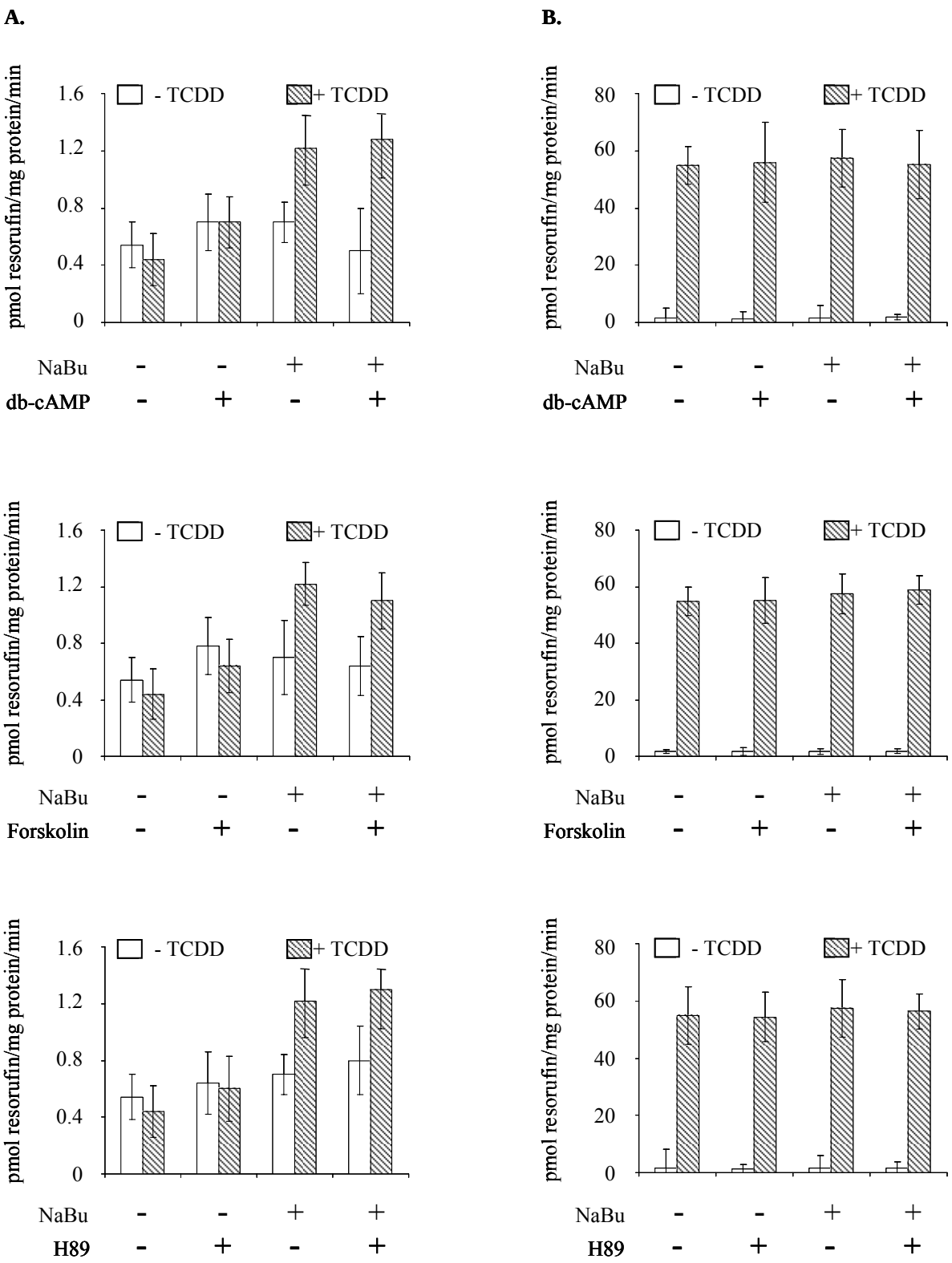


Figure 7-22. Effect of modulators of the PKA activity on NaBu-stimulated EROD activity. Cells were treated with or without NaBu in concentrations of 3 mM for HeLa cells **(A)** and 1 mM for Hepa cells **(B)** in the presence or absence of 50 µM db-cAMP, 5 µM forskolin or 5 µM H89 for 16 hours followed by the addition of 2 nM TCDD to the cell culture medium and incubation of the cells for another 16 hours. After treatment, EROD activity was estimated in the cells. Each value represents the mean of six experiments.

two cell lines also failed to modulate the NaBu mediated increase in the *CYP1A1* expression in any significant manner. These results indicate that the NaBu effect on the *CYP1A1* gene activity is probably not due to changes in the serine/threonine phosphorylation.

8 DISSCUSION

Cytochrome P450 1A1 (CYP1A1) monooxygenase is carefully regulated in the organism, mostly at the transcriptional level. This enzyme is not expressed in the absence of its exogenous substrates while increased transcription of the *CYP1A1* gene occurs upon exposure to such xenobiotics like PAHs or HAHs. In most cases oxidation of these compounds by the activated enzyme constitutes an initial step of their metabolism, which is a defense against these potentially toxic environmental contaminants. On the other hand, induction of the oxidative metabolism by CYP1A1 can also generate reactive intermediate metabolites that bind to DNA and thus may contribute to mutagenesis. Transcriptional activation of the *CYP1A1* gene by xenobiotic inducers is mediated by AHR, a ligand-activated transcription factor that regulates the expression of a battery of genes encoding xenobiotic metabolizing enzymes. Ligand binding to AHR causes activation of the receptor, leading to its accumulation in the nuclear compartment where it forms a heterodimer with ARNT, binds to the DRE motif in the regulatory regions of the responsive genes and activates their transcription. The most potent known inducer of the AHR-dependent gene transcription is TCDD. Although the exogenous ligand-dependent way of transcriptional activation of genes by AHR is well established, several studies have reported an enrichment of the nuclear AHR which binds to DNA without exposure to an exogenous ligand, indicating a possible regulation of the AHR function by an endogenous AHR ligand or other mechanisms (Chang and Puga 1998; Singh et al. 1996).

8.1 Identification of the TCDD-independent AHR/ARNT complex on DRE in HeLa cells

This study investigated mechanisms controlling the response to TCDD, in particular those concerning the activity of the CYP1A1 enzyme in two cell lines of different species and tissue origin: Hepa (mouse hepatoma) and HeLa (human cervix carcinoma). In Hepa cells the activation of AHR followed by increased transcription of the *CYP1A1* gene is highly inducible by TCDD (Whitlock et al. 1989). In HeLa cells instead, a significant amount of AHR is

associated with the nuclear fraction and AHR dimerization with ARNT is observed in the absence of an exogenous ligand. Moreover, proteins isolated from untreated HeLa cells form a specific complex on DRE (Singh et al. 1996).

As expected, in Hepa cells the AHR/ARNT interaction and binding to DRE were observed only in TCDD treated cells. This study also confirmed the TCDD-independent formation of the AHR/ARNT heterodimer in HeLa cells and extended this finding by identification of AHR and ARNT proteins in the protein complex constitutively present on DRE in these cells. But in contrast to Hepa, no influence of TCDD on these processes was observed in HeLa cells. Singh (1996) reported a strong additional activation of the AHR/ARNT heterodimer formation in TCDD treated HeLa cells compared to the control group. However this TCDD-dependent AHR/ARNT interaction was observed after incubation of the cells with 10 nM TCDD for 2 hours. In this study, the same conditions that induced the AHR/ARNT dimerization in Hepa cells (2 nM TCDD present for 1 hour in cell culture medium) were maintained in the HeLa cell culture for better comparison of TCDD effects in these two cell types. But this treatment seems to be not sufficient to induce an enhancement of the AHR binding to ARNT in HeLa cells.
The presence of the TCDD-independent AHR/ARNT complex on DRE in HeLa cells suggests an endogenous mechanism that activates AHR in the absence of exogenous ligand. Thus these cells provided a suitable model to search for such a mechanism.

8.2 Transcriptional repression of the *CYP1A1* gene in HeLa cells

A physiological consequence of the AHR activation by an exogenous ligand is a transcriptional activation of target genes. To ascertain whether the constitutive increase in the activated form of AHR in HeLa cells leads to the enhanced transcription of CYP1A1 enzyme, the activity of CYP1A1 (measured as EROD activity) was analysed. Interestingly, although AHR as a dimer with ARNT bound to DRE, this binding did not resulted in any elevated EROD activity in these cells as compared to Hepa cells. In addition HeLa, unlike Hepa cells, did not respond with an induction of *CYP1A1* expression even in the presence of TCDD. This observation can not be explained by a species specific regulation of the *CYP1A1* gene since TCDD was able to induce the *CYP1A1* transcription in other human cells, e.g. HepG2 (hepatoma). This observation is in agreement with a report that describes the TCDD-dependent inducibility of the *CYP1A1* gene in HepG2 and also in human breast carcinoma (MCF-7) cells (Nakajima et al. 2003). Thus the *CYP1A1* gene is suppressed by a cell type-specific *trans*-acting

factor that modulates the AHR/ARNT function, resulting in an interference with the TCDD induced signalling pathway in HeLa cells, but this is not a general mechanism for the human *CYP1A1* gene regulation. This factor was not only active towards human *CYP1A1* regulatory sequences but was also able to specifically repress *CYP1A1* of mouse origin. In transient transfection experiments reporter constructs containing the luciferase gene connected to the mouse *CYP1A1* regulatory sequences were activated by TCDD in both hepatoma cell lines, Hepa and HepG2, but not in HeLa cells. Therefore the repression is probably due to the recruitment of the *trans*-acting factor to the *CYP1A1* sequences common for both human and mouse genes.

8.3 Role of cAMP-activated PKA in the regulation of the AHR-mediated *CYP1A1* transcriptional control

In several studies the PKA-dependent phosphorylation has been implicated in the activation of metabolism by CYP1A1 and expression of the *CYP1A1* gene in human and rat cells (Hornsby et al. 1985; Zhang et al. 1997). An accumulation of AHR in the nucleus after activation of PKA in Hepa cells has been shown by Oesch-Bartlomowicz. Although the binding of a high molecular protein complex to DRE has been described, a presence of the AHR and ARNT proteins in this complex has not been conclusively proved. Moreover, the DRE core motif has been shown to be not crucial for the formation of the unique PKA-dependent protein-DRE complex (Oesch-Bartlomowicz et al., manuscript submitted). These data suggest that the PKA-dependent phosphorylation modulates the activation of AHR in Hepa cells.

By analogy to the TCDD-independent activation of AHR in Hepa cells, the PKA dependent phosphorylation as an endogenous mechanism triggering the constitutive AHR/ARNT binding to DRE in HeLa cells was speculated. But no alterations in the AHR interaction with ARNT or binding of AHR and ARNT to DRE after activation of PKA by db-cAMP or forskolin were observed in these cells. Also in Hepa cells the activation of PKA did not influence these processes. Thus, an effort to further evaluate the role of PKA in events that take place subsequentially to the translocation of the AHR to the nucleus was not successful.

Considering published data, the transcriptional consequences of the PKA-dependent regulation of the AHR function are unclear. The TCDD-dependent expression of the mouse *CYP1A1* gene appears to be suppressed in the presence of an increased cAMP level as the TCDD-induced activity of reporter gene containing regulatory sequences of the mouse *CYP1A1* gene is decreased in the presence of db-cAMP or forskolin (Oesch-Bartlomowicz et al.,

manuscript submitted). But in rat IEC-18 cells an activation of the *CYP1A1* gene upon treatment of the cells with db-cAMP has been shown (Zhang et al. 1997). The cAMP-induced activation of metabolism by CYP1A1 has also been observed in cultured fetal human adrenocortical cells. Treatment of these cells with adrenocorticotropin, forskolin or cholera toxin stimulates the metabolism of the classical CYP1A1 substrate benzo[a]pyrene (Hornsby et al. 1985). These data suggest that both inhibition and activation of the *CYP1A1* gene by the PKA-dependent phosphorylation is possible and this response is likely species and/or cell type specific.
Using of Hepa and HeLa cells of various species and tissue origin aimed in an enlightening of the ambiguous regulation of the CYP1A1 transcription by PKA. Since the basal as well as TCDD-induced EROD activity analysed in these cells after treatment with activators (db-cAMP or forskolin) or an inhibitor (H89) of the kinase were not significantly changed as compared to the untreated cells, this issue could not be convincingly elucidated.

Thus an attempt to further evaluate the role of cAMP-dependent phosphorylation in the AHR-mediated regulation of the CYP1A1 transcriptional control did not succeed in the course of this study. Also the question regarding an endogenous mechanism involved in AHR-mediated regulation of the CYP1A1 transcription stays open. A further search for such a mechanism should take a possible presence of an endogenous AHR ligand in HeLa cells into account. It has been shown that in mutant hepatoma cells that do not contain a functional CYP1A1 enzyme, AHR is constitutively enriched in the nuclear fraction and forms the AHR/ARNT complex on DRE that activates transcription of the *CYP1A1*-driven reporter gene. Activation of the AHR-dependent transcription is presumably due to the accumulation of an endogenous AHR inducer (Chang and Puga 1998; RayChaudhuri et al. 1990). Lack of a basal CYP1A1 enzyme activity in HeLa cells creates conditions where such an accumulation of an endogenous CYP1A1 substrate, that is able to activate AHR, is possible.

8.4 Contribution of HDAC to the repression of the *CYP1A1* gene

As mentioned above, HeLa cells did not respond to the TCDD treatment with an increased expression of CYP1A1 enzyme even though the AHR/ARNT complex is formed on DRE. The repressional character of this complex in these cells provoked further investigation of the mechanism that inhibits the activity of the *CYP1A1* gene. Binding of transcription factors to their responsive elements in the absence of ligands is observed in the case of nuclear receptors, that under these conditions are associated with corepressors (Aranda and Pascual 2001). The

corepressors assemble with a multisubunit complex containing histone deacetylase, whose activity is responsible for the repressional character of the complex (Nagy et al. 1997). Since *in vitro* studies have shown that the corepressor SMRT is able to bind AHR (Nguyen et al. 1999; Rushing and Denison 2002), it was presumed that the HDAC complex may repress the *CYP1A1* gene via interaction with the AHR/ARNT heterodimer in HeLa cells.

Analyses of the CYP1A1 expression in Hepa and HeLa cells treated with the HDAC inhibitors NaBu or TSA delivered an important evidence for the HDAC contribution to repression of the *CYP1A1* gene. Levels of both, the CYP1A1 enzyme measured as EROD activity and the *CYP1A1* mRNA analysed in Northern blots, were increased by the synergistic action of the AHR ligand-TCDD and an HDAC inhibitor. The additional induction of the *CYP1A1* transcription caused by treatment of Hepa cells with TSA or NaBu prior to the addition of TCDD was rather weak, probably due to the high TCDD responsiveness in these cells, where further induction might not be expected to be significant. In HeLa cells instead, the synergistic action of the inhibition of histone deacetylase and TCDD-induced activation of AHR on the transcription of the *CYP1A1* gene was apparent. TSA and NaBu restored the *CYP1A1* transcriptional response to TCDD in a dose dependent manner. However, this response did not reach the level of the TCDD-inducible *CYP1A1* expression in Hepa cells, suggesting that additional factors are involved in the repression of *CYP1A1* in HeLa cells. These findings are consistent with a report published during the course of this study showing the TSA-mediated restoration of *CYP1A1* and *CYP1B1* inducibility by TCDD in HeLa cells (Nakajima et al. 2003). Similar to the native *CYP1A1*, repression of the mouse *CYP1A1*-driven reporter genes transfected into HeLa cells proved to be due to the deacetylation of histones or other proteins since treatment of the cells with NaBu or TSA increased the basal and TCDD-induced reporter gene activity.

There are several mechanisms by which alteration of intracellular acetylation could stimulate transcription. One is an increase of the total cellular concentration of the transcription factor. The regulation of the gene coding for AHR has also been reported to be influenced by HDAC inhibitors (Garrison et al. 2000), and an increase of the constitutive and TCDD-induced expression of the *CYP1A1* gene after treatment of HeLa cells with TSA has been correlated with an elevated expression of AHR (Nakajima et al. 2003).

Thus, the possible induction of AHR and ARNT expression in cells treated with HDAC inhibitors in concentrations that stimulate TCDD-dependent transcription of the *CYP1A1* gene was tested. Western blot analyses excluded that an activation of the *CYP1A1* gene was due to the increased level of functional AHR or ARNT, as no alterations in the amounts of these proteins

were observed in Hepa and HeLa cells after their treatment with the HDAC inhibitors.
The published observations indicate a possible additional effect of an increase in the cellular amount of AHR on the TSA stimulated transcription of the *CYP1A1* gene. But, an increase of the AHR transcription has been observed upon treatment of HeLa cells with 300 nM TSA for 48 hours (Nakajima et al. 2003), while in this study 120 nM TSA present in the cell culture for 27 hours sufficed to stimulate TCDD-dependent expression of the *CYP1A1* gene, but without a detectable modulation of the AHR concentration. This indicates that prolonged treatment of the cells with a higher concentration of TSA modulates the AHR amount within the cells, which likely contributes to the enhanced expression of the *CYP1A1* gene under these conditions. However, the NaBu-and TSA-mediated restoration of the TCDD inducibility in HeLa cells seems to be primarily associated with the events occurring on the *CYP1A1* gene that are not a result of an increase in availability of the transcription factor.

8.5 Role of the enhancer region of *CYP1A1* in the effects of TSA and NaBu on the transcriptional control of the gene

The *CYP1A1* regulatory elements involved in the HDAC-mediated regulation were analysed in transient expression experiments using reporter genes containing either the entire 1700 bp fragment upstream of the mouse *CYP1A1* or only the enhancer region of that gene. Both constructs transfected into HeLa cells were activated in the presence of TCDD and NaBu or TSA in a similar way. Since deletion of the basic transcriptional element, the negative regulatory element and the *CYP1A1* promoter did not abolish the effect of the HDAC inhibitors, the repression of the *CYP1A1* driven reporter gene was mediated by the motifs enclosed in the enhancer region of the gene namely the DREs, the Sp1 binding site, the guanine-cytosine-rich element or surrounding sequences.
Since DREs are responsible for the AHR and ARNT binding, the influence of the HDAC complex on the *CYP1A1* gene might be mediated by AHR, ARNT or both of these proteins in a way resembling repression of genes by nuclear receptors. The physical interaction of nuclear receptors with the members of the HDAC complex is critical for their function so the binding of AHR and/or ARNT to the HDAC associated proteins or HDAC itself would be a likely event in HeLa cells. An attempt to visualise these putative interactions was however not successful since the HDAC1, SMRT, and NCoR proteins were not detected in the protein complexes precipitated from HeLa cells extracts with α-AHR or α-ARNT antibodies.

Also Sp1-like proteins constitute a very interesting protein family, which might mediate this effect. Both, AHR and ARNT interact with the Sp1 transcription factor (Zhao et al. 2003). The AHR/ARNT complex and Sp1 synergistically enhance the TCDD-induced expression of a reporter gene driven by the *CYP1A1* promoter (Kobayashi et al. 1996). The transcriptional regulation of genes mediated by this interaction is an example of the AHR involvement in the regulation of genes that are not involved in the metabolism of xenobiotics. In the absence of an exogenous ligand, AHR/ARNT and Sp1 regulate the expression of cathepsin D via specific motifs in its proximal promoter, which contains the Sp1 binding site and also DRE (Wang et al. 1998; Wang et al. 1999). Sp1 exerts its regulatory function via an interaction with HDAC (Kaczynski et al. 2001; Zhang et al. 2001; Zhao et al. 2003). Given that the Sp1 binding site is one of the recently identified "NaBu responsive elements", which are necessary for the transcriptional effect of NaBu (Yang et al. 2001), it is possible that this sequence plays a role in the NaBu-mediated activation of the *CYP1A* gene.

The abilities of NaBu and TSA to stimulate TCDD-dependent transcription of the native human *CYP1A1* and a reporter gene containing mouse *CYP1A1* regulatory region suggest that the HDAC inhibitors modulate the activity of both genes together with a factor which is recruited to the gene in the presence of TCDD. Besides similarities, some differences in this regulation imply species specific variation in the HDAC inhibitors-mediated activation of the human and mouse genes. The human gene was only modestly activated by TSA as compared to the activation by NaBu and both inhibitors were able to increase the transcription only in the presence of TCDD. Although a synergistic action of an HDAC inhibitor and TCDD was observed also in the case of the mouse sequences containing reporter gene, both TSA and NaBu were able to significantly increase the reporter activity even in the absence of TCDD. Interestingly, the reporter gene was activated much stronger by TSA than by NaBu.

The phenomenon of a different modulation of these two genes might be attributed to the specific chromatin versus plasmid DNA structure that possibly generates different accessibility conditions for the transcriptional machinery. But the species-specific variation in the TSA/NaBu-responsive regions of the *CYP1A1* gene should also be taken into account. A high activation of the reporter gene in the presence of TSA (a strong competitive inhibitor of histone deacetylase) indicates a deacetylation mediated repression. Still, the effect of this compound on the human gene was week, which suggests a contribution of another mechanism in the repression of the later. This mechanism is likely mediated by a regulatory element either present exclusively in the human gene or, if present in the mouse *CYP1A1* gene as well, located behind the sequences analysed in the here presented reporter assays. Although the human and rodent *CYP1A1* genes

are similar, there are some apparent variabilities concerning the negative regulatory mechanism, which involve the species specific structure of the negative regulatory element, binding of nuclear proteins and consequently, the inhibitory function of this element (Boucher and Hines 1995; Boucher et al. 1993). A role of the negative regulatory element in the *CYP1A1* gene regulation by TSA and NaBu was excluded in reporter assays but an involvement of other regions in this effect is also possible. A functional analysis of human and mouse *CYP1A1* genes revealed a presence of several G-rich domains in their enhancer region of the human gene and one G-rich domain in the mouse gene. One of the human G-rich regions, located at about 450 nucleotides upstream of the transcriptional start which has no homologue in the mouse gene, is constitutively occupied by proteins (Kress et al. 1998). On the other hand, the only one G-rich motif in the mouse *CYP1A1* enhancer region identified so far is occupied by proteins in a TCDD- and AHR-dependent manner (Watson and Hankinson 1992). Although the human gene contains a homologue of this sequence, no constitutive or TCDD-induced binding of proteins to this sequence has been observed (Kress et al. 1998). It appears possible that these different species-specific protein-DNA interactions that control the constitutive and TCDD-induced transcription of the human versus mouse *CYP1A1* gene may be involved in the different NaBu and TSA action on these genes.

8.6 Role of corepressors in the regulation of the *CYP1A1* gene

Further effort focussed on the identification of members of the histone deacetylase complex that may be involved in the repression of the *CYP1A1* gene.

An implication that in fact NCoR is the corepressor contributing to the different regulation of the *CYP1A1* genes in Hepa and HeLa cells was achieved by Western blot analysis of the expression of HDAC and associated proteins in these cells. HDAC1 as well the corepressors mSin3A and SMRT were present in both cell types, while NCoR was expressed exclusively in HeLa cells. Thus the high inducibility of the *CYP1A1* gene in Hepa cells may be due to the absence of NCoR in these cells while repression of the *CYP1A1* gene in HeLa cells might be correlated with the presence of NCoR supporting the repression of genes by histone deacetylase. Such a correlation of the cell specific repression of genes by HDAC with the level of NCoR has been reported for genes controlled by nuclear receptors (Zhang J. et al. 1998).

To verify the hypothesis of the NCoR being a mediator of the *CYP1A1* gene repression, the activity of the reporter constructs containing the mouse *CYP1A1* regulatory sequences

cotransfected with the expression constructs coding for NCoR, HDAC1 or SMRT was analysed in Hepa cells. But an overexpression of NCoR did not decrease the reporter gene activity. In cells cotransfected with the HDAC1 expressing vector even an increase of the constitutive and TCDD-induced reporter activity was observed. The expected inhibition of the luciferase activity was observed solely in the case of SMRT that slightly reduced the constitutive and TCDD-induced reporter gene expression. Known corepressors exert their effects on genes not isolated but in large multiprotein complexes where the interactions between particular proteins are essential for the repressor activity. Therefore an overexpression of a single member of the complex may not resemble the native situation due to lack of this interactions. A simultaneous expression of various members of the HDAC complex should optimize the conditions enabling its repressional function on the *CYP1A1*-driven reporter gene activity. But no further effects were observed in Hepa cells when the two corepressors NCoR and SMRT were coexpressed, and also the additional presence of the HDAC1 expressing vector in the transfection mix did not alter this situation.

The same SMRT-expressing construct, kindly received from R. Evans, has been used in independent studies of AHR-mediated transcriptional regulation in two other laboratories (Nguyen et al. 1999; Rushing and Denison 2002). However, the observations described by these two groups serve vague conclusions as an inhibition as well as an enhancement of the *CYP1A1* containing reporter genes was observed. The effect of SMRT seems to depend on the origin of the *CYP1A1* regulatory sequences (human versus mouse), the host cell context (species and cell type differences) but also the amount of the expression plasmid used for transfection. Considering current studies reporting a rather weak effect of SMRT, the role of SMRT in the regulation of the AHR function still remains to be defined.

8.7 Mechanisms of TSA- and NaBu-mediated transcriptional regulation of the *CYP1A1* gene

TSA is a potent and specific histone deacetylase inhibitor which regulates the activity of the enzyme through binding to its active core (Finnin et al. 1999). Although the mechanism of the NaBu action is less clear, also this compound is believed to modulate gene expression by its ability to inhibit HDAC (Riggs et al. 1977). Therefore an increase of the *CYP1A1* gene activity in the presence of these compounds supported a role of acetylation of histones or other proteins as a regulatory mechanism contributing to the expression of these genes.

In addition to an increased acetylation, NaBu has been shown to have multiple effects on the cell physiology, such as changes in phosphorylation and the calcium status of the cells (Rivero and Adunyah 1996; Whitlock et al. 1980). Investigation of the diverse processes activated by NaBu in the cells that may also contribute to the activation of the *CYP1A1* gene appeared an interesting aim, because a better understanding of the NaBu action could lead to the identification of the endogenous factors responsible for regulation of the AHR function.

NaBu activated gene expression might be mediated through specific serine/threonine phosphatases since inhibition of phosphatase activity in cells treated with okadaic acid or calyculin A blocks the transcriptional induction of genes by NaBu (Cuisset et al. 1998; Cuisset et al. 1997). This phosphorylation-mediated effect of NaBu is probably not associated with regulation of the HDAC activity as hyperphosphorylation of histone deacetylases (observed upon treatment of cells with ocadaic acid) leads to disruption of the interactions between the histone deacetylase and corepressors, thus preventing the establishment of repressive complexes on regulatory sequences (Galasinski et al. 2002).

The phosphatase inhibitors calyculin A and ocadaic acid are able to increase the TCDD-dependent transcription of the reporter gene containing DREs. The increased serine/threonine specific phosphorylation stimulates the AHR/ARNT-dependent transcription at a level subsequent to the DRE binding since the TCDD-induced formation of the AHR/ARNT complex on DRE is not affected in the presence of phosphatase inhibitors (Li and Dougherty 1997). On the other hand, a formation of a protein complex on DRE is observed upon activation of PKA-dependent serine phosphorylation (Oesch-Bartlomowicz et al., manuscript submitted). Until now, it is not clear which protein is a target for either phosphatases or kinase A in the AHR-mediated regulation of the *CYP1A1* gene and whether these enzymes act antagonistically in this process. Considering an association of the NaBu action with a modulation of phosphatase activity, it was interesting to investigate the possible mediation of NaBu-activated *CYP1A1* transcription by PKA. Activation of PKA by db-cAMP or forskolin as well as inhibition by H89 however failed to affect the NaBu-stimulated TCDD-dependent activation of EROD activity in Hepa and HeLa cells. Thus in contrast to phosphatases, protein kinase A is probably not a mediator of the effect of NaBu on *CYP1A1* gene transcription.

8.8 Concluding comments

This study implicates the HDAC-mediated repression of *CYP1A1* as a carefully controlled mechanism that contributes to the xenobiotic-induced expression of this gene in a tissue specific manner. Silencing of the *CYP1A1* gene even in the presence of a xenobiotic inducer is necessary in tissues that are not provided with a complete set of enzymes required for an efficient detoxification. Without repression mechanisms, an exposure to the xenobiotic inducer would lead to an increased production of the toxic intermediates and deleterious effects in these tissues. At times of increasing use of compounds with an HDAC-inhibitor activity as therapeutics in leukaemia and other diseases, the importance of an understanding of these mechanisms becomes apparent. A therapy involving HDAC inhibitors combined with an exposure to AHR activating agents, which is actually unavoidable considering an overall distribution of PAHs or HAHs, might lead to a high activation of metabolism by CYP1A1. This will stimulate the production of reactive metabolites that bind to DNA and may lead to mutagenesis and a consequent increased risk of cancer. Elucidation of these processes will lead to a better prediction and prevention of the adverse effects of such a complex exposition.

It seems obvious that the repressional mechanism evolved in HeLa cells to prevent the activation of the *CYP1A1* gene even in the presence of a potent AHR inducer, such as TCDD. If this mechanism can be extended to the in vivo situation, this would evoke the question what function AHR has in the cells if not the transcriptional activation of the *CYP1A1* gene. A high level of the constitutive DRE bound AHR in these cells suggests its active role in transcriptional control. Although in the case of the *CYP1A1* gene binding of AHR to DRE does not induce transcription, it can not be excluded that AHR activates transcription of other genes in these cells. In fact, an increasing amount of studies report the presence of functional DREs in the regulatory regions of genes which products are not associated with the metabolism of xenobiotics. In addition, observations in AHR-deficient mice suggest a role of AHR in cell proliferation and differentiation. Extended studies aimed in the recognition of an endogenous mechanism that activates AHR to its DNA binding form and, on the other hand, aimed in the identification of target genes that are probably activated by AHR in HeLa cells will contribute to the evaluation of the AHR role in normal cell physiology.

9 LITERATURE

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