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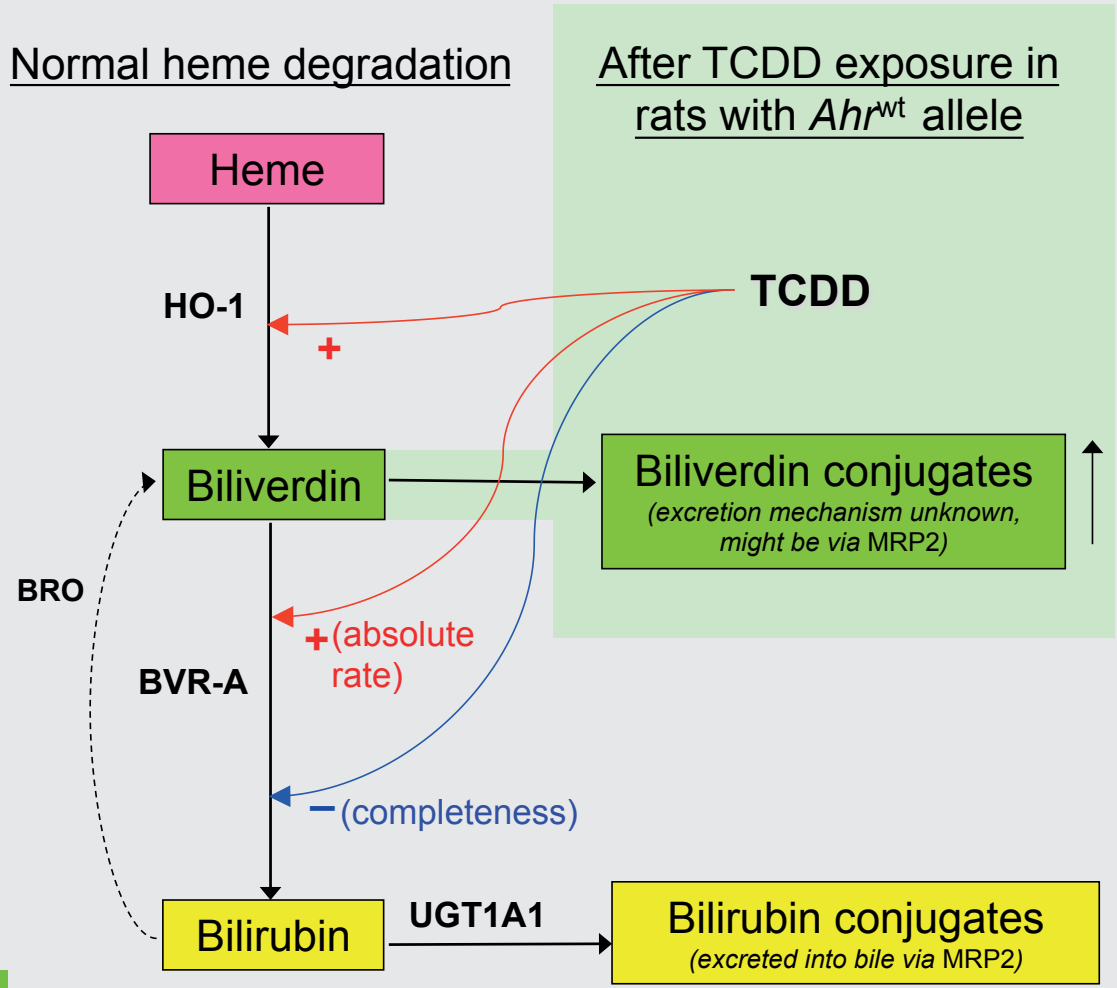
Marjo Niittynen

TCDD-Induced Accumulation of the Heme Degradation Product Biliverdin in Rat Liver

RESEARCH



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TCDD-Induced Accumulation of the Heme Degradation Product Biliverdin in Rat Liver

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Health Sciences of the University of Eastern Finland for public examination in Medistudia Auditorium MS301, Yliopistonranta 1A, on Friday 24th of October, at 12:00.

National Institute for Health and Welfare,
Department of Environmental Health, Kuopio, Finland
and
University of Eastern Finland, School of Pharmacy, Kuopio, Finland

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Cover photo: A summary of the probable TCDD-induced events leading to hepatic accumulation of biliverdin, bilirubin and their conjugates in rats expressing wild-type AHR. Redrawn from the original version published in Basic & Clinical Pharmacology & Toxicology (Niittynen et al. 2014).

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To my family

Abstract

Marjo Niittynen. TCDD-induced accumulation of the heme degradation product biliverdin in rat liver. National Institute for Health and Welfare (THL). Research 133. 203 pages. Helsinki, Finland 2014.
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Polychlorinated dibenzo-*p*-dioxins and -furans (PCDD/Fs), "dioxins", are widespread and persistent environmental pollutants. Due to their lipophilicity and stability, dioxins accumulate in the food chain finally ending into human diet. In Finland, the part of population eating plenty of fish from the Baltic Sea receives highest dioxin exposures. Health risk assessment of dioxins is challenging as their mechanisms of action are very complicated and not fully understood.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent dioxin congener and the prototypic compound used in toxicological studies. In laboratory animals, TCDD elicits multiple biochemical and toxic responses, which are mainly mediated by the aryl hydrocarbon receptor (AHR). *AHR*- and yet unidentified gene *B* -genotypes have major influence on the TCDD-sensitivity of a rat: Han/Wistar (hw)-type alleles confer increased resistance relative to wild-type (wt) alleles of these genes. Here the pathogenesis and mechanism of a novel TCDD-induced toxicity syndrome, accumulation of green pigment in rat liver, was studied in order to increase understanding of the mechanisms of TCDD toxicity. In liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis, the pigment was found to consist of the heme degradation product biliverdin and its conjugates.

Heme is the prosthetic group of hemoglobin and several other proteins. At the time of degradation of these proteins, the heme part must also be degraded. The rate-limiting enzyme heme oxygenase (HO-1) first converts heme to biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase (BVR-A). Increased serum bilirubin is a well-known effect of TCDD in rats. In contrast, TCDD-induced biliverdin accumulation has been reported only in specific rat strains of our laboratory, foremost in semi-TCDD-resistant line B rats carrying wt alleles of *AHR* but TCDD-resistance alleles (hw) of gene *B*. Notably, biliverdin accumulation has never been detected in TCDD-resistant Han/Wistar (*Kuopio*) or line A (*Kuopio*) rats, which express only mutated AHR (genotype $AHR^{hw/hw}$).

It was hypothesised that biliverdin formation is either increased and/or its metabolism is decreased due to TCDD-induced changes in HO-1, BVR-A or δ-aminolevulinic acid synthetase 1 (ALAS-1; the rate-limiting enzyme of heme synthesis). In order to probe the hypothesis and further characterize the syndrome, following issues were studied: 1) Incidence of the macroscopic biliverdin

accumulation syndrome and effect of TCDD on hepatic biliverdin level in differentially TCDD-sensitive rat strains, 2) Liver histopathology, 3) Dose-responses of TCDD exposure and hepatic biliverdin and bilirubin accumulation, serum bilirubin and bile acids, 4) Effect of TCDD on hepatic and splenic expression and/or activity of ALAS1, HO-1 and BVR-A, and 5) Effect of TCDD on bilirubin excretion. In addition, acute toxicity of TCDD and another dioxin congener, 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD), were compared in line A, line B and H/W rats. Further, the effect of TCDD on the expression of 18 commonly used housekeeping genes was investigated in order to find, inter alia, a suitable normalization gene for the point 4 above.

At 28 days in line B rats, doses ≥100 µg/kg of TCDD significantly elevated hepatic biliverdin, whereas in line A rats hepatic biliverdin remained at the control level even after 10,000 µg/kg of TCDD. In TCDD-sensitive Long-Evans rats (*Turku*/AB; genotype $AHR^{wt/wt}$ $B^{wt/wt}$), hepatic biliverdin level increased slightly in response to TCDD treatment. In correlation analysis between hepatic bile pigments, biliverdin conjugates correlated best with biliverdin suggesting it to be their immediate precursor. TCDD enhanced bilirubin excretion suggesting that excretory problems are not the principal cause for increased serum bilirubin. In general, TCDD had moderate inducing effect on hepatic HO-1 and BVR-A, however, curious exceptions such as late inhibition of BVR-A in line B rats were detected. TCDD repressed hepatic expression of ALAS1 and splenic expression of HO-1. About 50% of the studied housekeeping genes were responsive to TCDD; most stable were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and phosphoglycerate kinase 1 (*Pgk 1*). HxCDD did not induce biliverdin accumulation. Instead, it caused severe hepatic steatosis, rapid body weight loss, and mortality by an AHR-independent mechanism. Both TCDD and HxCDD induced hepatic peliosis.

In conclusion, a fundamental cause behind both TCDD-induced biliverdin accumulation and increased serum bilirubin is increased hepatic heme degradation. Concurrent incomplete conversion of biliverdin to bilirubin – possibly due to complexities in the function of the catalyzing enzyme, BVR-A – is the most likely reason for biliverdin accumulation. Biliverdin conjugates are most probably secondary products of biliverdin and they form the main part of the accumulating pigment. Homozygocity for AHR^{hw} provided complete protection against TCDD-induced biliverdin accumulation, indicating necessity of the wild-type AHR transactivation domain for the syndrome. For B^{hw} two options remain: B^{hw} may be unrelated to biliverdin metabolism *per se* but crucial for macroscopic manifestation of the syndrome via increasing rat´s TCDD-resistance, or B^{hw} may be a necessary factor for substantial biliverdin accumulation to be possible. Further, it was found that in addition to the classical AHR-mediated toxicity of PCDDs, there is another

mechanism of PCDD toxicity characteristic for HxCDD but not activated at all or only very weakly by TCDD.

Keywords: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin, biliverdin, bilirubin, rat, hepatotoxicity, heme, heme oxygenase, biliverdin reductase, sinusoidal distension, hepatic peliosis, housekeeping genes, steatosis

Tiivistelmä

Marjo Niittynen. TCDD:n aiheuttama biliverdiinin kertyminen rotan maksaan. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 133. 203 sivua. Helsinki, Finland 2014.
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Polyklooratut dibentso-*p*-dioksiinit ja -furaanit (PCDD/F:t), ”dioksiinit”, ovat laajalle levinneitä, pysyviä ja ravintoketjussa kertyviä ympäristömyrkkyjä. Suomessa runsaasti Itämeren kalaa käyttävä väestönosa altistuu suurimmille dioksiinipitoisuuksille. Dioksiinien riskinarviointia vaikeuttaa niiden vaikutusmekanismien monimutkaisuus ja puutteellinen tunteminen.

2,3,7,8-Tetraklooridibentso-*p*-dioksiini (TCDD) on voimakkain dioksiinijohdos ja käytetyin malliyhdiste dioksiineja koskevissa toksikologisissa tutkimuksissa. Koe-eläimissä TCDD aiheuttaa monia biokemiallisia ja toksisia vasteita, jotka välittyvät pääasiassa aryylihiilivetyreseptorin (AHR) kautta. *AHR*:n ja tuntemattoman *B*-geenin genotyypit määrittävät rotan TCDD-herkkyyden: Han/Wistar (hw) -tyypin alleelit tuottavat suuremman kestävyyden kuin villityypin (wt) alleelit. Tässä työssä pyrittiin lisäämään ymmärrystä TCDD:n vaikutusmekanismeista tutkimalla aivan uutta TCDD:n aiheuttamaa toksista vaikutusta: vihreän pigmentin kertymistä rotan maksaan. Nestekromatografia-massaspektrometria (HPLC-MS/MS) -analyysi osoitti pigmentin koostuvan hemin hajoamistuotteesta biliverdiinistä ja sen konjugaateista.

Hemi on hemoglobiinin ja eräiden muiden proteiinien prosteettinen ryhmä. Näiden proteiinien hajotessa elimistön on hajotettava myös hemiosa. Tässä tarvitaan kahta entsyymiä: hemioksigenaasi (HO-1) katkaisee hemin rengasrakenteen ja muodostaa biliverdiiniä, jonka puolestaan biliverdiinireduktaasi (BVR-A) pelkistää bilirubiiniksi. Kohonnut seerumin bilirubiinipitoisuus on tunnettu TCDD:n vaikutus rotissa. Sen sijaan TCDD-altistuksen suurentamia maksan biliverdiinipitoisuuksia on kuvattu ainoastaan tietyissä laboratoriossamme kehitetyissä rottalinjoissa, ennen kaikkea TCDD:tä keskinkertaisesti kestävissä B-linjan rotissa, joilla on villityypin alleelit *AHR*-geenistä mutta hw-tyypin kestävyyttä lisäävät alleelit *B*-geenistä. On huomionarvoista, että biliverdiinin kertymistä ei ole koskaan havaittu erittäin TCDD-kestävissä Han/Wistar (*Kuopio*) tai A-linjan (*Kuopio*) rotissa, jotka ilmentävät ainoastaan mutatoitunutta AHR-proteiinia (genotyyppi $AHR^{hw/hw}$).

Työhypoteesin mukaan biliverdiinin kertymisen taustalla olisi joko sen muodostumisen lisääntyminen ja/tai metabolian hidastuminen johtuen TCDD-altistuksen aiheuttamista muutoksista HO-1:n, BVR-A:n tai hemin synteesitien 1. entsyymin, δ-aminolevulinaattisyntetaasi 1:n (ALAS-1) ilmenemisessä tai aktiivisuudessa. Hypoteesin testaamiseksi ja biliverdiinin kertymisilmiön lisäluonnehtimiseksi

tutkittiin seuraavia asioita: 1) silminhavaittavan syndrooman ilmaantuvuus sekä TCDD:n vaikutus maksan biliverdiinipitoisuuksiin TCDD-herkkyydeltään erilaisilla rottakannoilla, 2) maksan histopatologia, 3) annos-vaste-suhteet TCDD-altistuksen ja maksan biliverdiini/bilirubiinipitoisuuden sekä seerumin bilirubiini- ja sappihappopitoisuuden välillä, 4) TCDD:n vaikutus ALAS1:n, HO-1:n ja BVR-A:n ilmenemiseen ja/tai katalyyttiseen aktiivisuuteen, ja 5) TCDD:n vaikutus bilirubiinin erittymiseen. Lisäksi vertailtiin TCDD:n ja 1,2,3,4,7,8-heksaklooridibentso-*p*-dioksiinin (HxCDD) akuuttia toksisuutta A- ja B-linjan sekä H/W-kannan rotissa. Tutkittiin myös TCDD:n vaikutusta 18 yleisesti käytetyn normalisointigeenin ilmenemiseen rotan eri kudoksissa mm. sopivan vaihtoehdon löytämiseksi kohtaan 4.

Maksan biliverdiinipitoisuus nousi B-rotilla TCDD-annoksella ≥100 µg/kg. A-rotilla pitoisuus säilyi kontrollitasolla jopa annoksen 10 000 µg/kg jälkeen. TCDD-herkillä Long-Evans (*Turku*/AB) -rotilla nähtiin lievä vaikutus. Sappipigmenttien välisessä korrelaatioanalyysissä biliverdiinikonjugaatit korreloivat parhaiten biliverdiinin kanssa, täten viitaten biliverdiinin olevan biliverdiinikonjugaattien välitön edeltäjä. TCDD tehosti bilirubiinin eritystä, joten eritysongelmat eivät selitä seerumin bilirubiinin nousua. TCDD lisäsi lievästi maksan HO-1:n ja BVR-A:n ilmenemistä ja/tai aktiivisuutta, poikkeuksia kuitenkin havaittiin kuten mm. BVR-A:n myöhäinen inhibitio B-rotilla. TCDD vähensi ALAS1:n ilmenemistä maksassa ja HO-1:n ilmenemistä pernassa. Noin 50 % tutkituista normalisointigeeneistä reagoi TCDD-altistukseen; stabiileimpia olivat glyseraldehydi-3-fosfaatti dehydrogenaasi (*GAPDH*) ja fosfoglyseraattikinaasi 1 (*Pgk 1*). HxCDD ei aiheuttanut biliverdiinin kertymistä. Sen sijaan se aiheutti vakavaa maksan rasvoittumista, nopeaa painonlaskua ja kuolleisuutta AH-reseptorista riippumattomalla mekanismilla. Sekä TCDD että HxCDD aiheuttivat maksapelioosia.

Tulokset osoittavat, että yhteinen taustasyy TCDD:n aiheuttamalle biliverdiinin kertymiselle sekä seerumin bilirubiinitasojen nousulle on lisääntynyt hemin hajotus. Samanaikainen biliverdiinin epätäydellinen pelkistyminen bilirubiiniksi – mahdollisesti johtuen BVR-A:n toiminnallisesta monimutkaisuudesta – on todennäköisin syy biliverdiinin kertymiselle. Biliverdiinikonjugaatit ovat luultavimmin biliverdiinin sekundäärituotteita ja muodostavat pääosan kertyvästä pigmentistä. Villityypin AHR-transaktivaatiodomeeni on edellytys TCDD:n aiheuttamalle biliverdiinin kertymiselle, sillä homotsygotia *AHR*^{hw}-alleelin suhteen esti kertymisen täysin. *B*^{hw}-alleelin osalta on kaksi mahdollisuutta: joko *B*^{hw}-alleeli ei suoranaisesti osallistu biliverdiinimetaboliaan mutta mahdollistaa syndrooman silmämääräisen havaitsemisen kasvattamalla rotan TCDD-kestävyyttä, tai *B*^{hw} voi olla välttämätön tekijä biliverdiinin huomattavalle kertymiselle. Lisäksi havaittiin, että klassisen AHR-välitteisen mekanismin lisäksi on olemassa toinen eräiden

PCDD-yhdisteiden toksisuutta välittävä mekanismi, joka on tyypillinen HxCDD:lle, mutta jota TCDD aktivoi hyvin vähän tai ei lainkaan.

Avainsanat: 2,3,7,8-tetraklooridibentso-*p*-dioksiini, 1,2,3,4,7,8-heksaklooridibentso-*p*-dioksiini, biliverdiini, bilirubiini, rotta, maksatoksisuus, hemi, hemioksigenaasi, biliverdiinireduktaasi, maksapelioosi, normalisointigeenit

Contents

List of original papers

I Niittynen M, Tuomisto JT, Auriola S, Pohjanvirta R, Syrjälä P, Simanainen U, Viluksela M, Tuomisto J. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-induced accumulation of biliverdin and hepatic peliosis in rats. Toxicological Sciences 2003; 71:112-123.

II Pohjanvirta R, Niittynen M, Lindén J, Boutros PC, Moffat ID, Okey AB. Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats. Chemico-Biological Interactions 2006; 160:134-149.

III Niittynen M, Simanainen U, Syrjälä P, Pohjanvirta R, Viluksela M, Tuomisto J, Tuomisto JT. Differences in acute toxicity syndromes of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin in rats. Toxicology 2007; 235:39-51.

IV Niittynen M, Tuomisto JT, Pohjanvirta R. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on heme oxygenase-1, biliverdin IXα reductase and δ-aminolevulinic acid synthetase 1 in rats with wild-type or variant AH receptor. Toxicology 2008; 250:132-142.

V Niittynen M, Simanainen U, Pohjanvirta R, Sankari S, Tuomisto JT. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) increases bilirubin formation but hampers quantitative hepatic conversion of biliverdin to bilirubin in rats with wild-type AH receptor. Basic & Clinical Pharmacology & Toxicology 2014; 114:497-509.

Abbreviations

ABCB6	ATP-binding cassette sub-family B member 6
AD	Alzheimer´s disease
AH	Aryl hydrocarbon
AHR	Aryl hydrocarbon receptor
AHR / *Ahr*	The gene encoding AHR
AHRR	Aryl hydrocarbon receptor repressor
ALAD	δ-Aminolevulinic acid dehydratase
ALAS	δ-Aminolevulinic acid synthetase
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
ARE	Antioxidant response element
ARNT	AH receptor nuclear translocator; the dimerization partner of AHR
ASAT	Aspartate aminotransferase
ATF2	Activating transcription factor 2
B	An unknown gene that affects TCDD sensitivity
bHLH/PAS	Basic Helix-Loop-Helix/homologous region of Periodic (PER), AHR nuclear translocator (ARNT), and Single-minded (SIM) proteins
BR	Bilirubin; yellow end product of heme degradation
BRO	Bilirubin oxidase
BV	Biliverdin; green to blue intermediate product of heme degradation
BVR	Biliverdin reductase; ubiquitous cytosolic enzyme that converts biliverdin to bilirubin
BVR-A	Biliverdin IXα reductase; predominant form of BVR in adult mammals. Highly selective for biliverdin IXα isomer.
BVR-B	Biliverdin IXβ reductase; fetal form of BVR. Reduces biliverdin isomers IXβ, IXγ and IXδ. Has flavin and ferric reductase activities in adult mammals.
b.w.	Body weight
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CO	Carbon monoxide
CoA	Coenzyme A
CPO	Coproporphyrinogen oxidase
CREB	cAMP response element-binding protein
CYP1A1	Cytochrome P4501A1, a phase 1 metabolizing enzyme
CYP1A2	Cytochrome P4501A2, a phase 1 metabolizing enzyme

DNA	Deoxyribonucleic acid
DRE	Dioxin response element in DNA; called also XRE, xenobiotic response element
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinases 1/2
EROD	Ethoxyresorufin-O-deethylase
FEC	Ferrochelatase
FR	Feed-restricted control group
FRC	Feed-restricted control group
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HHV-6	Human herpesvirus 6
HIV-1	Human immunodeficiency virus 1
HO	Heme oxygenase
HO-1	Inducible form of HO present in all tissues. Induced during cellular stress.
HO-2	Constitutively expressed form of HO. Expressed mainly in vasculature and nervous system.
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
H/W	Han/Wistar (*Kuopio*) rat strain. The most TCDD-resistant laboratory animal known.
hw	H/W type; the deviant alleles of *AHR* and *B* genes from the H/W rat; mediates TCDD-resistance
HxCDD	1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin
IARC	International Agency for Research on Cancer
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-2	Interleukin 2
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRK	Insulin receptor tyrosine kinase
IRS-1	Insulin receptor substrate 1
LD50	Lethal dose 50%; the dose that is estimated to kill 50% of animals in an acute toxicity test
LDL	Low-density lipoprotein
L-E	Long-Evans (*Turku*/AB) rat strain. The most TCDD-sensitive rat strain known.
LOAEL	Lowest observed adverse effect level

MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
mRNA	Messenger RNA
MRP2	Multidrug resistance protein 2
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells; transcription factor which is activated upon T cell stimulation
NF-κB	Nuclear factor kappa B
Nrf2	NF-E2-related factor-2; redox-responsive transcription factor
PBGD	Porphobilinogen deaminase
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
Pgk 1	Phosphoglycerate kinase 1
PKC-βII	Protein kinase CβII
PKCδ	Protein kinase Cδ
PPAR	Peroxisome proliferator-activated receptor
PPO	Protoporphyrinogen oxidase
PXR	Pregnane X receptor
qRT-PCR	Quantitative real-time reverse transcription PCR
RB	Retinoblastoma protein
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
Ser/Thr/Tyr	Serine/Threonine/Tyrosine
SLC25A11	Mitochondrial 2-oxoglutarate/malate carrier protein
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; the most toxic congener of dioxins
TEF	Toxic equivalence factor
TEQ	Toxic equivalent quantity
TNF/D-GalN	Tumor necrosis factor/D-galactosamine
UCoS	Uroporphyrinogen III cosynthase
UGT1A1	Uridine diphosphate (UDP) glucuronosyltransferase 1A1
UROD	Uroporphyrinogen decarboxylase
U.S. EPA	United States Environmental Protection Agency
WHO	World Health Organization
wt	Wild type; the typical rat alleles of *AHR* and *B* genes
XRE	Xenobiotic response element

1 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), collectively called "dioxins", are a group of persistent environmental contaminants. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent dioxin congener, having also reputation as being the most toxic man-made chemical. Dioxins are formed in various combustion and industrial processes as unwanted by-products. They are widespread in the environment, good news being that the concentrations have been declining since 1970's mainly due to improvements in burning processes. Over 90% of human exposure to dioxins occurs via food; in Finland a major source is fatty fish from the Baltic Sea followed by meat and dairy products. Due to their ubiquitous presence, persistence, potent toxicity and ability to accumulate in the food chain, dioxins constitute an environmental hazard for human health. Vast amount of research on dioxins has been conducted during past decades, and a connection between several human health effects and dioxin exposure has been suggested, including cancer, developmental defects, endocrine and reproductive disturbances as well as metabolic diseases. The current background exposure levels probably do not increase cancer incidence and the more relevant concern is indeed comprised by developmental defects. In addition to low background levels to which all people are exposed, some severe industrial accidents have occurred causing higher human exposures to dioxins. Worth mentioning is also dioxins' presence as impurities in the herbicide Agent Orange during Vietnam war, due to which a large number of people are still suffering from serious developmental defects and other adverse health effects.

The mediator of dioxin effects in the body is the aryl hydrocarbon receptor (AHR). Binding of dioxin to this receptor affects the expression of hundreds or even thousands of genes. Therefore, dioxins are able to disturb many physiological processes. A characteristic feature of TCDD's toxicity is large sensitivity differences between species and even between the strains of the same species. The largest intraspecies difference is the 1000-fold sensitivity difference towards acute lethality of TCDD between Han/Wistar (*Kuopio*) and Long-Evans (*Turku*/AB) rats. The reason for the exceptional resistance of Han/Wistar rats is that they possess mutated allelles of *AHR*. In addition there is another, yet unknown gene *B*, affecting TCDD sensitivity. Despite huge amount of toxicological research on TCDD, the biochemical pathways leading to dioxin-induced lethality and other toxic effects are still insufficiently understood, which complicates risk assessment.

In this study, the pathogenesis and mechanism of a novel toxic effect of TCDD, hepatic biliverdin accumulation, has been addressed. This work started due to quite

dramatic observations of green/black livers in so called line B rats after TCDD exposure. Line B rats are intermediately TCDD resistant as they possess resistant alleles of unknown gene *B* influencing dioxin sensitivity. It was hypothesised that elucidating the biochemical mechanism of biliverdin accumulation would help in identifying gene *B* and thus benefit general understanding of the toxic mechanisms of TCDD. Biliverdin is an intermediate product in heme degradation and it has distinct green color. Normally biliverdin is rapidly turned to bilirubin, a yellow pigment, which is excreted. Cases of biliverdin accumulation are rare but existent in humans; in rats no previous cases were found in literature.

In order to clarify the mechanism of biliverdin accumulation, the obvious study objects (among others) were the enzymes responsible for heme degradation, heme oxygenase (HO-1) and biliverdin reductase (BVR-A). HO-1 is an essential, inducible enzyme involved in cellular defence against various stressful insults. It has been a subject of growing interest during past decades. Quite interestingly, during just a few past years, BVR-A has been recognized to participate in cellular signaling and regulation in complex ways. On the other hand, both bilirubin and biliverdin, "bile pigments", are physiologically important, possibly essential molecules due to their antioxidant and other biological activities. Therefore, in addition to the toxicological dioxin literature, the following literature review includes a look on heme metabolism. An overall theory is presented on how TCDD induces biliverdin accumulation.

2 Review of the literature

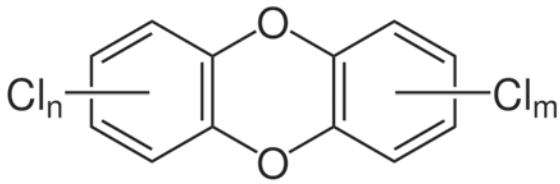
2.1 Overview to dioxins and their toxicity

2.1.1 General properties of dioxins

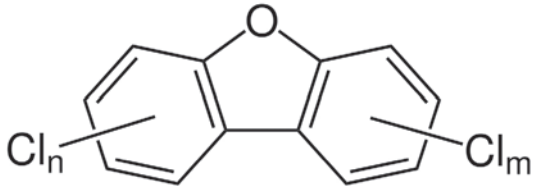
Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are persistent environmental toxicants collectively called as "dioxins" (Figure 1). Coplanar polychlorinated biphenyls (coplanar PCBs) have similar biological effects and thus are often classified as dioxins. Sometimes the term "dioxin" is used as a synonym of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent congener among the PCDD/Fs (Figure 2). When the biological effects of dioxins are investigated, TCDD is the most often used model compound.

The term congener refers to compounds with the same basic structure but different number of substituents. Altogether there are 75 possible PCDDs and 135 possible PCDFs. Chlorine at lateral positions 2, 3, 7 and 8 results in the characteristic "dioxin-like" effects. Hence, those 7 PCDDs and 10 PCDFs that have chlorine at positions 2, 3, 7 and 8, are toxicologically the most important. (Lindén et al. 2010; Poland and Knutson 1982; Safe 1986).

Dioxins are non-volatile, highly lipophilic molecules and resistant to chemical and biological degradation. Due to these properties dioxins are an environmental problem, as they bioaccumulate in the food chain ending into tissue fat of higher trophic level animals such as fish, birds, and mammals (Loonen et al. 1996). Dioxins are able to cross the placenta so the developing embryo or fetus may be exposed (Kreuzer et al. 1997). The lipophilicity and stability of dioxins increase with increasing number of chlorine substituents in the molecule. The half-lives of different PCDDs in adult humans vary from 5 to 13 years, for TCDD the half-life is approximately 7.2 years (Milbrath et al. 2009; Tuomisto et al. 2011). In infants, the half-life of TCDD is about 5 months (Kreuzer et al. 1997). Half-lives of dioxins are species-specific: in rats the half-life of TCDD is about 3 to 4 weeks (Rose et al. 1976).



PCDD



PCDF

Figure 1. General structures of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

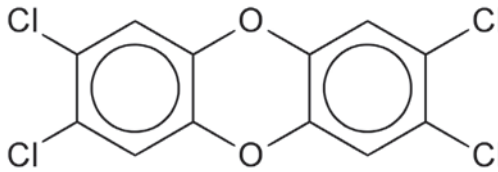


Figure 2. Chemical structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

2.1.2 Comparing the potency of dioxins – TEF concept

All 2,3,7,8-substituted PCDD and PCDF congeners induce similar biological effects through the AHR, although they are observed at vastly different doses. The potency differences are due to differences in pharmacokinetics and affinity to AHR (Poland and Knutson 1982; Simanainen et al. 2002). Toxic equivalence factor (TEF) is used to compare the potency of each congener to that of TCDD (Van den Berg et al. 1998, 2006). The TEF approach is based on following assumptions: the compounds must show a structural relationship to the PCDDs and PCDFs, bind the AHR, elicit AHR-mediated biochemical and toxic responses, be persistent and accumulate in the food chain. Typically, the rank order of potency is TCDD (TEF = 1) > 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (TEF = 1) > hexachlorodibenzo-*p*-dioxin (3 congeners;

TEF = 0.1) > 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (TEF = 0.01) > octachlorodibenzo-*p*-dioxin (TEF = 0.0003) (Van den Berg et al. 2006).

By summing the normalized TEF concentrations of each dioxin-like compound in a sample, total amount of dioxin-like compounds can be calculated and expressed as toxic equivalents (TEQs). Although TEF concept includes assumptions and limitations, it has turned out to be a useful approach in risk management of dioxin-like compounds (Safe 1997; Van den Berg et al. 2000).

2.1.3 Sources and human exposure

Dioxins have no industrial or other uses; instead they arise as unwanted by-products in various combustion and industrial processes where chlorine and metal catalysts are present. Main sources of dioxins include waste incineration in too low temperatures, metal smelting and refining, and chlorine bleaching of cellulose pulp. They also occur as impurities among PCBs, chlorophenols and some other chlorinated chemicals. Chemical accidents may result into formation of dioxins and lead to exposure of workers or nearby residents, as happened in Seveso, Italy, 1976, in context of explosion of a chemical plant producing trichlorophenol. (Bertazzi et al. 1998; Lindén et al. 2010).

Dioxins are widespread in the environment. Highest levels can be found in some soils, sediments and food of animal origin, whereas concentrations in plants, water and air are generally low. Dioxins are also invariably present in humans constituting so called “background exposure” (WHO 2010). Dioxin emissions have been decreasing since 1980’s due to improved waste incineration and industrial techniques (U.S. EPA 2006). It is probable that exposure of general human population to dioxins is continuously declining. For example, decrease of dioxin concentrations in mother´s milk has been observed during 1988 to 2007 (WHO 2009).

Food, especially fatty items, is the major source of human exposure to dioxins and PCBs contributing more than 90% of the average intake (Liem et al. 2000). Fish is a major source of dioxin intake in Finland, comprising about 72–94% of the total exposure (Kiviranta et al. 2004). The Baltic Sea is very contaminated with dioxins thus contributing to high dioxin levels in e.g. Baltic herring and salmon (Hallikainen et al. 2011). In some other European countries, dairy products, meat, fats and oils constitute the main exposure route. Average daily intake of dioxins in many countries is about 1 pg/kg/d (TEQ per b.w.) or about 50–100 pg/d per person (Tuomisto et al. 2011).

The WHO recommendation for tolerable daily intake level is 1 to 4 pg/kg/day (in TEQ per b.w.) during continuous exposure (WHO 2000). Breast-fed infants are a high exposure group of a special concern, as their exposure may be one to two orders of magnitude larger (Päpke 1998). Another high-exposure group is comprised by those consuming plenty of fish, especially Baltic herring and salmon. However, no adverse effects due to dioxin exposure could be found in fishermen having on average twofold higher dioxin blood levels as compared to general population; in contrast, decreased mortality and risk of cardiovascular diseases were observed (Turunen et al. 2008).

Although dioxin concentrations in the environment are declining, there are aspects that warrant solid follow-up of their presence in the environment and food items also in the future. Since dioxins are present everywhere, virtually all people are exposed to low amounts of them throughout life. This is somewhat alarming since margin of exposure in regard to some developmental and reproductive effects is very small (Miettinen 2006; U.S. EPA 2012; WHO 2000). Another issue is that there have been several cases of acute food safety problems due to dioxin-contaminated feed in some European countries and US during 1997-2011 (EU 2011; WHO 2010). In addition to potential human health risk, these kinds of incidents tend to have substantial economical effects (Covaci et al. 2008; Lascano-Alcoser et al. 2011).

2.1.4 Effects on humans

A possible link between exposure to dioxins/TCDD and multiple human health effects has been suggested, including developmental dental defects, cancer, cardiovascular disease, insulin resistance and diabetes, neurological symptoms, and endocrine and reproductive effects such as impaired semen quality, decreased male/female ratio of births, endometriosis and changes in neonatal thyroid function (Akhtar et al. 2004; Alaluusua et al. 1996, 1999, 2004; Baccarelli et al. 2008; Bertazzi et al. 1998, 2001; Birnbaum and Cummings 2002; Chang et al. 2010; Cranmer et al. 2000; Eskenazi et al. 2002, 2010; Feeley and Brouwer 2000; Lee et al. 2006; Mayani et al. 1997; Mocarelli et al. 2000, 2011; Pesatori et al. 2003, 2009; Rennert et al. 2012; Remillard and Bunce 2002; Steenland et al. 2004; Sweeney and Mocarelli 2000; Urban et al. 2007; Warner et al. 2011, 2013). Dioxins are prototypical endocrine disrupting compounds, that is, they cause adverse effects via affecting endocrine systems (Birnbaum 1995; Birnbaum and Fenton 2003; Mocarelli et al. 2008, 2011; WHO/IPCS 2002). After very high exposure to dioxins, as in cases of chemical accidents or intentional poisoning, a skin disease called chloracne is the characteristic consequence in humans (Geusau et al. 2001; Saurat et al. 2012). It is not known that any human being would have died due to acute effects of TCDD or other dioxins.

The most worrying health effects of dioxins in humans are possibly those on the development, as evidence exists that background exposure via mother´s milk can cause dental aberrations in children (Alaluusua et al. 1996, 1999). Although dioxin concentrations in the environment have declined, the concern of potential effects of even low doses during the fetal period or early childhood remains. Another concern has been the carcinogenic potential of dioxins. The International Agency for Research on Cancer (IARC) has classified TCDD as a class I human carcinogen (IARC 1997), however, the current background exposure levels are supposed to have minor or even negligible effect on cancer incidence or mortality (Tuomisto and Tuomisto 2012).

2.1.5 Effects on laboratory animals

TCDD exposure induces numerous toxic and adaptive responses in experimental animals. There are wide inter- and even intraspecies differences in the manifestations of dioxin toxicity. In terms of acute lethality, the most sensitive mammal is guinea pig and the most resistant hamster. In rats and mice, strains with widely different sensitivities towards acute lethality of TCDD exist. The mechanisms of intraspecies differences vary, but seem to involve aryl hydrocarbon receptor (AHR), a transcription factor and main mediator of dioxin toxicities. The complex features of dioxin toxicity make the risk assessment very challenging. (Lindén et al. 2010; Pohjanvirta and Tuomisto 1994).

The toxic responses to TCDD are dependent on many factors, such as dose, species, strain, age and gender of the animal, and the target tissue. Typical pathophysiological responses caused by TCDD in rats include thymic atrophy, immune dysfunction, wasting syndrome, mortality, hepatic damage and steatosis, increase in serum bilirubin, reproductive and endocrine effects, embryonic teratogenesis, and tumor promotion. (Birnbaum and Tuomisto 2000; Pohjanvirta and Tuomisto 1994; Poland and Knutson 1982; Uno et al. 2004). Some of these effects are seen in both sensitive and resistant rat strains, whereas others are seen mostly only in sensitive rats.

In mammals, the most sensitive responses to TCDD exposure seem to be increased susceptibility towards infections (Lindén et al. 2010; Pohjanvirta and Tuomisto 1994; Thigpen et al. 1975), male reproductive effects (Gray et al. 1997; Mably et al. 1992a,b,c) and effects on tooth development (Kattainen et al. 2001; Miettinen et al. 2002). In a recent study (Lensu et al. 2011), aversion to novel food items was shown to be another extremely sensitive endpoint of TCDD toxicity.

2.1.6 AHR, the principal mediator of dioxin toxicity

The principal mediator of the biological effects of dioxins and related aromatic hydrocarbons in the body is the cytosolic protein called aryl hydrocarbon receptor (AHR; Poland and Knutson 1982). AHR is a ligand-activated transcription factor belonging to Helix-Loop-Helix/Periodic, AHR nuclear translocator, Single-minded (bHLH/PAS) protein family. Several genes coding the phase I and phase II drug-metabolizing enzymes are direct targets of the AHR-driven transcriptional activation and have been named the AHR gene battery (Table 1; Nebert et al. 2000). In addition, TCDD has been shown to modify (either up- or downregulate) the transcription of several hundreds of genes in adult rat or mouse liver, and in human hepatoma cells, presumably in an AHR-mediated way (Boverhof et al. 2005, 2006; Boutros et al. 2008, 2011; Fletcher et al. 2005; Puga et al. 2000). Basically, transcriptional targets of AHR include genes involved in xenobiotic detoxification and homeostatic control of endobiotics, as well as genes involved in control of cell proliferation, differentiation and inflammation (Bock and Köhle 2009). Findings on AHR knockout mice support the role of AHR in normal vascular and hepatic development (Nguyen and Bradfield 2008). On the other hand, AHR knockout mice have proved to be unresponsive to all major effects of TCDD (Lindén et al. 2010). The role of AHR in biology and toxicology is a very wide theme recently covered by a book: Pohjanvirta (ed.) 2012.

Table 1. Typical target genes of AHR-mediated transcriptional activation (the AHR gene battery; Nebert et al. 2000)

Enzyme	Acronym
Cytochrome P4501A1	CYP1A1
Cytochrome P4501A2	CYP1A2
NAD(P)H:quinone oxidoreductase	NQO1
Glutathione transferase	GSTA1
UDP glucuronosyltransferase 1A6	UGT1A6
Cytosolic aldehyde dehydrogenase 3A1	ALDH3A1

2.1.6.1 Mechanism of AHR action

In the absence of ligand, AHR resides in the cytosol as a part of a multiprotein complex including heat shock protein 90 dimer, tyrosine kinase c-src, hepatitis virus X-associated protein 2, and co-chaperone p23 (Haarmann-Stemmann and Abel 2012). After ligand binding, AHR dissociates from its cytosolic partners and translocates into nucleus, where it forms a dimer with aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the bHLH/PAS-family. AHR-ARNT heterodimer binds to specific sites of genome called dioxin/xenobiotic responsive elements (DREs or XREs) situated upstream of target genes (Figure 3). Consequently, the expression of target genes is induced or sometimes repressed. A prototypical representative of this classical AHR-mediated signaling pathway is cytochrome P450 1A1 (CYP1A1) induction as a response to dioxin exposure (Lindén et al. 2010; Ma 2012).

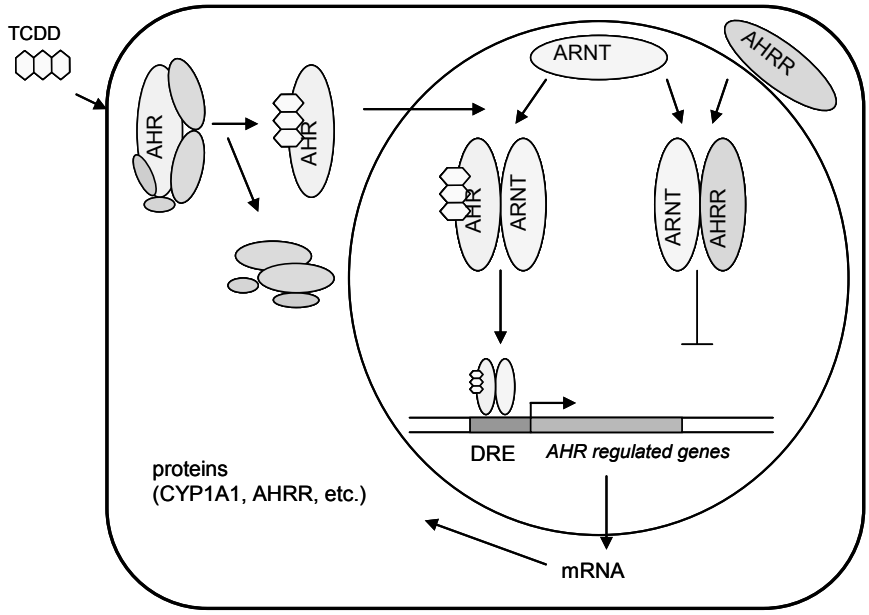


Figure 3. The classical pathway of AHR-mediated gene expression and negative feedback regulation by AHRR. (Illustration by M. Korkalainen; with permission).

In addition to the interactions with ARNT, AHR functions in protein-protein interactions with many other transcription factors. This crosstalk leads to mutual modulation of the ability of AHR and the interacting transcription factors to regulate the expression of their target genes. The best characterized crosstalk mechanisms

include interactions between AHR and aryl hydrocarbon receptor repressor (AHRR), estrogen receptor (ER), nuclear factor kappa B (NF-kB), retinoblastoma protein (RB)/E2F1 and specificity protein 1 (SP1). Physiologically these interactions may have consequences in the areas such as ER signaling, cell cycle progression and inflammatory response. (Swanson 2012).

So called non-genomic pathways of AHR signaling are also likely to exist as described by Matsumura (2012). One of such pathways results from the release of tyrosine kinase c-src upon ligand binding to AHR. The released c-src subsequently phosphorylates the epidermal growth factor receptor (EGFR) leading to downstream activation of mitogen-activated protein kinases (MAPKs), with further activation of the gene expression of proinflammatory enzymes. (Haarmann-Stemmann and Abel 2012).

2.1.6.2 AHR ligands

Various structurally diverse exogenous and endogenous compounds can bind to AHR and activate AHR mediated gene expression (Lindén et al. 2010; Nguyen and Bradfield 2008). Dioxins and PCBs, environmental contaminants of anthropogenic origin, are the most potent class of AHR ligands. Polycyclic aromatic hydrocarbons, including e.g. 3-methylcholantrene and benzo(a)pyrene, are another major group of contaminants that can bind AHR. However, they produce clearly milder effects than dioxins most probably due to their relatively rapid metabolic inactivation (Lindén et al. 2010).

Majority of naturally occurring AHR ligands are dietary plant-derived chemicals such as flavonoids. Most of them are relatively weak ligands of AHR (Nguyen and Bradfield 2008). An exception is indolo(3,2-b)carbazole present in some cruciferous vegetables, as it binds AHR strongly. However, it does not cause toxicity most probably due to its rapid metabolism (Pohjanvirta et al. 2002; Lindén et al. 2010). Mammalian endogenous compounds that can bind and activate AHR include e.g. tryptophan and its metabolites, indigo, indirubin, bilirubin, biliverdin, arachidonic acid metabolites such as lipoxin A4, several prostaglandins and retinoids (Gambone et al. 2002; Lindén et al. 2010; Seidel et al. 2001). On the other hand, an endogenous compound 7-ketocholesterol has antagonist effects on AHR-mediated gene expression (Savouret et al. 2001). The existence of a wide variety of compounds capable to bind and activate/inhibit AHR supports the view that AHR has multiple roles in normal physiology, embryonic development and adaptive response to xenobiotics, although no high-affinity endogenous ligand has yet been found. In some cases, AHR may be activated ligand-independently, e.g. via cyclic adenosine monophosphate (cAMP) or low density lipoprotein (LDL; Nguyen and Bradfield 2008).

2.2 Rat models

2.2.1 H/W and L-E rats

Han/Wistar (*Kuopio*; H/W; LD50 > 10,000 µg/kg) and Long-Evans (*Turku*/AB; L-E; LD50 10-20 µg/kg TCDD) rats are the most TCDD-resistant and the most TCDD-sensitive rat strains, respectively. They display the largest known intraspecies difference, about 1000-fold, in TCDD-induced acute lethality. This makes H/W rats the most TCDD-resistant mammals known, whereas L-E rats are slightly more sensitive than e.g. Sprague-Dawley rats (LD50 25-60 µg/kg, Beatty et al. 1978). The exceptional TCDD resistance of H/W rats is related to a mutated AHR allele (AHR^{hw}) and to another, unknown gene *B* allele (B^{hw}) (Pohjanvirta 1990, 1998; Tuomisto et al. 1999b). The AHR^{hw} allele possesses a point mutation that results in an abnormal splicing variant and C-terminus transactivation domain and a smaller AHR protein in H/W rats compared with the wild-type receptor present in TCDD-sensitive strains such as Sprague-Dawley and L-E (~98 kDa versus 106 kDa, respectively) (Elferink and Whitlock 1994; Pohjanvirta et al. 1998; Pohjanvirta et al. 1999). The identity of gene *B* has not yet been determined, but it may encode a protein participating in the AHR signaling pathway.

In H/W rats, the binding of TCDD to the AHR and the subsequent binding of the receptor complex to the DNA occur normally (Lindén et al. 2010). However, due to alternative splicing in the transactivation domain of the receptor protein, the initiation of the transcription of some, but not all, genes is ineffective. As a consequence, some responses, called Type I endpoints, of H/W rats to TCDD are same as in sensitive rat strains, whereas other responses are clearly attenuated and called Type II endpoints (Table 2; Lindén et al. 2010; Simanainen et al. 2002, 2003). Very little is known about the particular protein-protein interactions that are disturbed/destroyed due to abnormal transactivation domain of AHR^{hw}, subsequently leading to ineffective transcription of some normally TCDD-inducible genes. Gene *B* affects Type II endpoints but not Type I endpoints. It is noteworthy that an attenuated Type II effect (if measurable) occurs at similar doses in H/W rats as the respective normal effect occurs in L-E rats. (Simanainen et al. 2002; Tuomisto et al. 1999b).

Table 2. Classification of TCDD-induced biochemical and toxic responses into Type I and Type II endpoints. The latter (Type II) endpoints are sensitive to alterations in the transactivation domain of AHR whereas the former (Type I) endpoints are not.

Type	Effect	Reference
I	CYP1A1 induction	Unkila et al. 1993
I	Thymic atrophy	Pohjanvirta et al. 1989
I	Fetotoxicity	Huuskonen et al. 1994
I	Dental defects (incisor tooth defect, 3rd molar depletion)	Alaluusua et al. 1993; Kattainen et al. 2001; Simanainen et al. 2002
II	Acute lethality	Pohjanvirta et al. 1993
II	Hepatotoxicity	Pohjanvirta et al. 1989
II	Wasting syndrome	Pohjanvirta et al. 1989; Simanainen et al. 2002
II	Liver tumor promotion	Viluksela et al. 2000
II	Hyperbilirubinemia	Simanainen et al. 2002, 2003; Tuomisto et al. 1999b; Unkila et al. 1994
II	Increased serum tryptophan level, free fatty acids level, aspartate aminotransferase (ASAT) activity	Pohjanvirta et al. 1989; Simanainen et al. 2002, 2003; Unkila et al. 1994
II	Lipid peroxidation	Pohjanvirta et al. 1990

2.2.2 Rat lines A, B and C

The two H/W-type TCDD resistance genes, AHR^{hw} and B^{hw}, have been segregated into separate rat lines by crossbreeding H/W and L-E rats (Tuomisto et al. 1999b). The resultant three new rat lines are called A, B and C (Figure 4) and they differ vastly in their TCDD-resistance: Line A has the mutated AHR^{hw} allele and the wild-type *B* allele (genotype $AHR^{hw/hw}$ $B^{wt/wt}$) and appears to be as TCDD-resistant as H/W. Line C has the wild-type alleles of both genes (genotype $AHR^{wt/wt}$ $B^{wt/wt}$) and is almost as TCDD-sensitive as L-E. Line B expresses wild-type AHR but is intermediately TCDD-resistant due to H/W-type resistance alleles of gene *B* (genotype $AHR^{wt/wt}$ $B^{hw/hw}$). Since lines A and B have only one of the H/W type resistance genes (either AHR^{hw} or B^{hw}), it has been possible to study the effects of each resistance gene independently from the other. This has provided a novel approach for resolving mechanisms of dioxin toxicity.

Lines A, B and C are similar in respect to the ethoxyresorufin-O-deethylase (EROD) activity (marker for CYP1A1 induction), dental defects and thymic atrophy, which are typical Type I effects. Instead, Type II effects such as mortality, body weight

loss and increased serum bilirubin show strain-dependence, line C being the most sensitive, line B intermediately sensitive and line A the most resistant to these effects. This is in concordance with the observations in H/W and L-E rats and confirms the conception that TCDD-induced short-term toxicity involves at least two different AHR-mediated signaling pathways leading to Type I and Type II endpoints, the wild-type transactivation domain being critical only for the latter effects. (Simanainen et al. 2002, 2003; Tuomisto et al. 1999b).

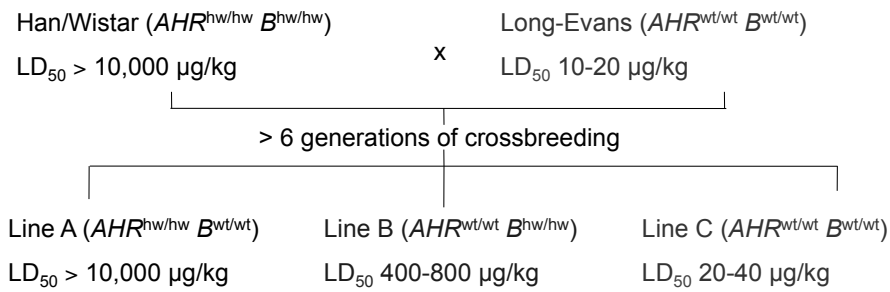


Figure 4. Crossbreeding of H/W and L-E rats, and the resultant novel rat lines A, B and C (Tuomisto et al. 1999b). *AHR* and gene *B* genotypes as well as LD50 values of TCDD are shown for each strain/line.

Line B and line C rats both express wild-type AHR. However, TCDD-induced Type II responses (e.g. mortality, wasting and hyperbilirubinemia) are weaker in line B than in line C rats. This implies that gene *B*, in addition to *AHR*, is somehow involved in the mechanistic pathways of these responses. Especially, B^{hw} allele seems to influence TCDD efficacy (magnitude of effect) for serum aspartate aminotransferase (ASAT) and bilirubin levels (Simanainen et al. 2003) but the reason for this is unknown.

Results from rats heterozygous in respect of both resistance alleles (genotype $AHR^{hw/wt}$ $B^{hw/wt}$) suggest that products of AHR^{hw} and B^{hw} interact in a way that increases resistance more than either heterozygous gene alone (Tuomisto et al. 1999b). Therefore, the product of B^{hw} could be a specific protein participating AHR signaling pathway. However, according to studies of Korkalainen et al. (2003, 2004) gene *B* is not the AHR dimerization partner ARNT, neither the negative regulator AHRR. Other possible candidates include, for example, the various coactivators interacting with the transactivation domain of AHR.

Some further information of the characteristics of gene *B* comes from the study where the temporal development of the resistance in line A and line B rats was studied. Both types of resistance were shown to develop postnatally (Simanainen et al. 2004). However, the time course of resistance development associated with AHR^{hw} and B^{hw} alleles is different. Line A rats are fairly resistant to TCDD already 2 days after birth and full resistance to acute lethality develops during the first week. In line B rats, complete resistance was gained at the age of 28 days in females and 42 days in males. Resistance associated with the B^{hw} allele starts to develop between days 14 and 28. Therefore, B^{hw} allele might be related to some physiological function that matures between the postnatal days 14 and 28 (Simanainen et al. 2004).

2.3 Selected features of (acute) dioxin toxicity

Acute dioxin toxicity in rats and other experimental animals includes numerous pathophysiological changes, most importantly, delayed mortality, wasting syndrome, hepatotoxicity and changes in clinical chemistry parameters. A short look is taken on some of these consequences, as well as on the TCDD-induced oxidative stress and the specific properties of HxCDD-induced acute toxicity, as these issues are essential background for *Results* and *Discussion*.

2.3.1 Acute lethality and wasting syndrome

Lethal dose of TCDD varies widely among animal species and strains, in addition, gender has a minor effect. Acute lethality caused by TCDD is typically delayed in nature, with the time to death from exposure being 2–5 weeks in rats (Pohjanvirta and Tuomisto 1994). Prior to death, animals reduce their feeding dramatically (a behaviour called hypophagia) and as a result, their body weight declines substantially, even by over 50%. Body fat stores and to some extent muscle mass are depleted. This phenomenon is known as the wasting syndrome. It is a rather uncommon manifestation of chemical toxicity. In addition to rats, TCDD-induced hypophagia and body weight loss have been reported in other animals such as mice, hamsters and guinea-pigs (Kelling et al. 1985; Lindén et al. 2010; Pohjanvirta and Tuomisto 1994).The biochemical basis of wasting syndrome is unclear; however, TCDD apparently leads to lowered body weight set point. Based on experiments including pair-fed rats, depletion of energy stores as a consequence of hypophagia is a major cause of acute mortality in dioxin-exposed rats. Yet other mechanisms must also exist since force-fed rats do not loose weight but still die at approximately same time after TCDD administration. (Lindén et al. 2010; Pohjanvirta and Tuomisto 1994).

From a biochemical point of view, wasting syndrome is associated with changes in energy metabolism including effects on gluconeogenesis, *de novo* fatty acid

synthesis, serum free fatty acids and triglycerides, as well as glycogen and fat storage in liver and adipose tissue, respectively (Croutch et al. 2005; Gorski et al. 1988, 1990; Muzi et al. 1989; Tuomisto et al. 1999a; Viluksela et al. 1999; Weber et al. 1991a,b). Reduced gluconeogenesis is probably due to inhibition of key enzymes of gluconeogenesis: phosphoenolpyruvate carboxykinase (PEPCK), pyruvate carboxylase and glucose 6-phosphatase (Weber et al. 1991a,b). Reduced gluconeogenesis leads to a lowering of blood glucose, reduction in plasma insulin and free insulin like growth factor (IGF-1) levels and activation of adenosine monophosphate-activated protein kinase (AMPK; Croutch et al. 2005).

At doses high enough to cause body weight loss, TCDD reduces circulating insulin levels in rats. Insulin, among other factors, is involved in the regulation of long-term energy balance but its possible role in wasting, if any, is undefined (Lindén et al. 2010). In addition to reduced gluconeogenesis, a possible cause for reduced insulin levels after TCDD exposure is impairment of the glucose-stimulated insulin secretion, an effect seen in isolated pancreatic islets from TCDD-treated rats (Novelli et al. 2005). On the other hand, the function of insulin may be enhanced by TCDD, as insulin sensitivity at the whole organism level has been shown to improve after TCDD administration in rats and mice (Lindén et al. 2010). This is in contrast with the observations in humans, where higher levels of dioxins and other endocrine disrupting compounds have been associated with insulin resistance (Alonso-Magdalena et al. 2011).

2.3.2 Hepatotoxicity of dioxins

A major target organ for TCDD toxicity in laboratory animals is liver (Boverhof et al. 2005, 2006; Chang et al. 2005; Goodman and Sauer 1992; Mann 1997; Pohjanvirta and Tuomisto 1994). Hepatic hyperplasia, fatty infiltration, and necrosis have been reported in several species (Birnbaum and Tuomisto 2000). In rats, the major features of TCDD´s hepatotoxicity are hepatocellular hypertrophy, multinucleate hepatocytes, steatosis and inflammatory cell infiltration. Abnormalities in hepatic plasma membrane and proliferation of both smooth and rough endoplasmic reticula have been reported (Pohjanvirta and Tuomisto 1994). Hepatotoxicity is also reflected by the altered serum transaminases, dehydrogenases and bilirubin (Birnbaum and Tuomisto 2000). Notable species and strain differences in TCDD´s hepatotoxicity exist: TCDD induces extensive hepatic necrosis in rabbit, whereas in rats and mice the effect is milder consisting of scattered, mainly centrilobularly located necrotic foci (Pohjanvirta and Tuomisto 1994). Further, H/W rats exhibit much milder liver lesion than L-E rats, hence, hepatotoxicity belongs to Type II dioxin effects (Pohjanvirta et al. 1989). TCDD decreases bile flow dose-dependently (Yang et al. 1977, 1983) and some dioxins other than TCDD have been shown to increase serum bile acid concentrations (Brewster et al. 1988; Couture et al.

1988). Bile acids are well-known hepatotoxicants and potent suppressors of PEPCK expression, therefore, altered bile acid synthesis and transport might contribute to TCDD-induced hepatotoxicity (Fletcher et al. 2005).

The wide range of hepatic effects may have its basis in the extensive changes in hepatic gene expression after TCDD exposure. For example, in adult rat or mouse liver, hundreds or even thousands of genes are affected. Both induction and repression of gene expression are consequences of TCDD exposure (Lindén et al. 2010). TCDD causes hepatic accumulation of porphyrins, especially uroporphyrin, in rats and mice (Goldstein et al. 1973, 1982). However, in rats, porphyria may occur predominantly during subchronic or chronic exposures and is not a major response during acute exposure. See the Section 2.7.2 for this effect.

In rats and other rodents, long-term TCDD treatment leads to the development of tumors of many sites, including liver. Female rats seem to be more prone to tumor-promoting activity of TCDD and this effect has been used as a basis for quantitative cancer risk assessment for TCDD. However, TCDD does not behave like a complete carcinogen (Knerr and Schrenk 2006; Viluksela et al. 2000). L-E rats are about 100-fold more sensitive than H/W rats to the tumor promoting effect of TCDD (Viluksela et al. 2000). Altered metabolism of endogenous molecules such as estradiol can lead to the formation of quinones and redox cycling. This has been hypothesized to play a role in the enhanced sensitivity of female rats to dioxin-induced liver tumors (Birnbaum and Tuomisto 2000).

2.3.2.1 Fatty liver

TCDD is known to cause fatty degeneration (steatosis) of the liver in rats and other species (Birnbaum and Tuomisto 2000; Poland and Knutson 1982). Fat is depleted from the white adipose tissue and concurrently accumulated in the liver (Pohjanvirta et al. 1990). However, it is unlikely that the mobilization of the peripheral adipose stores would alone cause fatty liver, since in a study on L-E rats, the TCDD-treated rats accumulated fat in the liver whereas the pair-fed controls did not (Pohjanvirta et al. 1990). Instead, the probable principal reason for fatty liver is the altered function of hepatocytes due to TCDD administration. This is supported by the reports of Kawano et al. (2010) and Lee et al. (2010) suggesting that hepatic steatosis induced by AHR activation is accompanied by upregulated CD36/fatty acid translocase and peroxisome proliferator-activated receptor (PPAR) expression, resulting in the upregulation of fatty acid uptake and accumulation of lipids in the liver. In addition, AHR activation was shown to result in inhibition of hepatic export of triglycerides and suppression of fatty acid oxidation, thus further favouring the development of steatosis (Lee et al. 2010).

2.3.3 TCDD-induced oxidative stress

Oxidative stress refers to an imbalance between the production and manifestation of reactive oxygen species (ROS), and the ability of a biological system to readily detoxify them or to repair the resulting damage. Oxidative stress is a well known consequence of TCDD exposure in rodents (Reichard et al. 2005; Senft et al. 2002, Shertzer et al. 1998; Stohs 1990; Stohs et al. 1990). A variety of mechanisms are suggested to be responsible for the TCDD-induced oxidative stress, as TCDD both reduces the expression levels of protective antioxidant enzyme systems, and induces the production of excessive amounts of ROS. Microsomes, mitochondria and leukocytes are typical sources of ROS following TCDD exposure (Latchoumycandane et al. 2003; Reichard et al. 2005; Stohs 1990; Wong and Cheng 2011). Types of ROS produced in response to TCDD exposure include superoxide anion, hydrogen peroxide, and possibly hydroxyl radical (Hassoun et al. 2003; Senft et al. 2002; Shertzer et al. 1998; Stohs et al. 1990).

The oxidative stress generated by TCDD seems to be dependent on the AHR genotype (Alsharif et al. 1994; Mohammadpour et al. 1988). L-E vs. H/W rat model supports this, as hepatic lipid peroxidation, an indicator of oxidative stress, was induced by TCDD dose-dependently in L-E but not in H/W rats (Pohjanvirta et al. 1990). In concordance with this, subtle induction of HO-1, an enzyme easily induced by oxidative stress (Alam and Cook 2003), was seen in L-E rats but not in H/W rats after TCDD exposure (Boutros et al. 2011). HO-1 has also been shown to be increased in hepatic macrophages of Sprague-Dawley rats dosed with 2 or 4 μg/kg TCDD (Nishimura et al. 2001). Most probably, oxidative stress can be classified as Type II dioxin effect, as it seems to be alleviated in rats resistant to acute toxicity of TCDD due to altered AHR.

Other indicators of oxidative stress include e.g. increased hepatic and macrophage deoxyribonucleic acid (DNA) damage, increased urinary excretion of malondialdehyde, decreased hepatic membrane fluidity, and decreased glutathione, nonprotein sulfhydryl and nicotinamide adenine dinucleotide phosphate (NADPH) contents of liver (Stohs 1990). For example, Stohs et al. (1990) showed that TCDD exposure first increased but later at 12 days decreased cytosolic NADPH content by 34% in female Sprague-Dawley rats, total decrease being even 50–70%. Antioxidants may alleviate, but not totally eliminate, the toxicity of TCDD. (Ciftci and Ozdemir 2011; Hassan et al. 1985; Pohjanvirta et al. 1990).

The precise role of the oxidative stress in the expression of the toxic manifestations of TCDD has not been clearly determined to date, but it may be relevant e.g. for the carcinogenic potential of dioxins (Lindén et al. 2010; Shertzer et al. 1998). According to a recent review by Wong and Cheng (2011), oxidative stress induced

by dioxin and some other environmental toxicants may be an important factor behind male reproductive dysfunction. The increase in oxidative stress disrupts the cell junctions between testicular cells leading to deleterious effects on male reproductive capability (Wong and Cheng 2011). TCDD has been shown to induce oxidative stress in epididymis of adult rats (Latchoumycandane et al. 2003). In addition, decreased epididymal sperm levels in F1 male rats is one of the most sensitive adverse effects of TCDD following a maternal dose of 0.064-1.0 µg/kg (Bell et al. 2010; Mably et al. 1992a,b,c).

2.3.4 Specific properties of HxCDD-induced toxicity

The potency of 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD) is about 10% of that of TCDD for typical AHR-mediated effects (TEF = 0.1; Van den Berg et al. 2006). Among some other hexa-substituted dioxins and furans, it belongs to the most toxic dioxin congeners after TCDD and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (for both, TEF = 1). For all mammals, as well as for avian and fish species studied so far, TEFs seem to be valid also for lethality (Van den Berg et al. 1998, 2000). Surprisingly, H/W rats have been shown to be more sensitive to HxCDD than to TCDD in terms of acute lethality (Table 3; Pohjanvirta et al. 1993), LD50 value of HxCDD being 2530 µg/kg for female H/W rats. For some characteristic short-term effects, the normal rank order of sensitivity holds even in H/W rats with TCDD being the most potent congener (Pohjanvirta et al. 1995; Simanainen et al. 2002). For L-E rats, the typical rank order between TCDD and HxCDD holds also for acute lethality, LD50 value of HxCDD being 132 µg/kg for female L-E rats (Table 3; Pohjanvirta et al. 1995).

Table 3. LD50 values of HxCDD and TCDD for female rats (Pohjanvirta et al. 1993, 1995). Highlighted value indicates the most potent congener in each case.

Rat strain	LD50 (µg/kg TCDD)	LD50 (µg/kg HxCDD)
H/W	>9600	2530
L-E	10	132

It has been hypothesised that the mechanism of acute lethality caused by large doses of HxCDD is different from the prime, canonical AHR-mediated action mechanism of dioxin-like compounds (Pohjanvirta et al. 1995). This second mechanism can only be detected using animals highly resistant to the prime mechanism, such as H/W rats. Other highly chlorinated dioxins with a relatively low affinity to AHR such as 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin may share this alternative toxic

pathway with HxCDD, but it appears to be especially characteristic to HxCDD (Pohjanvirta et al. 1995).

Interestingly, in a study where the levels of 121 endocrine disrupting compounds were studied in breast milk samples from Denmark and Finland, HxCDD, as one of six chemicals out of 121, and the only PCDD/F, was found to have significantly higher levels in Danish than in Finnish samples. In less stringent statistical analysis also TCDD and many other endocrine disrupting compounds exhibited higher levels in Denmark than in Finland. It remains to be clarified, whether these findings are somehow linked to much higher incidence of male reproductive disorders in Denmark than in Finland (Krysiak-Baltyn et al. 2010).

2.4 Heme and its synthesis

Heme is a complex of protoporphyrin IX and ferrous iron (Figure 5). It is a functionally essential prosthetic group of various proteins. These so called *hemoproteins* have diverse biological functions including transportation and storage of diatomic gases (as exemplified by hemoglobin and myoglobin), chemical catalysis (cytochrome P450 superfamily, catalase, peroxidases, etc.), diatomic gas detection (neuronal PAS2, soluble guanylyl cyclase), and electron transfer (e.g. cytochromes C, B5 and B558). In addition, heme acts as a cellular messenger via heme-regulated proteins such as Bach1 and δ-aminolevulinic acid synthetase (ALAS1). (Furuyama et al. 2007; Gozzellino et al. 2010).

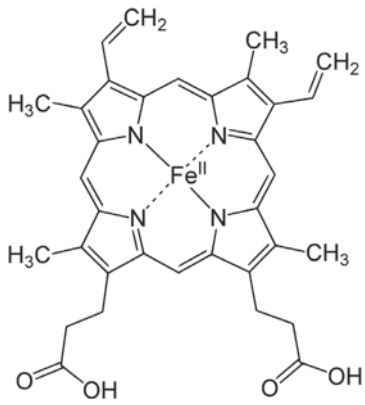


Figure 5. Chemical structure of heme.

Heme is a necessary molecule for all aerobic organisms. The two largest pools of hemoproteins in the body are hemoglobin in red blood cells and myoglobin in muscles. According to some estimates about 80-85% of total heme of the body is in

hemoglobin, 12-17% in myoglobin and 3% in other hemoproteins. (Gozzellino et al. 2010; Ponka 2003; Shibahara et al. 2002). Heme is synthesized virtually in all tissues, but the principal sites are erythroid cells (about 85%) and hepatocytes (nearly all the rest; Ponka 1997).

In mammals, eight enzymes are involved in heme biosynthesis (Figure 6). δ-Aminolevulinic acid synthetase (ALAS) is the first and the rate-limiting enzyme of this pathway and it exists as two isozymes. ALAS1 (hepatic or non-specific ALAS) is expressed in all tissues, whereas the expression of ALAS2 is restricted to erythroid cells. Both ALAS isozymes catalyze the formation of δ-aminolevulinic acid from glycine and succinyl coenzyme A (succinyl CoA) in mitochondria. δ-Aminolevulinic acid is transported to cytosol, where the subsequent four enzymatic steps take place. The resultant coproporphyrinogen III is transported back into mitochondria by the transport protein ATP-binding cassette sub-family B member 6 (ABCB6) or mitochondrial 2-oxoglutarate/malate carrier protein (SLC25A11), and the last three steps of heme biosynthesis occur in mitochondria. Finally, heme molecule is transported to cytosol by an unknown heme transporter. (Furuyama et al. 2007).

Free heme is a potent pro-oxidant due to its reactive iron atom that can catalyze the formation of hydroxyl radical from hydrogen peroxide. Since free heme concentration exceeding 1 μM is toxic, intra- and extracellular free heme levels must be precisely maintained. Intracellular control is achieved through the regulation of the synthesis and catabolism of heme. Extracellular, systemic free heme levels are maintained at non-toxic area by help of specific plasma proteins such as haptoglobin and hemopexin, which bind cell-free hemoglobin and free heme, respectively. Hemoglobin-haptoglobin and heme-hemopexin complexes are recognized and taken up via endocytosis by e.g. macrophages and liver parenchymal cells. (Eskew et al. 1999; Furuyama et al. 2007; Gozzellino et al. 2010; Sassa 2004).

Heme itself has the capability of determining the rate of its synthesis and degradation. Heme regulates ALAS1 expression in a negative feedback manner (Furuyama 2007). On the other hand, free heme induces the heme-degradative enzyme HO-1 (see the next section) and thus enhances its own degradation. A number of mechanisms are suggested to be involved in the negative feedback regulation of ALAS1 expression by heme. These include reduction of transcription (Yamamoto et al. 1982, 1988) and translation (Sassa and Granick 1970; Yamamoto et al. 1983), destabilisation of mRNA (Hamilton et al. 1991) and inhibition of mitochondrial transport of precursor protein (Yamauchi et al. 1980). ALAS2 expression is not suppressed by heme as large amounts of heme are needed for the efficient production of hemoglobin during erythroid differentiation.

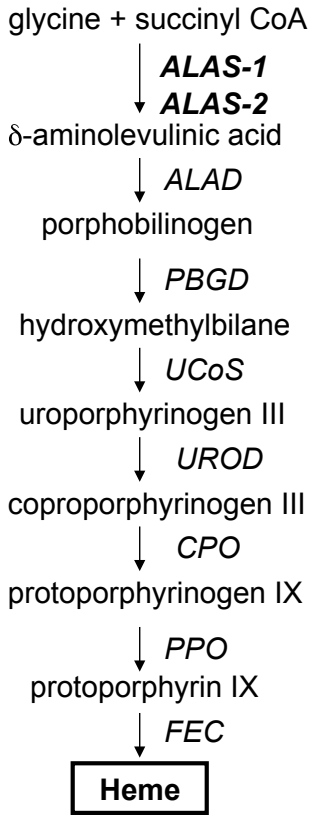


Figure 6. Heme synthesis pathway. Rate-limiting enzymes are in bold. Abbreviations: ALAD, δ-aminolevulinic acid dehydratase; ALAS, δ-aminolevulinic acid synthetase; CPO, coproporphyrinogen oxidase; FEC, ferrochelatase; PBGD, porphobilinogen deaminase; PPO, protoporphyrinogen oxidase; UCoS, uroporphyrinogen III cosynthase; UROD, uroporphyrinogen decarboxylase.

2.5 Heme degradation

Heme degradation is a two-step process, where heme oxygenase (HO) first cleaves the porphyrin ring of heme to a linear tetrapyrrole biliverdin. Subsequently, biliverdin is rapidly reduced to bilirubin by biliverdin reductase (BVR; Figure 7). These compounds have characteristic colors: heme is red, biliverdin blue to green and bilirubin yellow. Biliverdin is water-soluble whereas bilirubin is lipophilic. (McDonagh et al. 2001).

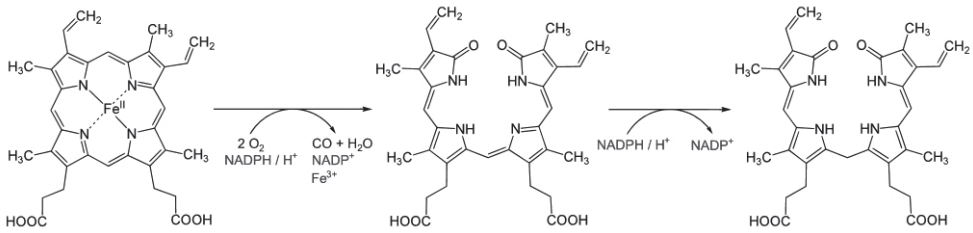


Figure 7. Degradation of heme. BVR, biliverdin reductase; HO, heme oxygenase. (Modified from Wikimedia Commons).

A majority, about 80% of plasma bilirubin originates from hemoglobin heme, the rest coming from myoglobin and other hemoproteins (Shibahara et al. 2002). Spleen is the major site of destruction of senescent erythrocytes, followed by liver and bone marrow. HO and BVR are ubiquitous enzymes, and apparently most, if not all, aerobic cells degrade at least small amounts of heme. The newly formed bilirubin is bound to plasma albumin and transferred to liver. As a lipophilic compound, it may enter hepatocytes by diffusion but other mechanisms may exist as well (Kamisako et al. 2000). In the hepatocyte, uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) adds two equivalents of glucuronic acid to bilirubin to produce the more water soluble bilirubin diglucuronide, which is subsequently excreted into bile. Due to their characteristic colors and presence in bile, bilirubin, biliverdin and their derivatives are often referred to as *bile pigments*. In medicine, heme synthesis and degradation processes are important due to various associated disorders such as porphyrias and neonatal as well as other hyperbilirubinemias.

Notably, bile pigments are not simply by-products of heme catabolism, but physiologically important, possibly essential molecules. This is due to a wide range of biological activities assigned to them during the last years and decades. Most importantly, bilirubin (and to some extent also biliverdin) is an efficient antioxidant, but bile pigments possess also anti-inflammatory, anti-viral, anti-apoptotic and anti-mutagenic properties (Bulmer et al. 2008; Stocker et al. 1987; Stocker 2004).

2.5.1 Heme oxygenase (HO)

Heme is oxidatively cleaved to biliverdin by the rate-limiting enzyme heme oxygenase which was first described by Tenhunen et al. (1968). Due to high regiospecificity of the reaction, biliverdin IXα is the predominant biliverdin isomer formed in vertebrates. Heme oxygenases are an evolutionarily conserved family of

enzymes found in bacteria, algae, plants and animals. Two catalytically active, structurally related forms exist, the inducible HO-1 and the constitutively expressed HO-2. In addition, HO-3 isoform with negligible catalytic activity has been identified. HO-1 and HO-2 have different expression profiles: while HO-2 is a constitutive enzyme expressed mainly in vasculature and nervous system, HO-1 responses to stress in all tissues. Induction of HO-1 is essential for cellular protection against various stressful situations, as evidenced by the severity of the few animal and human cases where HO-1 is lacking (Foresti et al. 2004; Gozzellino et al. 2010; McDonagh 2001; Poss and Tonegawa 1997; Radhakrishnan et al. 2011; Shibahara et al. 2002; Tenhunen et al. 1968; Yachie et al. 1999).

In addition to biliverdin, equimolar amounts of carbon monoxide (CO) and ferrous iron are released in the reaction catalyzed by HO (Shibahara et al. 2002). Biliverdin is then rapidly converted to bilirubin by biliverdin reductase. All products of heme degradation are biologically active. CO is a vasoactive molecule and a potential neural messenger; in addition it may possess anti-inflammatory, anti-apoptotic and anti-proliferative effects (Foresti et al. 2004). Free ferrous iron (Fe^{2+}) leads to rapid upregulation of iron-sequestering protein ferritin, but iron has also many other regulatory functions, including activation of specific genes (Foresti et al. 2004; Öllinger and Pratscke 2010). Importantly, free iron, like free heme, is a potent pro-oxidant, and for this reason its amount in cells must be strictly regulated. Iron is either stored in ferric state within ferritin or it is transported to bone marrow to be recycled for heme synthesis in erythroid cells (Gozzellino et al. 2010; Shibahara et al. 2002). Biological activities of biliverdin and bilirubin are covered in the next sections (2.5.2–2.5.4).

The expression level of HO-1 is normally low in cells other than macrophages, but various initiators of stress markedly activate HO-1 in virtually all cell types (Abraham and Kappas 2008; Foresti et al. 2004; Poss and Tonegawa 1997). These include e.g. hyperthermia, heme, heavy metals, oxidized lipoproteins, ultraviolet irradiation, hydrogen peroxide, inflammatory cytokines, and hypoxia (Alam and Cook 2003; Choi and Alam 1996; Ewing and Maines 1991; Murphy et al. 1991; Poss and Tonegawa 1997; Siow et al. 1995). TCDD exposure has been shown to induce HO-1 in the liver of L-E rats and hepatic macrophages of Sprague-Dawley rats (Boutros et al. 2011; Nishimura et al. 2001). The ability of HO-1 to respond to many stress signals is probably associated with the various stress-activated regulation elements within the HO-1 promoter, including activator protein 1 (AP-1) sites, CCAAT/enhancer-binding protein sites, phorbol ester response elements, heme response elements, and antioxidant response elements (Alam and Cook 2003; Inamdar et al. 1996). Further, several xenobiotic response elements (XREs) are present in the regulatory region of HO-1 but they have not been functionally

characterized (Bock and Köhle 2010). Characteristic features in the regulation of HO-1 expression are remarkable interspecies and inter-individual differences. In some specific situations, repression of HO-1 activity may be protective; especially, this may be a defense strategy developed in humans (Shibahara et al. 2003).

Altogether, heme catabolism by HO-1 is an important cellular protection mechanism against oxidative and other stressful insults. Active HO-1 diminishes the amount of the pro-oxidant heme, and, on the other hand, the reaction products are strongly antioxidant, inhibit cell death, apoptosis, and aberrant proliferation (Gozzellino et al. 2010; Idriss et al. 2008). Direct evidence for the protective role of HO-1 comes from the study of Poss and Tonegawa (1997), which showed that oxidation of macromolecules and tissue injury arise spontaneously in genetically modified mice deficient of HO-1. Further, the two known humans with verified HO-1 deficiency suffered from severe symptoms such as endothelial injury and general inflammation and eventually died at the age of 6 and 15 years (Radhakrishnan et al. 2011; Yachie et al. 1999).

In recent years, HO-1 has received attention as a potential therapeutic target. HO-1 was found to be up-regulated in disease models such as endotoxemia and ischemia in rodents (Rizzardini et al. 1994; Takeda et al. 1994) and in pathological states such as atherosclerosis, acquired immunodeficiency syndrome, diabetes mellitus and Parkinson´s and Alzheimer´s disease in humans (Alam and Cook 2003; Abraham and Kappas 2008). It has been suggested that heme degradation pathway, especially the ratio of HO-1 and BVR-A, might be a promising serum biomarker source for the early detection of Alzheimer´s disease (Mueller et al. 2010). HO-1 has complex effects on apoptosis and cell cycle in carcinogenesis, therefore, HO-1 is a possible target for anti-cancer treatment (Fang et al. 2004). In humans, expression of the HO-1 gene is modulated by two functional polymorphisms in the promoter. A short dinucleotide repeat (<25 GT) in the HO-1 gene promotor is associated with enhanced transcriptional activity whereas the longer dinucleotide repeat has been associated with increased susceptibility to atherosclerosis and some lung diseases, however, contradictory results exist (Abraham and Kappas 2008; Kikuchi et al. 2005; Yamada et al. 2000; Yasuda et al. 2006). Induction of HO-1 has been shown to be protective in transplantation in many experimental models but it is yet unclear whether this property can be utilized clinically (Öllinger and Pratscke 2010).

2.5.2 Biliverdin

In vertebrates, biliverdin is continuously produced from catabolism of senescent or damaged erythrocytes and of the heme released during turnover of cytochrome P450 enzymes and other heme containing proteins (Shibahara et al. 2002). Some vertebrates – birds, amphibians and reptiles – excrete biliverdin from the liver

directly into bile, but most mammals reduce most or all of it regiospecifically to bilirubin IXα. Biliverdin has a wide occurrence in the animal kingdom. It occurs e.g. in the plasma of many marine fish, in the blood of tobacco hornworm, the wings of moth and butterfly, the shell of bird eggs, the serum and egg of frog, the placenta of dog (Fang and Bada 1990), the milk of buffalo and sheep (Jandal 1995; Kumar et al. 1983), and in human amniotic fluid (Krasner et al. 1969, 1971). In addition, biliverdin is found in plants, cyanobacteria and algae as a biosynthetic precursor for essential photoresponsive bilin pigments. Biliverdin is also used for camouflage or possibly photoprotection in insects and other lower organisms (McDonagh 2001). In normal human plasma, biliverdin was long unrecognized, but according to a recent report where sensitive analysis methods were used, its plasma level in control women was assessed to be 0.12 μM (Nytofte et al. 2011). Higher plasma values (0.9–6.5 μ M) were reported for normal healthy individuals by Gåfvels et al. (2009). Conjugated biliverdin has been found in *post mortem* bile of humans, and it is a major pigment in rabbit bile (Garay et al. 1965; Muñoz et al. 1986). However, it is unclear whether conjugated biliverdin is a product of the enzymatic conjugation of free biliverdin or a result of the oxidation of bilirubin conjugates (Garay et al. 1965; Noir et al. 1965). According to Garay et al. (1965), in many animal species conjugation of both biliverdin and bilirubin may occur simultaneously. Conjugated biliverdin was recently found also in normal human serum, and its level was increased in patients suffering from hepatocellular carcinoma or cirrhosis (An et al. 2010).

In human blood, bilirubin is bound with serum albumin, which prevents its toxic effects (Ahlfors 1981; Blauer and Wagniere 1975). Also biliverdin binds to albumin to the same binding site, yet somewhat less tightly than bilirubin (Ahlfors 1981). Concordantly, in serum of fish and frogs as well as in eggs of frogs, biliverdin is tightly bound to a carrier protein, although the nature of this binding is different from that in humans (Fang and Bada 1990). Tight binding to proteins explains why biliverdin is retained in serum of certain marine fish. The concentration of biliverdin in some fish species has been reported to be 1.1–6 mg/100 ml (= 19–103 μM; Fang and Bada 1990). These are extraordinary high concentrations in comparison with other vertebrates.

All natural biliverdins seem to be formed by ring cleavage of the heme molecule, instead of being synthesized from smaller units (McDonagh 2001). Depending on at which carbon bridge the cleavage occurs, four structural isomers are possible; IXα, IXβ, IXγ and IXδ. In vertebrates biliverdin IXα is the predominant type of biliverdin, however, very small amounts of the IXβ isomer and even smaller amounts of the IXγ and IXδ isomers are produced in humans and other mammals. Due to conformational flexibility and depending on the environment, the color of

biliverdins can vary from blue to green. Biliverdin is easily excreted (McDonagh 2001). For this reason, it has been wondered why biliverdin is reduced in an energy consuming reaction to lipophilic, not excretable bilirubin in mammals. At least two possible explanations exist. One is related to the development of placental animals, since McDonagh et al. (1981) showed that only bilirubin, not biliverdin, can readily pass through the placenta between fetus and the mother. This is supported by the fact that many non-mammalian vertebrates such as several birds, reptiles and amphibians, predominantly excrete unconjugated biliverdin (Himes and Cornelius, 1975; Lin et al. 1974; McDonagh et al. 1981). Another benefit resulting from the reduction of biliverdin to bilirubin is the potent antioxidative capacity of bilirubin (Doré et al. 1999; Stocker et al. 1987; Stocker 2004).

2.5.2.1 Biological effects of biliverdin

In normal mammalian metabolism, biliverdin is rapidly and quantitatively reduced to bilirubin as the responsible enzyme, biliverdin reductase, is ubiquitously present in tissues (McDonagh 2001). Because of this rapid conversion of biliverdin to bilirubin it is difficult to identify a function that is exclusive to biliverdin. Nevertheless, various biological effects have been assigned on biliverdin (Table 4). Like bilirubin, also biliverdin is a potent antioxidant via its peroxyl radical scavencing activity. Often the action of biliverdin is inhibitory in nature. For example, it is an inhibitor of protein kinase Cδ (PKCδ), a suppressor of alloimmune responses, and it interferes with virus replication (Table 4). Biliverdin has been shown to function as a ligand of the AH receptor (see Section 2.6.1).

Biliverdin may also have a critical function in embryogenesis. It is necessary for the proper dorsal axis development in *Xenopus laevis* embryos (Falchuk et al. 2002), suggesting that biliverdin is a gene regulator. It is present in the cytoplasm of the oocyte, eggs and stage 1 embryos affecting mRNA synthesis (Falchuk et al. 2002). However, in addition to the reports of Falchuk et al. (2002) and Montorzi et al. (2002), no further studies were found that would confirm the effects of biliverdin on embryogenesis. Biliverdin is also present in human amniotic fluid peaking at about 16 weeks (Krasner et al. 1969, 1971). This probably reflects the gradual development of fetal heme catabolism.

2.5.2.2 Green jaundice and biliverdinaemia in humans

The yellowish skin/tissue color characteristic to jaundice is caused by bilirubin. A form of jaundice exists where the color of skin is green instead of yellow. This is usually a sign of biliverdinaemia, that is, elevated levels of biliverdin in serum. (Gåfvels et al. 2009; Huffmann et al. 2009; Larson et al. 1947; Tickner and Gutteridge 1978).

Table 4. Biological effects of biliverdin.

Effect of biliverdin	Species where studied	Reference
Antioxidant (peroxyl radical scavenger)	*In vitro*	Stocker 2004
Anti-mutagen	*Salmonella* mutagenicity test; cultured mammalian cells	Bulmer et al. 2007
Confers resistance against chemoterapeutics	Human pancreatic cancer cells	Nuhn et al. 2009
Determinant factor of dorsal axis development	*Xenopus laevis*	Falchuk et al. 2002; Montorzi et al. 2002
Inhibitor of protein kinase Cδ (PKCδ)	*In vitro*	Gibbs et al. 2012
Inhibits Toll-like receptor-4 expression	*In vitro* (mouse macrophages)	Wegiel et al. 2011
Protects against acute liver damage in mouse TNF/D-GalN model (BVR and eNOS required)	Mouse	Wegiel et al. 2011
Endogenous ligand and activator of AH receptor	Human, rat and guinea pig cell lines	Phelan et al. 1998
Inhibitor of virus replication (hepatitis C, HIV-1, HHV-6)	*In vitro* (various cell lines)	Lehmann et al. 2010; Mori et al. 1991; Nakagami et al. 1992; Zhu et al. 2010
Suppressor of alloimmune responses (Prolongs cardiac allograft survival. Synergistic effect with CO.)	Mouse	Yamashita et al. 2004
Suppressor of IL-2 and IFN-γ production through inhibition of NFAT and NF-κB activation (i.e., nuclear translocation).	*In vitro* (mouse splenocytes)	Gibbs and Maines 2007; Yamashita et al. 2004
Suppressor of T-cell proliferation	*In vitro* (mouse splenocytes)	Yamashita et al. 2004
Elevates hepatic DNA synthesis and mitotic index in hepatotectomized rats	Rat i.p.	Okazaki et al. 1978
Activator of ornithine decarboxylase in liver	Rat	Matsui et al. 1979
Tumor promoter: promotes neoplastic cell formation in liver epithelial cells after they	*In vitro* (liver epithelial cells)	Lafarge-Frayssinet et al. 1983

are pre-incubated with aflatoxin B1 (This effect is possibly related to the up-regulation of ornithine decarboxylase activity by biliverdin.)		

Abbreviations: AH, aryl hydrocarbon; BVR, biliverdin reductase; CO, carbon monoxide; eNOS, endothelial nitric oxide synthase; HIV-1, human immunodeficiency virus-1; HHV-6, human herpesvirus 6; IL-2, interleukin 2; IFN-γ, interferon gamma; i.p., intraperitoneally; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B; TNF/D-GalN, tumor necrosis factor/D-galactosamine.

Whereas jaundice is a common clinical finding, green jaundice is a rare condition (Huffmann et al. 2009). Green jaundice and biliverdinaemia have been described only in humans. Although some fish species may have extremely high serum levels of biliverdin, it belongs to their physiology (Fang and Bada 1990). The only reported animal cases found in literature which might be interpreted as abnormally high levels of biliverdin are the situations where the bile color of cod and salmon changed from yellow to green under starvation. It is also known that fish such as *Anguilla japonica* (freshwater eel) deactivate the enzymes BVR and uridine diphosphate glucuronosyltransferase (UGT) during starvation and excrete unconjugated biliverdin directly into bile (Fang and Bada 1990). This skipping of the reduction of biliverdin is suggested to be a way of preserving energy under conditions of malnutrition.

In 1947, Larson et al. studied a large number of jaundice patients for the presence of biliverdin. On the basis of this study and other reports on green jaundice or biliverdinaemia from the past 70 years, it seems that most often green jaundice is seen in the context of obstructive jaundice, especially that of carcinomatous origin (An et al. 2010; Larson et al. 1947; Tickner and Gutteridge 1978; Wardle and Williams 1981). Biliverdinaemia has been reported also in context of hepatitis, hepatic necrosis due to paracetamol intoxication, malnutrition, (alcoholic) cirrhosis, multisystem POEMS syndrome and biliary atresia (Fenech et al. 1967; Greenberg et al. 1971; Gåfvels et al. 2009; Huffman et al. 2009; Prichard 1972; Wardle and Williams 1981; Yasuda et al. 1994). In the above-mentioned studies, the highest plasma biliverdin concentration, 98 μM, was reported in case of obstructive jaundice (Wardle and Williams 1981). However, since biliverdinaemia is such a rare symptom, it most probably does not always accompany these diseases. In some rare cases, biliverdinaemia has been seen without increases in serum bilirubin values (Gåfvels et al. 2009; Yasuda et al. 1994). Interestingly, biliverdinaemia is usually not detected in the jaundice of the indirect type, i.e., in hemolytic cases (Larson et al. 1947; McDonagh 2001). This is in accordance with the view that biliverdin reductase is not the limiting factor of heme catabolism, instead it is present in tissues in surplus amounts relative to basal need (McDonagh 2001).

The pathogenesis, or the immediate molecular cause, of biliverdinaemia is in most cases unclear. Suggested reasons include failure of change in biliverdin to bilirubin in liver and/or other tissues (Fenech et al. 1967; Greenberg et al. 1971; Larson et al. 1947). This might be due to cellular damage such as, for example, necrosis in context of paracetamol intoxication, or inhibition of BVR e.g. by bile acids during obstructive jaundice. In the paracetamol-induced necrosis decreased activity of BVR was indeed detected (Wardle and Williams 1981). Two case reports exist where mutation in the gene coding for BVR-A (the main type of BVR in adults; see Section 2.5.3) explains biliverdinaemia. In one case, where the patient suffered from alcoholic cirrhosis without jaundice, a heterozygous nonsense mutation in *BVR-A* was shown to underlie biliverdinaemia (Gåfvels et al. 2009). Mutation in *BVR-A* might lead to decreased capacity of BVR-A, although biliverdinaemia manifests only after compromised liver function. Similarly, Nytofte et al. (2011) reported two unrelated cases where biliverdinaemia was observed during obstructive cholestasis due to gallstones. In both patients, investigations revealed the same homozygous mutation in *BVR-A*. In cases of malnutrition, deficiency of NADH or NADPH might result in decreased rate of biliverdin to bilirubin conversion (Greenberg et al. 1971). Oxidation of bilirubin back to biliverdin has been mentioned as a possibility but seems not to be the most likely reason (Larson et al. 1947).

2.5.3 Biliverdin reductase (BVR)

In mammals, biliverdin is rapidly and quantitatively reduced to bilirubin by the ubiquitous cytosolic enzyme biliverdin reductase (BVR), which was first described by Singleton and Laster (1965). Bilirubin is subsequently transferred to liver for conjugation and excretion. Biliverdin reductase (BVR) exists in two forms, biliverdin IXα reductase (BVR-A) and biliverdin IXβ reductase (BVR-B). However, BVR-A is the predominant form while BVR-B is postulated to have major metabolic significance only during fetal growth. BVR-A is highly selective for biliverdin IXα isomer, whereas BVR-B reduces the other biliverdin isomers (IXβ, IXγ and IXδ). In addition, BVR-B has flavin and ferric reductase activities in adult mammals. BVR-A and BVR-B are encoded by different genes and have distinct molecular weights and sequences. (McDonagh 2001; Pereira et al. 2001).

BVR-A catalyzes the regiospecific addition of hydrogen to the –HC(10)=C-N group of biliverdin IXα. The reaction mechanism of both BVR-A and BVR-B is compulsory ordered kinetic mechanism, in which NADPH must bind first followed by the substrate. After the reduction of biliverdin molecule, there is sequential release of the product and oxidized cofactor. Both biliverdin and bilirubin at sufficient high concentrations can inhibit BVR-A. (Kutty and Maines 1981; McDonagh 2001; Rigney et al. 1989; Rigney and Mantle 1988).

For a long time the catalysis of the reduction of biliverdin IXα to bilirubin was the only known function of BVR. However, currently BVR-A is thought to have multiple functions in cellular signaling due to its function as a transcription factor and serine/threonine/tyrosine (Ser/Thr/Tyr) kinase, intracellular transporter of regulators such as heme, and as a scaffold/adaptor protein. (Ahmad et al. 2002; Florczyk et al. 2008; Gibbs et al. 2012; Maines 2005; Tudor et al. 2008)

BVR-A is evolutionarily highly conserved in its primary structure and molecular properties (Maines 2005). However, it undergoes post-translational modifications resulting to variants with different molecular weights (Huang et al. 1989a, 1989b). BVR-A is the only known dual pH and dual cofactor dependent enzyme: with NADH as cofactor it exhibits a peak of activity at pH 6.7, whereas with NADPH as cofactor the peak of activity resides in the basic range (pH 8.7) (Franklin et al. 2007; Maines 2005). Franklin et al. (2007) have shown that the apparent peak of activity observed at neutral pH with NADH as cofactor is an anion-dependent activation, where inorganic phosphate mimics the role of 2´-phosphate of NADPH thereby stabilizing the interaction between NADH and the BVR-A enzyme. Still another rare feature is that BVR-A needs to be autophosphorylated for its activity (Maines 2005; Salim et al. 2001). On the other hand, BVR-A phosphorylates serine and threonine moieties in proteins such as insulin receptor substrate 1 (IRS-1), protein kinase CβII (PKC-βII) and HO-2, and activates oxidative stress-responsive transcription factors c-Jun, c-Fos, activating transcription factor 2 (ATF2), cAMP response element-binding protein (CREB) and NF-kB of the mitogen activated protein kinase (MAPK) pathway (Tudor et al. 2008). Phosphorylated BVR-A interacts with members of MAPK family, in particular with the extracellular signal-regulated kinases 1/2 (ERK1/2), and regulates the expression of inducible nitric oxide synthase (iNOS; Barone et al. 2012). Due to its wide spectrum of functions, BVR-A is postulated to participate in central cellular processes of growth, apoptosis, oxidative response and gene expression (Florczyk et al. 2008). The reductase function of BVR-A is considered cytoprotective.

BVR-A was long considered to be solely a cytosolic protein; today it is known to exist in other cellular parts as well. In response to specific stimulus such as bromobenzene or bacterial lipopolysaccharide (LPS), BVR-A has been shown to undergo nuclear translocation. In nucleus, BVR-A forms dimers and binds to regulatory sites such as AP-1 of genes such as HO-1 and ATF-2, thus regulating cellular stress-response (Ahmad et al. 2002; Kravets et al. 2004; Maines 2005). On the other hand, BVR-A has been shown to exist in external plasma membrane of macrophages, where it quickly converts biliverdin to bilirubin. This starts a signaling cascade through tyrosine phosphorylation at the cytoplasmic part of BVR-

A, and is one way for biliverdin to modulate inflammatory response (Wegiel et al. 2009). Concomitantly with HO-1, BVR-A has been found in caveolae of rat pulmonary artery endothelial cells, and it is suggested that by facilitating caveolae mediated signaling cascades heme degradation pathway may confer cellular protection (Kim et al. 2004). Similarly, BVR-A has been found to colocalize with HO-1 in mitochondria (Converso et al. 2006).

Via its kinase activities, BVR-A may have a significant role in glucose metabolism. BVR-A itself is a substrate for insulin receptor tyrosine kinase (IRK) activity, whereas BVR-A directly phosphorylates IRS-1 peptides at sites known to negatively affect glucose uptake. Insulin-mediated glucose uptake was increased in cultured cells when BVR-A expression was knocked down using small interfering RNA. Thus, BVR-A seems to have potential antagonistic role in the insulin signaling pathway. (Lerner-Marmarosh et al. 2005).

Currently many agents or situations are known to modulate the expression, localization and/or catalytic activity of BVR-A (Table 5). The promoter region of rat BVR contains recognition sites for several regulatory proteins connected with oxidative stress and development, including e.g. activator protein 1 (AP-1; McCoubrey et al. 1995). Interestingly, TCDD is a known transcriptional activator of AP-1-regulated genes. BVR-A promoter organisations differ between humans and rats, but mouse and human genes share the same structural features and in the promoter, the sequences of consensus regulatory elements are more similar (Gibbs et al. 2010).

The product of BVR-A, bilirubin, is cytoprotective due to its potent antioxidant capacity. Barañano et al. (2002) have suggested that BVR-A functions in bilirubin–biliverdin antioxidant cytoprotectant cycle, which would have a major role in intracellular antioxidant defense. However, this view has been strongly questioned by other researchers. For example, Maghzal et al. (2009) showed that many experimental models of bilirubin oxidation did not yield biliverdin at all or only in low amounts. Debate is still ongoing (Jansen and Daiber 2012; Maghzal et al. 2009; McDonagh 2010a, 2010b; 2010c; Sedlak and Snyder 2009; Stocker and Maghzal 2009).

Table 5. Inductive, dual and inhibitory effects of different agents on BVR-A.

Agent or situation	Effect on BVR-A	Species	Organ	Reference
Inductive effect				
Lipopolysaccharide	Translocation into nucleus ↑	Rat	Kidney	Maines et al. 2001
Lipopolysaccharide	Increase in surface expression on macrophages	Mouse	Macrophages	Wegiel et al. 2009
Smokers with primary spontaneous pneumothorax (mechanism: oxidative stress via HIF-1α)	Induction of mRNA and protein levels (concomitant with HO-1 and H-ferritin)	Human	Lung macrophage	Goven et al. 2010
Bromobenzene	Translocation into nucleus ↑	Rat	Kidney	Maines et al. 2001
Heat shock (6 hours post-hyperthermia)	Elevation of mRNA levels	Rat	Kidney	McCoubrey et al. 1995
Renal carcinoma	Elevation of mRNA, protein and reductase activity levels	Human	Kidney	Maines et al. 1999
Hypoxia	Activation of hBVR promoter	Human		Gibbs et al. 2010
Acute myeloid leukemia	mRNA upregulated	Rat	Spleen	Papiez et al. 2009
H_2O_2, insulin, sodium arsenite, cobalt protoporphyrin	Stimulation of kinase activity of BVR-A	*In vitro*	Kidney cells	Lerner-Marmarosh et al. 2005; Miralem et al. 2005
Dual effect				
Alzheimer´s disease/ mild cognitive impairment	Protein levels increased BUT activity decreased	Human	Hippocampus	Barone et al. 2011a
Inhibitory/repressive effect				
Biliverdin	Inhibition of hBVR promoter	Human		Gibbs et al. 2010
Mercuric chloride	Inhibition of activity	Rat	Kidney	Kutty and Maines 1983

Biliverdin (> 5 μM)	Substrate inhibition	Rat / *In vitro*	Enzyme purified from rat liver	Kutty and Maines 1981
Bilirubin (> 0.5-1.0 μM)	Competitive inhibition			
Hematin	Inhibition			
Zinc protoporphyrin	Inhibition			
Sulfhydryl reagents	Inhibition			
TNFα-activated NF-κ B	Suppression of hBVR promoter	Human		Gibbs et al. 2010
Bromobenzene	Selective suppression of enzyme variants in liver and kidney but not in spleen	Rat	Liver and kidney	Huang et al. 1989c; Huang and Maines 1990

Abbreviations: hBVR, human biliverdin reductase; HIF-1-α, hypoxia-inducible factor 1-α, NF-κB, nuclear factor kappa B; TNFα, tumor necrosis factor α

It is notable that BVR-A is upregulated in certain cancers, and that in experimental models, BVR-A overexpression protects mouse fibroblasts against the effect of tested chemotherapeutics (Florczyk et al. 2011). Heme degradation pathway has been proposed for a promising serum biomarker source for the early detection of Alzheimer´s disease (Mueller et al. 2010). Intriguing recent results are that although total BVR-A protein levels were increased in the hippocampus of patients with Alzheimer´s disease (AD) or mild cognitive impairment (MCI), the activity of BVR-A was reduced (Barone et al. 2011a). This discrepancy is apparently due to differences in the phosphorylation status of BVR-A, and underlines the importance of phosphorylation for BVR-A activity. Post-translational modifications such as phosphorylation of critical Ser/Thr/Tyr residues seem to play a major role in the regulation of the neuroprotective and metabolic activities of BVR-A. The reduced phosphorylation of Ser/Thr/Tyr residues of BVR-A in hippocampus of patients with AD or MCI might contribute to the impaired glucose metabolism seen in both disorders (Barone et al. 2011a).

2.5.4 Bilirubin

Bilirubin is a yellow, lipophilic end product of heme degradation and the main pigment of bile. 250–400 mg of bilirubin is formed every day in adult humans primarily from hemoglobin degradation in spleen. Bound to serum albumin, bilirubin is transported to liver, where it is transferred from blood into hepatocytes both actively and passively. At the luminal side of the hepatocyte endoplasmic reticulum, bilirubin is conjugated with glucuronic acid in a reaction catalyzed by UGT1A1, resulting in water-soluble bilirubin mono- and diglucuronides. Depending on species, glucose and xylose derivatives are also found (Stocker 2004). Conjugated bilirubin is excreted into bile via multidrug resistance protein 2 (MRP2) export transporter proteins. UGT1A1 is regulated by various transcription factors

including AHR. (Bock 2011a, 2011b; Bock and Köhle 2010; Jansen and Bittar 2004).

Bilirubin was long considered solely as a waste product of the body, whose high levels were known to be potentially toxic. Nowadays bilirubin is recognized as a powerful antioxidant, i.e., scavenger of free radicals, and it has been shown to possess immunomodulatory, anti-inflammatory and antiapoptotic activity thus having beneficial effects in the body. Despite being cytoprotective at low concentrations, bilirubin at high concentrations is cytotoxic and induces apoptosis. (Bock and Köhle 2010; Kapitulnik 2004; Oakes and Bend 2005; Ryter 2012; Stocker et al. 1987; Stocker 2004).

Jaundice is a common clinical finding caused by increased bilirubin levels. It may originate from hepatic failure/primary hepatocellular damage, blockage of biliary outflow or increased degradation of hemoglobin (hemolysis; Guyton 1986; Gåfvels et al. 2009). Depending on which form of bilirubin, conjugated or unconjugated, is elevated, the underlying cause of elevation may be evaluated. Under physiological conditions, plasma bilirubin concentration in humans ranges from 4 to 20 μM, most of which is unconjugated pigment bound to albumin. When bilirubin concentration exceeds 43 μM (2.5 mg/decilitre), jaundice will occur (Jansen and Bittar 2004). In newborns, plasma unconjugated bilirubin is normally elevated due to hemolysis and immaturity of UGT1A1. If neonatal hyperbilirubinemia worsens up to total serum concentration of 428 to 514 μM or more, the risk increases that unconjugated bilirubin accumulates in focal brain regions leading to neuronal damage and cell death (Bock and Köhle 2010). Phototherapy and exchange transfusion are effective ways to treat hyperbilirubinemia (Bock and Köhle 2010). A healthy human liver is capable of handling three times the normal daily load of bilirubin without abnormal elevations in its concentration in the blood (Okazaki et al. 1978). In rats plasma total bilirubin is about 4 μM, of which about 30% is unconjugated (Unkila et al. 1994).

Both the free unconjugated, albumin-bound, and conjugated forms of bilirubin have been shown to exhibit antioxidant activity, which is based on a pair of reactive hydrogen atoms. Via hydrogen donation, bilirubin can scavenge radicals such as lipid peroxyl radical followingly: $LOO\cdot$ + bilirubin → LOOH + bilirubin$\cdot$. Bile pigments have been shown to scavenge both 1e and 2e oxidants, for example, singlet oxygen, quinones, peroxyl radicals, α-tocopheroxyl radical, hypochlorous acid, nitric oxide, peroxynitrite and nitroxyl radical, but they do not act on superoxide anion radical or hydrogen peroxide. (Stocker 2004).

After the water-soluble antioxidants ascorbate and ubiquinol-10, albumin-bound bilirubin is proposed to represent a second-line antioxidant defence against lipid peroxidation in extracellular space. Bilirubin has also been shown to protect plasma proteins from oxidative damage. In cell membranes, bilirubin (and biliverdin) inhibit membrane lipid peroxidation in synergy with α-tocopherol, the most important lipid-soluble antioxidant in humans. Although much of the evidence for the importance of bilirubin as an antioxidant *in vivo* is indirect, like induction of HO-1 as a response to oxidative stress, there is also more direct evidence. For example, it has been shown that hyperbilirubinemia results in reduced oxidative injury in rats, and that serum samples from patients with Gilbert´s syndrome display higher total antioxidant capacity than serum from individuals with normal bilirubin levels. (Bulmer et al. 2008; Dennery et al. 1995; Ryter 2012; Stocker et al. 1987; Stocker 2004).

It has been suggested that bilirubin would be an important intracellular antioxidant via BVR-A-catalyzed cycling of bilirubin-biliverdin (Barañano et al. 2002; Sedlak and Snyder 2009). However, as bilirubin concentration in cells has been estimated to be very low, around 50 nM, and biliverdin seems to be only a minor product of bilirubin oxidation (except when bilirubin is albumin-bound), the existence and importance of this antioxidant cycle has been questioned. Established cellular antioxidants such as glutathione and ascorbate exist in much higher concentrations in cells. On the other hand, it should be noted that in pathological conditions such as in pancreatic cancer cells, induction of HO-1 has been observed, leading to clearly elevated levels of biliverdin/bilirubin and thus making cancer cells resistant to oxidative stress generated by chemoterapeutics. (Jansen and Bittar 2004; Maghzal et al. 2009; Nuhn et al. 2009; Stocker 2004).

Both bilirubin and biliverdin have been shown to have protective effects in animal models of tissue injury and organ transplantation. Beneficial effects of bilirubin have also been observed in several epidemiological studies. According to them, natural elevations of bilirubin or elevations due to Gilbert´s syndrome are associated with protection against cardiovascular diseases, cancer, diabetes and lung disease. (Ryter 2012; Zelenka et al. 2012).

2.6 Interactions between heme metabolism and AHR

2.6.1 Biliverdin and bilirubin as AHR ligands

Bilirubin is one of several potential endogenous ligands of mammalian AHR (Nguyen and Bradfield 2008). The possibility that bilirubin might be an AHR ligand was first suggested by Kapitulnik and Gonzalez (1993), who found that microsomal CYP1A1/1A2 mRNA and protein levels were constitutively elevated in the livers of Gunn rats, a strain of rats that are congenitally jaundiced, thus suggesting that

bilirubin is an endogenous inducer of the CYPs. Later Sinal and Bend (1997) reported that a substantial dose-dependent increase in CYP1A1 mRNA and EROD activity occurred after treatment of Hepa 1c1c7 cells with either hemin, biliverdin or bilirubin, bilirubin being the most potent of the three compounds. This effect was absent in the AHR- or ARNT-deficient mutant cells, indicating that the effect required functional AHR. Phelan et al. (1998) showed that bilirubin and biliverdin competitively inhibited [^{3}H]TCDD specific binding to the cytosolic AHR, thus revealing that these compounds truly are AHR ligands. They also showed that physiological concentrations (1–10 μM) of bilirubin and biliverdin induced DRE-driven luciferase gene expression in stably transfected mouse, guinea pig, rat, and human cells. Phelan et al. (1998) suggest that bile pigments, among other endogenous, weak ligands of AHR, may have significance in inducing transient changes in gene expression in order to achieve appropriate cellular response in specific physiological and biochemical situations.

2.6.2 AHR-mediated induction of HO-1 and UGT1A1

AHR, which is embedded in complex signaling networks (Puga et al. 2009), seems to participate in regulation of HO-1 and UGT1A1 in crosstalk with other transcription factors including for example NF-E2-related factor-2 (Nrf2), constitutive androstane receptor (CAR) and pregnane X receptor (PXR; Bock and Köhle 2010).

Typical inducers of HO-1 include its substrate heme and various factors causing oxidative stress. The latter type of induction is mediated e.g. by the ubiquitous transcription factor Nrf2, which binds to antioxidant response elements (AREs) present in the regulatory region of HO-1. In addition, several xenobiotic response elements (XREs) are present in regulatory region of HO-1 although they have not been functionally characterized (Alam et al. 1999; Alam and Cook 2003; Bock and Köhle 2010). Crosstalk has been shown to take place between AHR and Nrf2 in regulation of several genes belonging to classical AHR battery (Yeager et al. 2009). Although HO-1 does not belong to that set of genes, it appears to be induced efficiently by mixed agonists of AHR and Nrf2 (Bock and Köhle 2010; Köhle and Bock 2006). *In vivo*, TCDD has been shown to induce HO-1 in a couple of studies (Boutros et al. 2011; Nishimura et al. 2001), however, no induction was seen in rats expressing only mutated AHR, supporting the participation of AHR in the regulation of HO-1.

Via its glucuronidation activity, hepatic UGT1A1 controls serum bilirubin level (Bock and Köhle 2010). UGT1A1 is inducible e.g. by nuclear receptors PXR and CAR, in addition, human UGT1A1 has a core XRE-element in its enhancer region, and AHR is one of the transcription factors mediating the induction of human

UGT1A1 (Yueh et al. 2003) but not that of rats (Bock 2011b). It has also been shown that bilirubin induces human UGT1A1 through AHR dependent pathway (Togawa et al. 2008). Via induction of HO-1 and UGT1A1, AHR may contribute both to local and systemic bilirubin homeostasis, respectively, and have impacts on e.g. atheroprotection. This might be one of the physiologic functions of AHR. (Bock and Köhle 2010).

2.7 Effect of TCDD on heme metabolism

The main known effects of TCDD on heme metabolism include 1) changes in ALAS1 expression and activity, 2) induction of porphyria and 3) increased serum bilirubin levels.

2.7.1 Expression and catalytic activity of ALAS1

About 40 years ago, TCDD was shown to be a potent inducer of hepatic δ-aminolevulinic acid synthetase (ALAS1) activity in chick embryo liver (Poland and Glover 1973). In mice, ALAS1 activity was found to increase in response to repeated exposure to 4 weekly doses of 25 µg/kg TCDD, but not with lower doses. A single dose of 150 µg/kg also induced the effect in 21 days (Goldstein et al. 1973). However, in rats a single dose of TCDD did not result in induction of ALAS1 either acutely or 16 weeks later (Goldstein et al. 1982; Tofilon and Piper 1982; Woods 1973), but after repeated exposure of Sprague-Dawley rats to a low dose (1 µg/kg/week) of TCDD for 16 weeks induction of ALAS1 was reported (Goldstein et al. 1982). Much later, Fletcher et al. (2005) showed that exposure of Sprague-Dawley rats to a single, high dose of TCDD (40 µg/kg) resulted in a marked reduction of hepatic ALAS1 mRNA expression at 7 days. In conclusion, species differences in TCDD´s effects on ALAS1 expression and activity seem obvious; otherwise, the mechanisms of these effects are quite unclear. One possible link between TCDD and ALAS1 is the transcription factor AP-1: Guberman et al. (2003) suggested that AP-1 complex has an inhibitory effect on ALAS-1 expression, and TCDD has been shown to induce AP-1 (Puga et al. 2000).

2.7.2 Porphyria

Porphyrias are a group of diseases caused by decreased activity (<50%) of one or more enzymes of the heme synthesis pathway (Figure 6). Typical consequences include urinary excretion and tissue deposition of porphyrins, the intermediate products of heme synthesis, leading to various clinical symptoms. Porphyrias are mostly genetic diseases, but acquired elements such as drugs, chemicals, heavy metals or hormones may be critical for the disease manifestation. Indeed, porphyrias are an example of disorders involving complex interactions between genes and chemicals. (Downey 1999; Smith and Elder 2010).

TCDD belongs to the most potent porphyrogenic agents known (Downey 1999; Goldstein et al. 1973). The porphyrogenic effect of TCDD has been detected both in humans and in laboratory animals such as mice and rats (Downey 1999; Goldstein et al. 1973; Kociba et al. 1978; Pelclova et al. 2006; Smith and Elder 2010). The effect seems to be more pronounced in mice than in rats: in susceptible mice already a single dose of TCDD induces porphyria, whereas chronic exposure seems to be required for TCDD-induced porphyria in rats (Cantoni et al. 1981; Goldstein et al. 1982; Smith and Elder 2010). Relatively high doses of TCDD are required for the manifestation of porphyria even in mice (Davies et al. 2008; Goldstein et al. 1973).

The porphyrogenic effect of TCDD results into accumulation of uroporphyrin in the liver, a condition termed "hepatic uroporphyria" (Goldstein et al. 1973) due to suppressed activity of the uroporphyrinogen decarboxylase (UROD). Inhibition of UROD is most likely caused by oxidative mechanism, and an inhibitor of UROD, uroporphomethene, which is an oxidation product of uroporphyrinogen, has been isolated from porphyric liver. In concordance with oxidative background, porphyrin accumulation is strongly potentiated by iron. Further, studies using transgenic knockout mice have demonstrated that CYP1A2 is required for TCDD-induced uroporphyria (Smith et al. 2001). AHR genotype has a major influence on the severity of porphyria as studied in mice (Davies et al. 2008; Smith and Elder 2010). In rats, ALAS1 induction and porphyria seem to be associated and occur related to chronic exposures, but not after a single, even high dose of TCDD.

2.7.3 Increased serum bilirubin

TCDD has profound effects on serum bilirubin in rats and some other rodents. In rats, total and/or conjugated forms have been shown to increase in response to acute, subacute or subchronic exposure to TCDD (Buu-Hoi et al. 1972; Kociba et al. 1976; Pohjanvirta and Tuomisto 1994; Simanainen et al. 2003; Tuomisto et al. 1999b; Unkila et al. 1994; Zinkl et al. 1973). Acute toxicity studies in L-E rats have shown that both total and conjugated bilirubin are progressively elevated with time, and the departure from control values is significant already 1 day after the exposure (Unkila et al. 1994). In L-E rats, serum bilirubin may rise to a level of approximately 100 times the concentration in control rats (Pohjanvirta et al. 1995). In contrast, in H/W rats expressing only mutated AHR, even extremely high dose of TCDD (9600 µg/kg) induced only modest, about 3-fold, increase in serum bilirubin, and interestingly, only unconjugated bilirubin was increased with no change in conjugated bilirubin (Unkila et al. 1994). In rat lines A, B and C, sensitivity to increased bilirubin levels after short-term TCDD exposure was in concordance with the order of the LD50 values of these rat lines (Simanainen et al. 2003; Tuomisto et al. 1999b). Increased serum bilirubin belongs to Type II dioxin endpoints.

The mechanism by which TCDD elevates serum bilirubin is not definitely proven. Bilirubin clearance from blood to liver and its conjugation seem to function properly (Pohjanvirta et al. 1995). Indications of hampered hepatobiliary excretory function due to TCDD treatment exist in the literature (Hwang 1973; Yang et al. 1977, 1983) but stagnant bile flow has not been generally found (Pohjanvirta et al. 1995; Tuomisto et al. 1999b). The ratio of unconjugated and conjugated forms seems to persist in most cases, suggesting increased formation of bilirubin (Simanainen 2004). Hepatic induction of HO-1 detected in a couple of studies is in concordance with this view (Boutros et al. 2011; Nishimura et al. 2001). Possibly the mechanism is somewhat different depending on the type of exposure (acute, subacute or subchronic). Wasting syndrome might contribute to the increased serum bilirubin as substantial body weight loss probably involves some loss of muscle mass and blood volume, which might lead to increased destruction of hemoglobin and myoglobin and consequently to increased formation of bilirubin. However, wasting syndrome does not fully explain bilirubinemia in TCDD treated rats, as pair-fed rats, which also loose their weight similarly due to restricted feeding, did not show significantly increased bilirubin levels (Unkila et al. 1994). Among the most toxic PCDDs (TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin, HxCDD and 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin), TCDD is clearly most potent in inducing increases in serum/plasma bilirubin (Pohjanvirta et al. 1995; Simanainen et al. 2002). In some specific cases such as in congenitally jaundiced Gunn rats, TCDD may reduce serum bilirubin by inducing its hepatic catabolism (Cohen et al. 1986).

3 Aims of the study

The aim of this study was to (1) solve the chemical composition of the green pigment accumulating in line B rats´ livers after TCDD exposure and (2) characterize the mechanism leading to the accumulation of that pigment. The hypothesis for point 1 was that the pigment constitutes of biliverdin, an intermediate product of heme degradation. As this hypothesis was proved true, the following more detailed questions were set in order to answer the point 2.

- How does TCDD affect the expression and activity of the enzymes responsible for the formation and elimination of biliverdin?
- What is the dose-response for the TCDD exposure and hepatic accumulation of biliverdin and bilirubin?
- Is the basic reason for elevated serum bilirubin, a well-known effect of TCDD, its induced formation or decreased elimination?
- Does HxCDD induce biliverdin accumulation?
- Are biliverdin levels elevated in line A or L-E rats after high/lethal doses of TCDD?

Answers to these questions would elucidate the mechanism of biliverdin accumulation and the roles of *AHR* and gene *B* in it.

4 Materials and Methods

4.1 Animals and animal husbandry

The rat strains/lines used in this thesis are shown in Table 6. All rats were obtained from the SPF (specific pathogen free)-barrier unit of the National Public Health Institute, Kuopio, Finland. Regular health surveillances consisting of bacteriological and serological screening as proposed by FELASA (FELASA 1996; FELASA 2002) indicated that the animals were free of typical rodent pathogens. Rats were housed in stainless-steel wire-mesh cages with pelleted (**I-V**) or powdered (**II**) R3 or R36 feed (Ewos, Södertälje, Sweden; Lactamin, Stockholm, Sweden) and tap water available *ad libitum* except for the feed-restricted groups of L-E rats (**II**). Rats were mainly kept in groups of 2–4 animals, but in some cases singly (**I, II, IV**). The temperature in the animal room was 21±1 °C, relative humidity 50±10%, and lighting cycle 12/12 hr light/dark. All study plans were approved by the Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government. The age of rats in the beginning of the experiments was usually 8–12 weeks; exceptions are mentioned. Body weights of the rats were recorded regularly throughout the experiments.

Table 6. Rat strains used in the study.

Rat strain/line	*AHR* genotype	*B* genotype	LD50 of TCDD (µg/kg); males	LD50 of TCDD (µg/kg); females	Reference
Long-Evans (L-E)	$AHR^{wt/wt}$	$B^{wt/wt}$	18	10	Pohjanvirta et al. 1993
Han-Wistar (H/W)	$AHR^{hw/hw}$	$B^{hw/hw}$	>9600	>9600	Unkila et al. 1994
Line A	$AHR^{hw/hw}$	$B^{wt/wt}$	>10,000	>2000	Tuomisto et al. 1999b
Line B	$AHR^{wt/wt}$	$B^{hw/hw}$	830	410	Tuomisto et al. 1999b
Line C	$AHR^{wt/wt}$	$B^{wt/wt}$	40	19	Tuomisto et al. 1999b

4.2 Chemicals

TCDD and HxCDD were purchased from UFA Oil Institute (Ufa, Russia) and were over 98% pure as analyzed by gas chromatography-mass spectrometry. They were

dissolved in corn oil. Biliverdin IXα (80% purity) was purchased from Sigma (St. Louis, MO; **I**) and from Chromatrin, Dublin, Ireland (**IV, V**); in Study **III** it was a gift from Dr. T. Mantle (Trinity College, Dublin, Ireland). Bilirubin was purchased from Sigma and was a mix of three bilirubin isomers, XIIIα, IXα, and IIIα in a proportion of 11%, 77% and 12%, respectively. Other chemicals used are given in the original publications.

4.3 Extraction of the hepatic pigment (I, III, V)

Before pigment extraction, the livers were usually perfused with buffer or saline in order to diminish the amount of blood in liver samples. Perfusion was made under ether anaesthesia (**I**) or in most cases (**IV, V**) right after decapitation of the rat. In Study **III** the pigment was extracted from non-perfused livers.

For mass spectrometric analysis (**I**) the extraction procedure of dark green/black liver pigment was as follows [modified from Bonkovsky et al. (1986)]. Liver homogenates (10%, w/v) were prepared in 0.25 M sucrose/20 mM Tris-Cl (pH 7.4). Proteins were precipitated by adding 0.8 ml 15% trichloroacetic acid to 0.5 ml of homogenate. After centrifugation, uncolored supernatant was discarded and the precipitate was suspended in 0.8 ml of ice-cold acetone/concentrated HCl (97.5:2.5, v/v), and centrifuged. Supernatants of ten parallel samples were combined and concentrated under nitrogen to a final volume of about 500 µl. At this stage, the mixture appeared in two phases. The lower one (volume 50-80 µl) contained the pigment and it was next applied to thin layer chromatography (TLC) plates for preliminary separation.

In later studies (**III, V**), the pigment extraction procedure was further developed for the purpose of quantitative high performance liquid chromatography (HPLC) analysis. The final version of the procedure differs from the above in that pigment was extracted from the protein precipitate with 0.8 ml of methanol. Resulting methanol extracts were filtered with Minisart SRP 15 filters having the pore size of 0.20 µm (Sartorius, Göttingen, Germany), and analyzed immediately with HPLC.

4.4 Preparative thin-layer chromatography (I)

Concentrated pigment was separated to its components by applying it on silica gel plates (SIL G-25, Macherey-Nagel, Düren, Germany). Development was done with chloroform:methanol:water (65:25:3). Bands were scraped off the plates and suspended in methanol (300 µl). Silica was removed by centrifugation. Methanol extracts were filtered using 0.45 µm Spartan 30/B filters (Schleicher&Schuell, Dassel, Germany) and stored at -20 °C for mass spectrometric analysis.

4.5 Liquid chromatography–mass spectrometry (HPLC-MS/MS) (I)

In liquid chromatographic separations before mass spectrometric detection the Rheos 4000 HPLC (Flux Instruments, Danderyd, Sweden) equipped with LaChrom autosampler L-7200 Merck Hitachi (Hitachi, Tokyo, Japan) and Spectroflow 757 UV-detector was used. Symmetry C18 column (5 µm; 4.6x150 mm) with a guard column (Waters) was used. Mass spectrometric analysis was performed using a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray ionisation source (San Jose, CA, USA).

The liquid chromatography conditions were as follows. Methanol containing 0.2% formic acid and water with 0.2% formic acid were used as eluents. A 20 min gradient was employed to increase methanol from 50 to 100%. Methanol was maintained at 100% for 5 min, after which the column was restored to initial conditions in 2 min. An automatic injection volume of 20 µl with a flow-rate of 1 ml/min was used. The eluate from HPLC column was divided in a 1:9 ratio. The smaller part was directed to the mass spectrometer and the larger to the UV-detector. UV-detection was carried out at 377 nm. Electrospray mass spectrometry was performed using positive ion mode. The capillary temperature was 225 °C, source voltage 4 kV, sheath gas flow 100 (arbitrary units, scale 0–100 units), capillary voltage 20 V and tube lens offset 10 V. Maximum ion time was 200 ms.

4.6 Quantitative HPLC (III, V)

Quantification of biliverdin, bilirubin and their conjugates in hepatic extracts was done by reverse phase HPLC (Waters, Milford, MA) equipped with Waters autosampler 717 and Waters diode array detector 996. Symmetry C_{18} column (5 µM; 4.6 x 150 mm, Waters) with a guard column was used. Eluent A was a mixture of 0.1 M ammonium dihydrogen phophate and methanol in 56:44 ratio, respectively (pH 3.4). Eluent B was methanol. A 27 min gradient was applied: the composition in the start was 70% eluent A, 30% eluent B; during 0–15 min eluent B was linearly increased to 100% and maintained for 10 min, after which the column was restored to initial conditions in 2 min. In Study **V** the 100% methanol was maintained 59 min instead of 10 min due to an unknown compound eluting at 53–56 min. An automatic injection volume of 25 µl with a flow-rate of 1 ml/min was used.

Biliverdin and bilirubin standards were prepared in methanol and dimethyl sulfoxide (DMSO), respectively. DMSO had to be used due to bilirubin´s low solubility in methanol. Biliverdin standard curves were constructed using 5 to 9 dilutions of biliverdin IXα corresponding to concentrations 0.02–4 µM. For standard curves of bilirubin isomers, 5 dilutions were prepared corresponding to 0.02–0.44 µM of

bilirubin XIIIα, 0.15–3.1 μM of bilirubin IXα, and 0.02–0.48 μM of bilirubin IIIα. The *r* values for all standard curves were >0.997.

Detection wavelengths were 377 nm for biliverdin and 460 nm for bilirubin. Identification of biliverdin and bilirubin in samples was based on the same retention time and similarity of the UV-VIS spectrum recorded at λ 250–750 nm with the respective standard compound. The peaks that had similar UV-VIS spectrum but different retention time compared with the standard biliverdin or bilirubin were considered conjugates of biliverdin or bilirubin, respectively. The peak areas were converted to concentrations in extracts using the formula:
concentration = (peak area – intercept)/slope.

The amounts of biliverdin and bilirubin conjugates were calculated using the standard curves of biliverdin IXα and bilirubin IXα, respectively, thus assuming that the molar absorption coefficients of the conjugates are the same as that of unconjugated biliverdin or bilirubin. This approach has been used previously e.g. by Lengyel et al. (2005) and it is justified as differences in the UV-VIS spectra of the parent bile pigment and its glucuronide conjugates are marginal (Brower et al. 2001; Lengyel et al. 2005; Noir et al. 1965; Wu 1983). Since there are no commercial standard preparations for bilirubin conjugates or biliverdin conjugates available, using bilirubin and biliverdin as calibrators for the respective conjugates was considered the best way to approximate their concentrations.

4.7 Histopathology (I, III)

In the histological examination of Study **I,** ten-week-old male rats of line B were used. The rats were dosed with 300 μg/kg of TCDD intragastrically and decapitated 2, 7, 14, 28, or 35 days later. In Study **III,** 2–4 animals from selected treatment groups (see the original publication) were used for histological examination.

Liver samples were preserved in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax and cut to the thickness of 5 μm. The tissue slices were mounted on glass slides and stained with Mayer's hematoxylin and eosin for general morphology or with Masson's trichrome stain for the verification of fibrosis. Two pathologists examined the slides using light microscope. Stainings were performed in accordance with standard protocols.

4.8 Concentration of HO-1 protein and catalytic activity of BVR-A (IV, V)

Hepatic and splenic concentrations of HO-1 protein were determined using the Rat Heme Oxygenase-1 solid-phase EIA Kit (Precoated; Takara Bio Inc., Otsu Shiga, Japan) based on a sandwich method utilizing two mouse monoclonal anti-rat HO-1

antibodies to detect rat HO-1 by a two-step procedure (**IV, V**). Analysis was carried out on a precoated 96-well plate according to the manufacturer's instructions. HO-1 concentrations were normalized to total protein concentrations of the supernatants determined by the Bradford method (1976).

BVR-A activity was determined in liver cytosols of line A and line B rats (**IV, V**) using Shimadzu UV-1601 spectrophotometer (Fennolab, Fenno Medical Oy, Kyoto, Japan) and Starna Brand 1 cm cuvets (Optiglass Ltd, Hainault, Essex, UK). BVR-A activity was identified as a spontaneous increase in absorbance at wavelength 460 nm, reflecting the formation of bilirubin in the solution. The initial composition of the reaction (volume = 3.08 ml) was 100 mM Tris (pH 8), 10 µM biliverdin, and 150 µl of cytosol. Immediately after mixing these components, the change in absorbance was followed for 10 x 1 min to record the blank rate. Thereafter, β-NADPH was added to the reaction at final concentration of 100 µM (final volume of the reaction was 3.1 ml), and the change of absorbance was followed again for 10 x 1 min to record the BVR-A activity. The amount of bilirubin formed in a min was calculated using the molar absorption coefficient of 52,500 $M^{-1}cm^{-1}$ (Rigney et al. 1989) and divided by the total protein concentration of the cytosol (determined by the Bradford method, 1976) to get the BVR-A activity expressed as the amount of bilirubin formed in nmol/min/mg total protein.

4.9 Clinical chemistry (V)

Total bilirubin was determined in serum, liver homogenate and bile samples by a modified diazo method using p-nitrobenzenediazonium salt (ThermoFisher Scientific, Vantaa, Finland) to give a corresponding azobilirubin. Direct bilirubin was determined in serum samples using diazotized sulfanilic acid (ThermoFisher Scientific). Total bile acids were determined in serum and liver homogenate supernatant samples using enzyme cycling amplification method (Total Bile Acids Assay Kit, Alere Ltd, Stockport, UK). Bilirubin and total bile acid analyses were performed with a clinical chemistry analyzer (Konelab 30i, ThermoFisher Scientific).

4.10 Analysis of hepatic crude fat (III)

A piece (about 4 g) of deep-frozen liver was weighed, minced with scissors and frozen in a pre-weighed bowl overnight at -20 °C. Subsequently, the tissue was dried in a Savant Modulyo freeze-dryer (Savant, Holbrook, NY) overnight and the weight of the dried tissue recorded. The dried tissue was powdered and extracted in a Soxhlet-apparatus with 300 ml toluene for 20 hr, after which the toluene was evaporated in a rotavapor. The sample was transferred to a pre-weighed decanter by rinsing with hexane, which was then allowed to evaporate. The decanter was kept at +40 °C overnight, allowed to cool in a desiccator, weighed, and the amount of fat was calculated.

4.11 mRNA expression levels (II, IV)

mRNA expression levels of ALAS1, HO-1, BVR-A, CYP1A1, CYP1A2 as well as of housekeeping genes β-actin, β-2 microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase, phosphoglycerate kinase 1 (Pgk 1), ribosomal protein L13A, succinate dehydrogenase complex A and TATA box binding protein were analyzed in line B liver and spleen samples using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)[1]. Analysis included following steps:

RNA-isolation and RT-PCR

Total RNA was isolated from homogenized liver and spleen samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). Isolated total RNAs were subjected to DNAse1 –treatment with the DNA-*free* kit (Ambion, Austin, TX). 1.2 μg (or 0.6 μg) of this RNA was then used for reverse transcription in a 20 μl (or 10 μl) reaction that was performed with Omniscript reverse transcriptase (Qiagen, Hilden, Germany) using random hexanucleotides (Roche, Mannheim, Germany) as primers. The reaction mixture was incubated at 37 ºC for 1.5 h. Resulting cDNAs were diluted in a 1:6 ratio with water and used in subsequent analysis.

Cloning of ALAS1, HO-1 and BVR-A gene fragments

169 to 284-nt-long fragments of ALAS1, HO-1 and BVR-A double-stranded cDNAs were cloned using line A rat cDNA as a template. PCR was performed with HotStarTaq DNA polymerase (Qiagen) on TGradient-thermocycler (Biometra, Göttingen, Germany). The "touchdown" method was applied: the annealing temperature was set at 63 °C for the first cycle and then decreased by 1 °C / cycle down to 57 °C. A total of 40 cycles were run. The primers were ordered from Sigma-Genosys Ltd., Cambridgeshire, UK, and are shown in the original publication (**IV**).

PCR products were purified from the agarose gel using Sigma GenElute Gel Extraction Kit (Sigma). They were cloned by blunt-end ligation into pCR-Script SK(+) Amp plasmids (Stratagene, La Jolla, CA). XLB-1 supercompetent cells (Stratagene) were used in transformations. The plasmids were purified by Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The inserts were sequenced with an A.L.F.express DNA sequencer (Amersham-Pharmacia Biotech, Uppsala, Sweden) using Thermo Sequenase CY5 Dye Terminator Kit (Amersham-Pharmacia Biotech). Previously prepared CYP1A1, CYP1A2 and β-

[1] In Study **II** the effect of TCDD on the expression level of 18 commonly used housekeeping genes was analysed. Here the methods used by the author of this thesis are described; the rest of the methods used in Study **II** are given in the original publication.

actin plasmids (Korkalainen et al. 2004; Korkalainen et al. 2005) as well as purified PCR products of the above named housekeeping genes [produced by R. Pohjanvirta as explained in the original publication (**II**)] were also used for production of external standard curves in mRNA quantitation.

Quantitative real-time RT-PCR (qRT-PCR)

The mRNA expression levels were analyzed using QuantiTect SYBR Green PCR Kit (Qiagen) and Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Mortlake, NSW, Australia). The 20 μl reaction mixture contained cDNA derived from 15 ng of total RNA, 0.5 μM each primer and 10 μl QuantiTect. The PCR was initiated with an incubation step of 15 min at 95 °C to activate HotStarTaq DNA polymerase. The "touchdown" method was applied: the annealing temperature was set at 63 °C for the first cycle and then decreased by 1 °C / cycle down to 57 °C. The cycling procedure was denaturation at 95 °C for 20 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s with fluorescence acquired. A total of 45 cycles were run. A melting curve was run at 55–99 ° C to verify the specificity of PCR products. Standard curve PCRs were carried out using 1–4 replicates of each dilution of plasmid/purified PCR product. The used dilutions varied between 10^{-4}–10^{-11} (relative to original plasmid at a concentration of 200 ng/μl). The Rotor-Gene 2000 version 4.6 software was employed to obtain the threshold cycle (C_t) for best fit (least squares method) of the standard curve. The C_t values were then converted to concentrations by the formula:

$$\text{Calculated concentration} = 10^{(\text{slope} \times C\text{t} + \text{intercept})}$$

The instrument analysis software was always allowed to carry out dynamic tube corrections. Reaction efficiencies were between 85 and 99%. All R values were >0.996. A negative control containing all the components of the reaction mixture but water replacing the template was included in each run.

4.12 Statistics

In general, for variables with homogenous variances ($p > 0.05$ in Levene´s test) groups of each strain/line were compared using one-way analysis of variance (ANOVA). If this test showed a significant difference, the least significant difference test or Duncan´s multiple range test (Study **II**) was used as a *post-hoc* test for detecting the deviating group(s). In case of non-homogenous variances (according to Levene´s test, $p < 0.05$), the non-parametric Kruskal-Wallis ANOVA was used and followed by the Mann-Whitney U test. P-values less than 0.05 were considered statistically significant. Student's t-test, or in case of non-homogenous variances, Mann-Whitney U test, were used to detect significant differences in comparisons involving no more than two groups. For line B rats of Study **V**, Pearson

correlations (*r*) were calculated for different combinations of hepatic biliverdin, bilirubin and their conjugates in dosing groups of 100–300 µg/kg of TCDD. In addition, Pearson correlations between hepatic bile pigments were calculated for different treatment groups of L-E rats.

4.13 Experimental designs

The used rat strains, dioxin treatments, analyzed tissues and follow-up times are outlined in Table 7.

Study I: Incidence of the "black liver" syndrome and identification of the pigment components

Incidence of the syndrome

The original observations of a new kind of toxicity, hepatic accumulation of dark green or black pigment, were made among a large number of rats (>300), which were tested with TCDD during the production, selection, and characterization of the new lines A, B, and C (for details, see Tuomisto et al. 1999b). These rats included the three new lines A, B and C, their hybrid offspring with L-E rats, and H/W x L-E F2 generation rats. They represented both genders and various age groups (4–57 weeks at the time of exposure) and were treated intragastrically with a wide range of single TCDD doses (10–2000 µg/kg). The results of these observations, that is, the incidence of the syndrome, are presented in the Study **I**.

Histopathology

The liver histopathology was studied in line B male rats using the appropriate dose (300 µg/kg) and duration of exposure (2, 7, 14, 28 and 35 days) in regard to pigment accumulation based on necropsy observations among over three hundred rats as explained above. The aim was to explore the possible histological alterations associated with pigment accumulation.

Identification of pigment components

The aim was to identify the components of the green liver pigment. Due to its distinctive green color it was hypothesized that the pigment consists of biliverdin, an intermediate product of heme catabolism. For pigment collection, line B female rats were dosed intragastrically with 300 µg/kg of TCDD at the age of 9–11 weeks. After 3 to 5 weeks, all rats were ether-anesthetized, livers were perfused and collected. Pigment was extracted as explained above (Section 4.3). It was pre-separated into its components using TLC (4.4). TLC bands were extracted from the plate and analysed using HPLC-MS/MS (4.5).

Study II: Applicable housekeeping genes for normalization of mRNA expression in TCDD-treated rats

In Study **II**, expression of a wide range of commonly-employed housekeeping genes was first determined with microarrays followed by verification of a selected subset by qRT-PCR. The used rat strains, TCDD doses, analyzed tissues and follow-up times are shown in Table 7. In L-E rats, TCDD treatment at lethal doses leads to a pronounced loss of body weight, which may indirectly influence hepatic gene expression. Therefore, a feed-restricted control group (FRC) of L-E rats was included into the study. Rats of the FRC group were put on a restricted feeding regimen intended to mimic TCDD-induced wasting; for detailed information see the original publication.

Study III: Differences in acute toxicity syndromes of TCDD and HxCDD

Study **III** consisted of two experiments. *Experiment I* involved male H/W, line A, and line B rats, and its purpose was to compare acute toxicity of TCDD and HxCDD in rat strains differing in their *AHR*- or gene *B* -genotype. H/W and line A rats were dosed with 2000 or 10,000 µg/kg of either TCDD or HxCDD. Line B rats received 200, 600 or 2000 µg/kg of TCDD or HxCDD or corn oil vehicle. Body weight change and mortality were monitored for 46 days. In *Experiment II*, which was carried out in order to supplement the data received from *Experiment I*, line B male rats were dosed with 1000 or 10,000 µg/kg of either TCDD or HxCDD. Randomly selected animals (n = 4) were euthanized on day 7 post-exposure for histology, and the remaining rats per group were monitored for 46 days. TCDD or HxCDD were administered to rats as a single dose, except when the dose was 2000 µg/kg or more. In that case, the substances were given as 1000 µg/kg boluses twice a day over 1 or 5 days.

The rats were weighed twice a week for the duration of the experiments. Animals were considered nonsurvivors and euthanized if their general condition was severely debilitated. Nonsurvivors, and on day 46 post-exposure all surviving rats were euthanized by decapitation. All animals were subjected to macroscopic examination at necropsy. General color of the liver and presence of green/dark spots was determined. Stomach and intestine were visually inspected for hemorrhage. From animals euthanized by decapitation, liver samples were collected for analysis of crude fat and biliverdin (stored at –80 °C), and for histology.

Study IV: Effect of TCDD on key enzymes of heme synthesis and degradation

The aim of Study **IV** was to examine whether the increased hepatic biliverdin concentrations in line B rats were associated with either augmented synthesis of heme or biliverdin (elevated ALAS1 or HO-1 expression, respectively) or with decreased metabolism of biliverdin (reduced BVR-A expression and/or catalytic

activity). The studied tissues were liver and spleen. Line A and line B rats were dosed with 300 μg/kg of TCDD and killed 2, 7, 14, 32 or 35 days later by decapitation. Controls received corn oil and were decapitated 2 or 35 days later. The dose 300 μg/kg TCDD was chosen because it is large enough to cause the syndrome of hepatic biliverdin accumulation at a fairly high incidence in line B rats during follow-up time of 3-5 weeks (**I**). mRNA expression levels of ALAS1, HO-1 and BVR-A were also analyzed in L-E rats used in Study **II**. Groups of L-E rats were control, TCDD-treated (100 μg/kg, 10 days), and feed-restricted control (FRC, 10 days).

Spleen and a piece of liver were rapidly removed after decapitation of the rats, flash-frozen in liquid nitrogen and stored at –80 °C for subsequent analysis. The remaining part of the line A and line B rats' livers was used for immediate cytosol preparation. When there were prematurely died rats, they were inspected at necropsy, but no samples were collected.

Study V: Dose-response of TCDD-induced effects on parameters of heme degradation; Effect of TCDD on excretion of bilirubin

The aim of Study **V** was to define the dose-response relationships between TCDD exposure and several parameters of heme degradation, as this might help in elucidating the biochemical mechanism of biliverdin accumulation. One of the key questions was the origin of conjugated biliverdin, i.e., is it formed via conjugation of free biliverdin or as a result of bilirubin oxidation.

Separate experiments were carried out:

- Line B female rats were used for defining the dose-responses of TCDD exposure and hepatic biliverdin, bilirubin, HO-1 protein and BVR-A activity levels, as well as serum bilirubin and bile acids.
- Hepatic accumulation of biliverdin is most probably related to increased serum bilirubin, a well-known effect of TCDD in rats. Does TCDD elevate serum bilirubin via increasing heme degradation or decreasing biliary excretion of bilirubin? Effect of TCDD on biliary bilirubin excretion was studied in line C rats.
- If studied using sensitive methodology (HPLC), is it possible to detect TCDD-induced changes in hepatic biliverdin in L-E rats, although no macroscopic syndrome has ever been observed? This was studied using the liver samples from the L-E rats of Studies **II** and **IV**.

Table 7. Overview to study designs.

Study	Rat strains	Congener	Doses (µg/kg)	Follow-up times (days)	Tissues analyzed	Objective / studied parameters	Analysis methods
I	A, B, C and their hybrid offspring with L-E; H/W x L-E F2	TCDD	10–2000	up to 42	Liver	Incidence of the syndrome; identification of the pigment components	Necropsy observations; histology; HPLC-MS/MS
II	L-E, H/W, B	TCDD	50, 100, 300	2, 4, 7, 10, 14, 32	Liver, spleen, hypothalamus	Finding stable housekeeping genes for dioxin studies	Microarray; qRT-PCR
III	H/W, A, B	TCDD; HxCDD	200, 600, 1000, 2000, 10,000	7, 46	Liver, intestine, stomach	Differences in acute toxicity syndromes of TCDD and HxCDD: body weight change, mortality (LD50); incidences of gastrointestinal hemorrhage, pale liver, green pigment in liver; concentrations of hepatic crude fat and biliverdin	Necropsy observations; histology; crude liver fat by Soxhlet-extraction; hepatic biliverdin by HPLC
IV	A, B, L-E	TCDD	100, 300	2, 7, 10, 14, 32/35	Liver, spleen	Effect of TCDD on - mRNA expression of ALAS1, HO-1, BVR-A, CYP1A1, CYP1A2 - expression of HO-1 protein - catalytic activity of BVR-A	qRT-PCR; commercial kit for HO-1 protein expression; kinetic assay of BVR-A activity; necropsy observations
V	B, C, L-E	TCDD	3, 10, 30, 100, 200, 300	10, 28	Liver, serum, bile	Hepatic concentrations of biliverdin, bilirubin and their conjugates; HO-1 protein expression; BVR-A activity; bilirubin excretion; serum bilirubin and bile acids	HPLC; BVR-A and HO-1 as above; standard clinical chemistry methods; necropsy observations

Note: Studies II, IV, V included a feed-restricted control (FRC or FR) group of L-E rats.

5 Results

5.1 Incidence of TCDD-induced "black liver syndrome" in rats (I, III-V)

A novel TCDD-induced syndrome, accumulation of green/dark pigment in rat liver ("black liver syndrome") was first detected during the development of new rat lines A, B and C (Tuomisto et al. 1999b). At that time, a large number of rats were tested with a wide range of TCDD doses and inspected at necropsy: the results of necropsy observations of more than 300 rats including the three new lines, their hybrid offspring with L-E rats, and H/W x L-E F2 generation rats are presented in Table 8.

The syndrome was never detected in rats expressing only mutated AHR, i.e., in line A or H/W x L-E F2 generation rats with verified $AHR^{hw/hw}$ genotype (Table 8). In addition, macroscopic syndrome has never been detected in H/W rats (**I, III**) or in L-E rats (**I, IV, V**). The majority of "black liver" cases were recorded in line B, line B x L-E or line A x L-E rats, that is, in rats with at least one resistance allele (AHR^{hw} or B^{hw}; **I**). In subsequent studies (**III, IV**), the incidence of the macroscopic syndrome in line B rats varied from 57 to 100% with TCDD doses of 200–2000 µg/kg, whereas no cases were recorded in line A rats even after 10,000 µg/kg of TCDD (**III, IV**).

Black liver syndrome was detected in rats exposed at widely different ages (4 to 57 weeks), therefore, the age of rat at exposure was not a critical factor in the development of the syndrome. Usually it took 3–5 weeks for macroscopic syndrome to develop, but as expected, in HPLC analysis of subsequent studies, hepatic biliverdin levels were found to be raised already before macroscopic signs (**V**; see Section 5.3). Depending on the TCDD dose and duration of exposure, mild and severe syndromes were seen. In mild/medium cases livers had few to numerous black spots. In severe cases, the overall color of liver was black and liver was swollen containing black or dark green fluid. Perfusion changed the color of affected livers from red with black spots to pale with green spots indicating that the pigment of interest was indeed green. Sometimes livers had yellowish color in addition to black spots, suggesting increased bilirubin levels.

Table 8. Number of rats with dark green or black pigment in their livers (**I**).

Rat strain/line	Genotype	Gender	Dose (µg/kg)	*n*	Green/dark spots in liver # (%)
H/W x L-E F2	$AHR^{hw/hw}$, *B* unknown	male	1000	6	0 (0%)
H/W x L-E F2	$AHR^{hw/hw}$, *B* unknown	female	1000	9	0 (0%)
A	$AHR^{hw/hw}$, $B^{wt/wt}$	male	700–2000	14	0 (0%)
A	$AHR^{hw/hw}$, $B^{wt/wt}$	female	700–2000	22	0 (0%)
A	$AHR^{hw/hw}$, $B^{wt/wt}$	male	12,000–30,000	3	0 (0%)
B	$AHR^{wt/wt}$, $B^{hw/hw}$	male	10–100	27	4 (15 %)
B	$AHR^{wt/wt}$, $B^{hw/hw}$	female	10–100	22	2 (9%)
B	$AHR^{wt/wt}$, $B^{hw/hw}$	male	300–2000	20	9 (45%)
B	$AHR^{wt/wt}$, $B^{hw/hw}$	female	300–2000	48	25 (52%)
C	$AHR^{wt/wt}$, $B^{wt/wt}$	male	10–100	40	2 (5%)
C	$AHR^{wt/wt}$, $B^{wt/wt}$	female	10–100	35	0 (0%)
A x L-E	$AHR^{hw/wt}$, $B^{wt/wt}$	male	10–100	5	0 (0%)
A x L-E	$AHR^{hw/wt}$, $B^{wt/wt}$	female	10–100	13	1 (8%)
A x L-E	$AHR^{hw/wt}$, $B^{wt/wt}$	male	300–2000	6	3 (50%)
A x L-E	$AHR^{hw/wt}$, $B^{wt/wt}$	female	300–2000	9	4 (44%)
B x L-E	$AHR^{wt/wt}$, $B^{hw/wt}$	male	50–2000	9	5 (56%)
B x L-E	$AHR^{wt/wt}$, $B^{hw/wt}$	female	50–2000	6	2 (33%)
C x L-E	$AHR^{wt/wt}$, $B^{wt/wt}$	male	10–100	9	5 (56%)
C x L-E	$AHR^{wt/wt}$, $B^{wt/wt}$	female	10–100	4	0 (0%)

Note: Age of rats at exposure was 4–57 weeks and time point of observation six weeks post-exposure or one day before the expected death.

5.2 Chemical composition of the green liver pigment (I)

The chemical composition of the green pigment accumulating in line B rats' livers after TCDD exposure is presented in Table 9. As indicated, pigment was found to consist mainly of biliverdin and its monoglucuronide; in addition, small amounts of other biliverdin conjugates were found.

Table 9. The compounds found in the green liver pigment extracted from the livers of line B female rats 35 days after a single intragastric TCDD dose (300 µg/kg; **I**)

Compound	Rough estimate of the contribution to the total amount of green pigment	Comments
Biliverdin	Major	
Biliverdin monoglucuronide	Major	
Unidentified biliverdin conjugate (= "Compound I" in **I**)	Minor	Molecular mass equals to biliverdin monoglucuronide – hydroxyl group
Biliverdin methyl ester	Minor	Not known, whether methylation has occurred *in vivo* or spontaneously in extractant (methanol)

Note: Analysis was made with HPLC-MS/MS. Biliverdin was identified using authentic standard. The molecular masses and daughter ion patterns implied that the other peaks absorbing light at the absorption maximum of biliverdin (377 nm) represent biliverdin conjugates named in the table. No commercial preparations of biliverdin conjugates exist.

5.3 Effect of TCDD on parameters of heme degradation: dose-response (V)

Effect of TCDD (3–300 µg/kg, 28 days) on hepatic biliverdin and bilirubin, HO-1 protein concentration and catalytic activity of BVR-A as well as serum bilirubin and bile acids in line B rats is summarized in Table 10. Part of the endpoints were assessed also in L-E rats (Table 10). Main results include:

Macroscopic observations

A few line B rats in the high dose groups had yellowish liver (3/6 in each of the dosing groups of 200 and 300 µg/kg TCDD) and some rats had black spots in liver (1/6, 2/6 and 2/6 in dosing groups 100, 200 and 300 µg/kg TCDD, respectively). All black liver cases were mild, that is, only few dark spots were detected in the liver tissue. No black spots or yellowish color were detected in the livers of L-E rats.

Body weights

In groups of line B rats treated with 30–300 µg/kg TCDD, body weight development differed significantly from control indicating wasting syndrome. In L-E rats, TCDD-treatment (100 µg/kg, 10 days) or restricted feeding resulted in weight losses of 32 ± 2% and 19 ± 3% relative to the day 0, respectively, whereas at the same time controls increased their weight 4 ± 3%.

Hepatic concentrations of biliverdin and bilirubin

In control line B rats, the mean hepatic biliverdin and its conjugate concentrations were 0.9 and 0.7 nmol/g, respectively; bilirubin and its conjugates were under the detection limit[2]. At doses of 100 µg/kg TCDD or more, hepatic concentrations of biliverdin, bilirubin and their conjugates were significantly elevated (Table 10). The maximal concentrations of biliverdin and its conjugates, 4.8 and 49.5 nmol/g (group means) were reached with the dose 200 µg/kg TCDD, whereas the highest concentrations of bilirubin and its conjugates were 22 and 28 nmol/g, respectively (group means; dose 300 µg/kg TCDD). Dose 100 µg/kg of TCDD increased hepatic biliverdin, biliverdin conjugate and total bilirubin concentrations significantly also in L-E rats, although the effect on the two latter analytes was clearly milder than in line B rats (Table 10). Further in L-E rats, feed-restriction induced similar increase in hepatic bilirubin level as TCDD exposure but had no influence on hepatic biliverdin (Table 10).

[2]The detection limit of the employed HPLC method for the three bilirubin isomers was not deliberately studied but the lowest detectable concentration of each isomer is presumably around 0.01 µM in the methanol extracts.

Serum bilirubin

In line B rats, the two lowest doses (3 and 10 µg/kg) had a slight diminishing effect on serum conjugated bilirubin, whereas the latter dose (10 µg/kg) elevated serum unconjugated bilirubin. All higher doses (30–300 µg/kg) dose-dependently increased the serum levels of both forms of bilirubin (Table 10).

Hepatic concentration of HO-1 and catalytic activity of BVR-A

Hepatic HO-1 protein expression was significantly increased at doses 100–300 µg/kg TCDD, whereas the catalytic activity of BVR-A was increased only with the dose 100 µg/kg (Table 10). Hepatic expression of HO-1 protein correlated strongly with serum total bilirubin level ($r = 0.918$, $p < 0.001$) whereas hepatic BVR-A activity did not, supporting the well-established view that HO-1 is the rate-limiting factor in heme degradation.

Bile acids

Compared with the control, the highest dose of TCDD (300 µg/kg) increased the level of serum bile acids about 2.6-fold in line B rats. Other doses did not have significant effects. In L-E rats, TCDD slightly decreased, whereas feed-restriction increased, the hepatic level of bile acids (Table 10).

Correlations between bile pigments

In line B rats dosed with 100–300 µg/kg of TCDD, correlation between hepatic bilirubin and its conjugates was strong ($r = 0.91$), whereas it was weak between biliverdin and bilirubin ($r = 0.01$), as well as between biliverdin conjugates and bilirubin conjugates ($r = -0.04$; Figure 8). Biliverdin conjugates correlated best with biliverdin ($r = 0.50$; Figure 8). In L-E rats, positive correlation between biliverdin and its conjugates was high in both control ($r = 0.78$) and TCDD-treated groups ($r = 0.78$). In addition, there was a good correlation between biliverdin and total bilirubin in control group ($r = 0.55$), but a negative correlation in the TCDD-treated group ($r = -0.43$). Thus it seems that the high positive correlation between biliverdin and its conjugates persists even after TCDD treatment, whereas TCDD treatment turns the positive correlation between biliverdin and total bilirubin into negative.

Table 10. TCDD (3−300 µg/kg) -induced changes in parameters of heme degradation. Exposure time was 28 days for line B rats and 10 days for L-E rats. The results are expressed as fold increases/decreases relative to the control (TCDD dose 0 µg/kg) or, if not possible, to the lowest detected value. Values in red and blue indicate statistically significant ($p < 0.05$) induction and repression, respectively. FR, feed-restricted control; ND, not detected. Absolute values of the analytes can be found in the original article (V).

	Tissue	**LIVER**								**SERUM**						
	TCDD dose (µg/kg)	0	3	10	30	100	200	300	0;FR	0	3	10	30	100	200	300
Analyte	***Rat strain***															
Biliverdin	Line B	1	0.6	0.5	0.5	3.4	**5.6**	**4.2**								
Biliverdin	L-E	1				**3.0**			1.0							
Biliverdin conjugates	Line B	1	0.3	0.4	1.7	**36**	**70**	**51**								
Biliverdin conjugates	L-E	1				**5.8**			1.5							
Bilirubin	Line B	ND	1	0.6	1.3	**24**	**37**	**52**		1	1.0	**1.6**	**1.8**	**17**	**41**	**53**
Bilirubin conjugates	Line B	ND	ND	1	ND	**91**	**173**	**262**		1	**0.6**	**0.5**	**1.7**	**16**	**49**	**72**
Total bilirubin	L-E	1				**1.7**			**1.7**							
HO-1 protein	Line B	1		0.8	1.1	**3.1**	**3.2**	**4.3**								
BVR-A activity	Line B	1		1.0	1.1	**1.4**	1.1	1.1								
Bile acids	Line B	1								1	0.9	0.6	1.9	1.8	2.1	**2.6**
Bile acids	L-E	1				**0.8**			**1.5**							

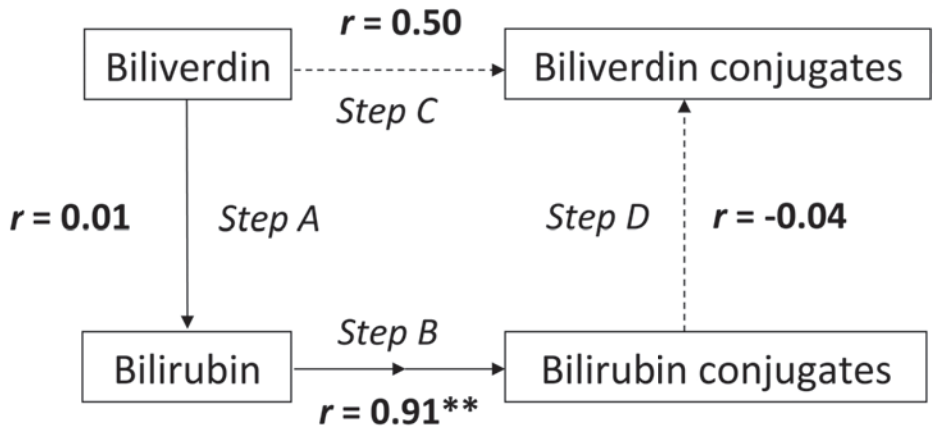


Figure 8. Pearson correlations between hepatic content of bile pigments in line B rats 28 days after 100–300 µg/kg TCDD. Solid arrows denote for known enzymatic steps (A and B), dashed arrows denote for hypothetical conversions (C and D). Asterisks (**) denote for statistical significance at the level of $p < 0.01$. Based on correlation coefficients, it seems that biliverdin conjugates are more likely formed enzymatically from biliverdin (step C) than oxidatively from bilirubin conjugates (step D). On the other hand, there might be problems in conversion of biliverdin to bilirubin, as their amounts do not correlate (step A), whereas there is a strong correlation between the substrate and product of another known enzymatic step, conjugation of bilirubin (step B). For original data, see Study **V**.

5.4 Effect of TCDD on HO-1, BVR-A and ALAS1; time-response (IV)

Effect of TCDD (100 or 300 µg/kg; time points 2, 7, 10, 14 and 32/35 days) on mRNA expression of HO-1, BVR-A and ALAS1, protein expression of HO-1, and catalytic activity of BVR-A in line A, line B and L-E rats is summarized in Table 11. Main points include:

Hepatic expression of ALAS1 mRNA
TCDD significantly decreased hepatic expression of ALAS1 mRNA in all studied rat strains (Table 11).

Hepatic expression of HO-1 mRNA and protein
In liver, HO-1 mRNA and protein displayed initial repression due to TCDD on postexposure day 2. At later time points, induction of HO-1 protein but not HO-1 mRNA was seen (Table 11).

Hepatic BVR-A activity

In both line A and line B rats, TCDD significantly increased hepatic BVR-A activity and mRNA expression on post-exposure day 14 (Table 11). However, at 5 weeks BVR-A activity was still induced in line A rats but reduced to 51% of control in line B rats (Table 11). At this time point, 5 out of 6 line A rats had yellowish liver, whereas in 4 out of 7 line B rats hepatic accumulation of dark green pigment ("black liver") was observed.

Splenic expression of HO-1 mRNA and protein

In spleen, TCDD strongly repressed the expression of HO-1 mRNA in line A and line B rats at all measured time points. Amount of HO-1 protein was also decreased (Table 11).

Table 11. Effect of TCDD on the expression/activity of ALAS1, HO-1 and BVR-A 2–35 days after exposure. TCDD dose was 300 µg/kg for line A and line B rats, and 100 µg/kg for L-E rats. The results are expressed as fold increases/decreases relative to the control (C). Values in red and blue indicate statistically significant ($p < 0.05$) induction and repression, respectively. Absolute values for the mRNA/protein expression or catalytic activity of the studied enzymes can be found in the original article (**IV**).

		Tissue	**LIVER**						**SPLEEN**					
		Time (days)	C	2	7	10	14	32/35	C	2	7	10	14	32/35
Enzyme	***Rat strain***	***Level***												
ALAS1	Line A	mRNA	1	0.64	**0.19**		**0.33**	**0.58**	1	0.92	0.90		1	1.41
ALAS1	Line B	mRNA	1	**0.37**	**0.20**		**0.14**	**0.20**	1	1.16	1.18		1.12	1.23
ALAS1	L-E	mRNA	1			**0.35**			1			0.98		
HO-1	Line A	mRNA	1	**0.17**	0.28		0.63	2.1	1	**0.19**	**0.15**		**0.17**	**0.17**
HO-1	Line B	mRNA	1	**0.22**	1.05		0.56-1.4	1.3	1	**0.29**	**0.32**		**0.37**	**0.26**
HO-1	L-E	mRNA	1			2.51			1			1.73		
HO-1	Line A	Protein	1				1.16	**6.52**	1				**0.77**	**0.80**
HO-1	Line B	Protein	1	**0.45**			2.81-**4.83**	4.08	1	0.90			**0.80**-0.91	**0.76**
BVR-A	Line A	mRNA	1	1.1	1.26		**2.44**	**3.34**	1	0.75	0.82		0.83	1.42
BVR-A	Line B	mRNA	1	0.92	**1.75**		1.33	0.83	1	1.14	1.16		1.18	2.16
BVR-A	L-E	mRNA	1			**2.2**			1			**1.72**		
BVR-A	Line A	Activity	1	**1.28**	1.16		**1.54**	**1.47**						
BVR-A	Line B	Activity	1	0.97	1.15		**1.46**	**0.51**						

5.5 Effect of TCDD on bilirubin excretion (V)

In line C rats, exposure to 10 μg/kg of TCDD significantly increased both serum and bile total bilirubin levels on post-exposure day 7, but decreased the amount of bile secreted per h per g body weight. Calculation of bilirubin clearance showed that despite the reduction in bile volume, the amount of excreted bilirubin per h per g body weight was 40% larger in TCDD-treated rats relative to control.

5.6 Differences in acute toxicities of TCDD and HxCDD (III)

The potency of HxCDD is about 10% of that of TCDD for typical AHR-mediated effects. The acute toxicity of TCDD and HxCDD was compared using H/W, line A and line B rats. A very high dose of HxCDD (10,000 μg/kg) reduced body weight more effectively than an identical dose of TCDD in all studied rat strains. In H/W and line A rats, the estimated LD50 values were >10,000 μg/kg and 2000–10,000 μg/kg for TCDD and HxCDD, respectively; for line B rats they were 480 μg/kg and 1000–2000 μg/kg, respectively. Thus, HxCDD was more potent than TCDD in inducing acute mortality in H/W and line A rats, contrary to what is predicted by TEFs. In line B rats, the expected rank order of potencies prevailed.

HxCDD (10,000 μg/kg) caused gastrointestinal hemorrhage and pale (fatty) livers in line A and H/W rats whereas TCDD did not. In line B rats, HxCDD (≥600 μg/kg) caused pronounced hepatic fatty degeneration, whereas TCDD (200–10,000 μg/kg) induced hepatic accumulation of biliverdin, detected both as black livers in necropsies and in quantitative biliverdin analysis of selected livers using HPLC. Mean hepatic biliverdin content was 6 nmol/g in controls, and 20 and 34 nmol/g in line B rats that received 200 μg/kg and 600 μg/kg of TCDD, respectively. The corresponding values for biliverdin conjugates were 0, 24 and 49 nmol/g.

No macroscopic accumulation of biliverdin was detected in H/W or line A rats even after 10,000 μg/kg of TCDD or in any rat dosed with HxCDD (200–10,000 μg/kg). For line A rats dosed with TCDD (10,000 μg/kg) and some line B rats dosed with HxCDD (200–600 μg/kg), absence of biliverdin accumulation was verified using HPLC. Both congeners caused peliosis-like sinusoidal distension in liver; most severe cases were detected in line B rats exposed to HxCDD (2000 μg/kg).

5.7 Applicable housekeeping genes for dioxin studies (II)

In Study **II**, 18 commonly used housekeeping genes were analysed in order to find a widely applicable housekeeping gene for acute toxicity studies of TCDD. About 50% of the studied housekeeping genes were responsive to TCDD in rat liver with the magnitudes of change up to nearly 10-fold (Table 12). Phosphoglycerate kinase 1 (*Pgk 1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) retained their

basal expression levels in all experimental settings (Table 12). Hepatic expression of β-actin, a commonly used housekeeping gene, increased in response to TCDD in L-E and line B rats if the exposure lasted 7 days or more. However, TCDD did not influence the expression of β-actin in spleen. Due to these findings, the housekeeping genes for the Study **IV** were selected as follows: β-actin mRNA was used for normalization of the gene expression data derived from spleen, whereas GAPDH mRNA was used for normalization of the gene expression data derived from liver.

5.8 Hepatic peliosis, a novel response to dioxin exposure (I, III)

In the histopathological examination of TCDD-treated (200 or 300 µg/kg) line B rats, a characteristic TCDD-induced liver response was seen (Studies **I** and **III**). This consisted of swollen hepatocytes with hydropic degeneration, formation of giant hepatocytes with multiple nuclei, necrosis of single cells or of small foci, fibrosis, vacuolization and infiltration of inflammatory cells in sinusoids. Additionally from day 28 on, hepatic sinusoids displayed a clear tendency towards distension reaching hepatic peliosis-like stage with membrane bound cysts by day 35. Some of these cysts contained erythrocytes or leukocytes. Mild cases of sinusoidal distension were recorded in line A and H/W rats after TCDD or HxCDD (2000 µg/kg) exposure; worst cases were recorded in HxCDD (2000 µg/kg, 23 days) -exposed line B rats (**III**). Peliotic process seems to start centrilobularly (**III**).

Table 12. The effect of TCDD on the expression of 18 housekeeping genes in rats in different experimental settings (described in top 5 rows). The results are expressed as fold increases/decreases relative to the control. Values in red and blue indicate statistically significant ($p < 0.05$) induction and repression, respectively. qPCR, quantitative real-time RT-PCR; FRC, feed-restricted control; MA, microarray; UC, unchanged (in microarray studies, the value between 0.75−1.25 was considered unchanged).

	Rat strain	L-E	L-E	H/W	LE	LE	LE	LE	B	B	B	B	LE	LE	LE
	Treatment	TCDD	FRC	TCDD	TCDD	FRC	TCDD	FRC	TCDD	TCDD	TCDD	TCDD	TCDD	FRC	TCDD
	(µg/kg)	100		100	100		100		300	300	300	300	100		50
	Tissue	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Spleen	Spleen	Hypothalamus
	Time (days)	4	4	4	4	4	10	10	2	7	14	32	10	10	10
	Method	MA	MA	MA	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR	qPCR
Gene acronym	**Gene**														
Alas1	Aminolevulinic acid synthetase 1	0.13	UC	0.31	0.11	0.82	0.30	1.86							
Alas2	Aminolevulinic acid synthetase 2	0.66	0.31	0.35											
Actb	Beta-actin	UC	UC	UC	0.78	0.69	1.53	0.53	0.83	1.64	1.86	2.95	0.91	0.96	1.08
B2m	Beta-2 microglobulin	0.71-1.0	UC	UC	0.99	0.94	0.99	0.81	0.56	0.55	0.67	0.62			
Ppia	Cyclophilin A (peptidylprolyl isomerise A)	UC	UC	UC	1.23	0.74	1.13	0.62							
Eef1a	Eukaryotic translation elongation factor 1 alpha 1 and 2	UC	UC	UC	1.06	1.11	1.22	0.85							
G6pdx	Glucose-6-phosphate dehydrogenase	2.46	0.22	4.59	2.28	0.29	2.29	0.40							
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	UC	0.66	UC	0.80	0.56	1.02	0.53	1.45	0.80	1.22	1.37	1.12	0.66	1.09
Hmbs	Hydroxymethylbilane synthase	UC	UC	UC											
Hprt	Hypoxanthine guanine phosphoribosyl transferase	UC	1.41	UC	0.77	1.49	0.86	0.85	0.50	0.86	1.04	0.82			
Pgk1	Phosphoglycerate kinase 1	0.62-1.0	UC	UC	0.66	0.79	1.01	0.61	0.73	0.56	0.75	0.89	1.26	0.84	1.07
Rpl13A	Ribosomal protein L13A	UC	UC	UC	0.95	1.04	1.18	0.75	1.08	1.40	1.76	0.89			
Rps18	Ribosomal potein S18	1.62	UC	UC	2.02	1.24	2.04	2.61							
Sdha	Succinate dehydrogenase complex, subunit A	UC	UC	UC	0.89	0.65	1.08	0.66	0.84	0.88	0.92	0.61	1.95	1.07	0.99
Tbp	TATA box binding protein	UC	UC	UC	0.74	0.80	1.1	0.91	0.73	0.49	1.09	1.08			
Tfrc	Transferrin receptor	0.47	UC	UC	0.48	0.70	0.33	0.20							
Ubc	Ubiquitin C	2.0	1.41	1.32											
Ube2b	Ubiquitin conjugating enzyme	UC	UC	UC											

6 Discussion

6.1 Overall conception of TCDD´s effects on heme degradation in rats

6.1.1 Occurence of TCDD-induced biliverdin accumulation

This thesis shows that TCDD is able to cause extensive hepatic accumulation of the heme degradation product biliverdin in both genders of line B, as well as in some other rat strains expressing wild-type AHR. This is a novel observation in terms of TCDD toxicity. Biliverdinaemia, i.e., increased serum biliverdin, has been described in humans associated to a variety of disease states, however, it is a rare phenomenon (Huffmann et al. 2009). A noteworthy property of TCDD-induced biliverdin accumulation in rats is its dependence on the genetic factors, *AHR* and unknown gene *B*, which makes the syndrome intriguing from the mechanistic point of view. Further, TCDD-induced biliverdin accumulation is associated with increased serum bilirubin, a well-known effect of TCDD. Biliverdin accumulation belongs to Type II effects of TCDD, as it is sensitive to alterations in the transactivation domain of AHR. At least one AHR^{wt} allele is required for TCDD-induced biliverdin accumulation, whereas AHR^{hw} allele in homozygous form seems to provide complete protection against this response.

Biliverdin accumulation was found to be an invariable response in line B rats as hepatic biliverdin (and also bilirubin) levels were elevated in all line B rats treated with the dose 100 µg/kg of TCDD or more (**V**). Apparently, the TCDD dose able to cause significant accumulation of biliverdin conjugates is between 30 and 100 µg/kg when exposure time is 28 days (**V**). Therefore, in line B rats, biliverdin accumulation is slightly less sensitive response to TCDD than wasting, a typical Type II response, which was significant with the dose 30 µg/kg of TCDD (**V**). The expression and/or activity of the enzymes responsible for heme degradation, HO-1 and BVR-A, was also increased with the dose 100 µg/kg of TCDD (**V**), hence coinciding biliverdin accumulation, whereas serum unconjugated bilirubin was slightly increased already with lower doses (10 and 30 µg/kg of TCDD). Possibly the dose 100 µg/kg of TCDD strongly increases heme degradation, whereas some enhancement of the process occurs even with lower doses.

In L-E rats, macroscopic cases of biliverdin accumulation have never been observed, although L-E rats are known to exhibit vast increases in serum bilirubin after TCDD exposure (Pohjanvirta et al. 1995; Unkila et al. 1994). However, when studied using sensitive analytical methods such as HPLC, acutely lethal dose of TCDD (100 µg/kg)

was found to increase hepatic biliverdin and bilirubin levels in L-E rats (**V**). In contrast to bilirubin, hepatic biliverdin was not increased in feed-restricted control L-E rats. This suggests that increased hepatic (and serum) bilirubin level after TCDD exposure may be partly secondary to weight loss, whereas increase in biliverdin seems to be a specific consequence of TCDD toxicity occuring e.g. in line B and L-E rats, and possibly other rats with wild-type AHR. Instead, rat strains expressing only mutated AHR, i.e., H/W and line A rats, were completely protected from the syndrome (**I, III, IV**). Therefore, the normal transactivation domain of AHR (Lindén et al. 2010; Pohjanvirta et al. 2012) is required for TCDD-induced biliverdin accumulation. As for many other effects of TCDD, the critical molecular events involving the transactivation domain and finally leading to specific toxicities (in this case, biliverdin accumulation) remain still unknown.

HxCDD (200–10,000 µg/kg) did not induce macroscopic biliverdin accumulation or elevations in hepatic biliverdin level as measured with HPLC in any of the studied rat strains (line A, line B, and H/W rats; **III**). According to the TEF concept, about 300–1000 µg/kg of HxCDD should induce an increase in hepatic biliverdin level in line B rats. As only part of the animals were studied using HPLC, it is not possible to say that HxCDD´s inability to induce biliverdin accumulation would have been definitely shown. Especially, the possibility remains that the effect is masked by the early death of HxCDD-treated animals due to other reasons; however, results of single animals do not support this. For example, two line B rats dosed with 600 µg/kg of HxCDD exhibited slightly reduced hepatic biliverdin level on postexposure day 46 (however, the difference to the control level was not statistically significant), and the only line B rat that survived 46 days after exposure to 2000 µg/kg of HxCDD did not show any macroscopic signs of biliverdin accumulation. Thus it seems that biliverdin accumulation is specific to TCDD, whereas HxCDD causes extensive hepatic steatosis with similar or slightly lower equivalent doses (absolute HxCDD dose 600 µg/kg = 60 µg/kg in TEQs), as studied in line B rats (**III**). As HxCDD has been shown to induce modest increases in serum bilirubin in rats (Pohjanvirta et al. 1995; Simanainen et al. 2002), the effect of TCDD and HxCDD on heme degradation is partly similar (both increase serum bilirubin, TEFs apply) and partly different (only TCDD induces biliverdin accumulation, TEFs do not apply).

6.1.2 TCDD elevates serum bilirubin by enhancing heme degradation

Increase in serum bilirubin is a well-known effect of TCDD in rats and some other rodents, but the underlying mechanism has been unclear (Pohjanvirta and Tuomisto 1994). In general, depending on which type of bilirubin is increased – unconjugated, conjugated or both – it is possible to evaluate the cause and pathology of jaundice (Guyton 1986). In this thesis, it is shown that in line B rats, TCDD induces dose-

dependent increases of both unconjugated and conjugated bilirubin in liver and serum finally reaching about 50 to 70-fold levels relative to control. Increase of both types of bilirubin suggests hemolytic type of jaundice, i.e., increased bilirubin formation (Guyton 1986). This is supported by the findings that TCDD increased bilirubin excretion into bile and did not markedly increase serum bile acid level, suggesting that cholestasis or other major unspecific decline in biliary excretory function is not a probable reason for increased serum bilirubin (**V**). Therefore, the principal reason for increased serum bilirubin levels detected in rats after TCDD exposure is most probably increased degradation of heme. This conclusion is further supported by the inducing effect of TCDD on the expression and/or activity of the enzymes responsible for bilirubin formation, HO-1 and BVR-A (**IV, V**). TCDD-induced hepatic peliosis (**I**, **III**) as well as repression of ALAS1 mRNA expression in liver (**IV**) are also in concordance with increased hepatic heme content and degradation: hepatic peliosis particularly stands for presence of blood-filled cysts in liver, whereas free heme is a potent repressor of ALAS1 expression (Tzirogiannis et al. 2004; Yamamoto et al. 1988). All these matters strongly suggest that for some unknown reason, TCDD increases hepatic content of heme, which leads to increased hepatic heme degradation and bilirubin formation. This is suggested to be the principal reason for increased levels of bilirubin in serum and liver after TCDD exposure in rats. However, this conclusion does not exclude the possibility that TCDD might also decrease bilirubin excretion in some situations, depending e.g. on the dose of TCDD or exposure time.

A peculiar finding detected both in line A and line B rats is the TCDD-induced repression of HO-1 mRNA and protein expression in spleen (**IV**). It has been suggested that HO-1 is normally induced in spleen due to large amounts of heme present (Braggins et al. 1986). Based on this, the simplest explanation for the repression of HO-1 in spleen would be that splenic content of heme is diminished due to TCDD exposure, which would lead to attenuation of the inducing effect of heme on the expression of splenic HO-1. Decreased splenic amount of heme might be associated with or even a consequence of the pooling of blood in liver, which occurs in hepatic peliosis, or related to the development of that condition. On the other hand, hypoxia has been shown to repress HO-1 expression in cultured human cells (Shibahara 2003), however, no literature was found on whether TCDD would induce splenic hypoxia. Altogether, the essential finding is that the consequences of TCDD exposure on HO-1 expression, and therefore on heme degradation, seem to be largely different in liver and spleen.

6.1.3 Origin of biliverdin monoglucuronide

The green liver pigment was found to consist mainly of biliverdin and biliverdin monoglucuronide, small amounts of other biliverdin conjugates were also found (**I**). In line B rats in Study **V**, the relative and absolute accumulation of biliverdin conjugates was remarkably stronger than that of unconjugated biliverdin. Therefore, the question is not only why biliverdin accumulates but also why and how its conjugates, mainly monoglucuronide, accumulate. Two theoretically possible sources of biliverdin conjugates exist: (1) enzymatic conjugation of biliverdin, and (2) oxidation of bilirubin conjugates. In Study **V**, it is shown that biliverdin conjugates are most likely formed via conjugation of biliverdin, not by oxidation of the respective bilirubin conjugates. The principal evidence is based on the correlations of hepatic bile pigments in line B rats treated with high doses (100–300 µg/kg) of TCDD. In these rats, a high correlation was detected between bilirubin and its conjugates, i.e., between the substrate and the products of an enzymatic reaction (Figure 8; **V**). In contrast, the correlation between biliverdin and bilirubin was surprisingly poor, suggesting that reduction of biliverdin does not obey 1st order kinetics in current conditions, and other products than bilirubin might be formed from biliverdin. Further, biliverdin conjugates correlated best with free biliverdin, resembling the situation between bilirubin and its conjugates (Figure 8). Additional support comes from the results on L-E rats, as TCDD disrupted the positive correlation between biliverdin and total bilirubin, but had no effect on the positive correlation between biliverdin and its conjugates (**V**).

Study **I** of this thesis is most probably the first to report the presence of conjugated biliverdin in rats. However, there are other studies showing the presence of conjugated biliverdin in mammals: it is the dominant green pigment in rabbit bile (Garay et al. 1965) and has recently been detected in human serum (An et al. 2010). Garay et al. (1965) suggested almost 50 years ago that in many animal species conjugation of both biliverdin and bilirubin may occur simultaneously. The issue of biliverdin conjugation has apparently not been thoroughly studied, but the results of this thesis support the above view of Garay et al. (1965). Although it has not been shown that biliverdin would be a substrate for UGT1A1, it might be possible as UGT1A1 has broad substrate specificity (Basu et al. 2004).

The other theoretical possibility, oxidation of bilirubin conjugates to biliverdin conjugates, seems less probable due to the observed poor correlation of their hepatic concentrations. Further support comes from the literature, as many studies suggest that in most conditions, bilirubin is not efficiently oxidized to biliverdin (Jansen and Daiber 2012; Maghzal et al. 2009; McDonagh 2010a, 2010c; Shishido et al. 2003).

6.1.4 Current hypothesis on TCDD-induced biliverdin accumulation

Current hypothesis on TCDD-induced biliverdin (and bilirubin) accumulation is formulated as follows. **(1)** The principal cause of increased serum and hepatic bilirubin levels after acutely toxic doses of TCDD is most probably increased bilirubin formation, i.e., increased degradation of heme, not decreased biliary excretion (**IV**, **V**). **(2)** In mammals, conversion of biliverdin to bilirubin is normally quantitative and rapid as BVR-A is present in excess (McDonagh 2001). Here it is postulated that in addition to enhancement of heme degradation, TCDD hampers the quantitative hepatic conversion of biliverdin to bilirubin to various degrees in line B and some other rat strains with AHR^{wt} allele but does not abolish it totally, leading to hepatic accumulation of biliverdin, bilirubin and their conjugates (Figure 9). In line A and H/W rats, which express only mutated AHR, conversion of biliverdin to bilirubin seems not to be hampered even after huge doses of TCDD (**I, III, IV**). Therefore, wild-type transactivation domain of AHR is a prerequisite for TCDD-induced hindrance of conversion of biliverdin to bilirubin. More detailed molecular causes of biliverdin accumulation might be related to BVR-A and they are discussed in the next section. **(3)** Biliverdin monoglucuronide and other biliverdin conjugates are most likely secondary products of biliverdin. **(4)** Since biliverdin accumulation was absent in feed-restricted L-E control rats, it mechanistically originates from other effects of TCDD than weight loss. **(5)** After acutely toxic doses of TCDD, hepatic biliverdin level is probably prone to increase in all rats expressing wild-type AHR. However, only in semi-TCDD-resistant rat strains (such as line B and the F1 offspring of the crosses line A x L-E and line B x L-E), the syndrome is able to develop to such a severe stage before the death of the animal that it becomes noticed at necropsies. In these semi-TCDD-resistant rats, the resistance is provided by one AHR^{hw} allele or one or two B^{hw} alleles, whereas the presence of at least one AHR^{wt} allele enables biliverdin accumulation.

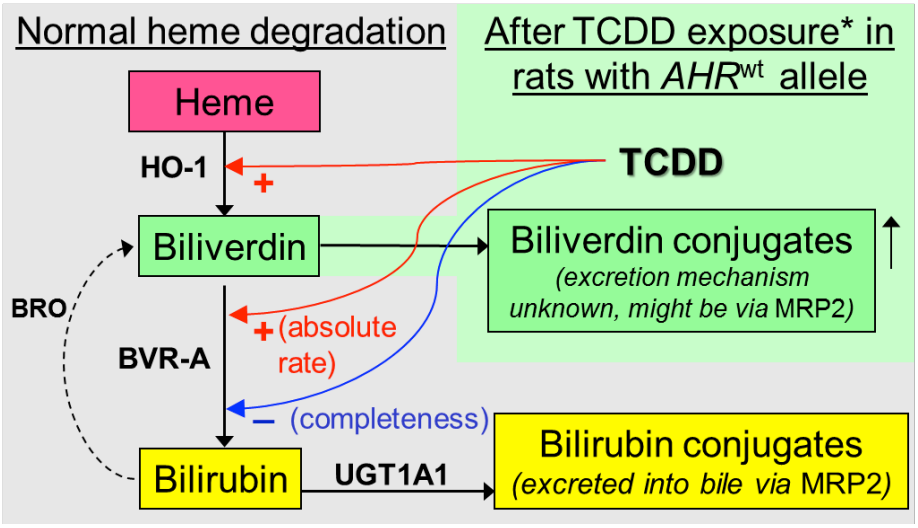


Figure 9. Suggested consequences of TCDD exposure on hepatic heme degradation in rats with *AHR*^{wt} allele, resulting in the accumulation of biliverdin, bilirubin and their conjugates. Presence of hepatic bilirubin oxidase (BRO) has been shown in rats, mice and humans (Abu-Bakar et al. 2011, 2012; Cardenas-Vazguez et al. 1986), however, it does not seem to play a major role in TCDD-induced biliverdin accumulation. *acutely toxic dose. (Original place of publication Basic & Clinical Pharmacology & Toxicology; Study **V**)

6.2 Further mechanistic considerations on biliverdin accumulation

6.2.1 Possible reasons for hampered conversion of biliverdin to bilirubin

The remaining key question is how TCDD would suppress conversion of biliverdin to bilirubin at the molecular level. Could it be possible that increased hepatic heme content and catabolism would simply exceed the normal capacity of BVR-A? The results on L-E rats do not support this as the increase in hepatic bilirubin was only 1.7-fold and yet biliverdin levels were raised (**V**). According to general understanding, there is remarkable excess capacity in biliverdin reduction in mammals (McDonagh et al. 2001). Further, results on feed-restricted L-E control rats show that 1.7-fold increase in hepatic bilirubin does not necessarily mean any change in hepatic biliverdin level (**V**). These aspects suggest that the question would not be simply an overload of normal enzymatic capacity.

Hepatic BVR-A activity was either normal or increased in TCDD-treated line B rats, except 32 days after 300 µg/kg when it was only 51% of control (Table 11). One

possible reason for decreased BVR-A activity might be substrate inhibition (Kutty and Maines 1981) as in necropsy, these rats exhibited severe hepatic swelling and leakage of dark green/black fluid, indicating biliverdin accumulation. At the same time-point, line A rats exhibited 1.5-fold increase in hepatic BVR-A activity and yellowish liver, suggesting increased hepatic content of bilirubin (**IV**). Concurrently, serum total bilirubin was significantly and similarly increased in both rat lines (mean values 45 μM and 36 μM in line A and line B rats, respectively; unpublished results). A possible interpretation for these findings is that hepatic heme degradation is strongly increased in both rat lines A and B, but only in line B rats part of biliverdin is conjugated rather than reduced to bilirubin. Why? A simple explanation would be that in line B, BVR-A might not function as efficiently as in line A under increased heme degradation caused by TCDD. BVR-A is known to be a versatile enzyme having pleiotropic functions, and its catalytic activity is critically regulated by phosphorylation state (Kapitulnik and Maines 2009; Maines 2005; Salim et al. 2001; see Section 2.5.3 for literature review on BVR-A). BVR-A is also prone to inhibition by biliverdin and bilirubin (Kutty and Maines 1981; Rigney and Mantle 1988). Therefore, although it is not possible to name the exact alteration, it might not be surprising if some specific difference would exist in the function or regulation of BVR-A between rat lines A and B, which would manifest under increased need for BVR-A-catalyzed reduction of biliverdin. This potential difference would have to be related to the AHR genotype, and in fact, it might be a difference e.g. in post-translational modification of BVR-A protein itself, or a difference in some regulator or interplayer of BVR-A. In humans, two case reports exist concerning hetero- and homozygous mutations in the gene of BVR-A. These individuals were phenotypically normal until an emergence of a specific stressor such as alcoholic liver failure or obstructive cholestasis due to gallstones, after which biliverdinaemia was observed (Gåfvels et al. 2009; Nytofte et al. 2011). The coding sequence of line B rat´s *BVR-A* was found to be equal to one in GenBank (Niittynen et al. 2003), however, there might be some post-translational change in rat line B affecting the function of BVR-A, which under a specific stressor – such as increased heme degradation due to TCDD – would lead to biliverdin accumulation.

When comparing line B and L-E rats in terms of biliverdin accumulation, two possibilities exist. A common part to both choices is the above conclusion, that after acutely toxic doses of TCDD, hepatic biliverdin level is probably prone to increase in all rats expressing wild-type AHR. However, only in semi-TCDD-resistant rat strains, such as line B, the syndrome seems to be able to develop to such a severe stage before the death of the animal that it becomes noticed at necropsies. In semi-TCDD-resistant rats, the resistance is provided by one AHR^{hw} allele or one or two B^{hw} alleles, whereas the presence of at least one AHR^{wt} allele enables biliverdin accumulation. Having this background in mind, two possible scenarios can be constructed:

- According to *Choice 1*, the role of B^{hw} in relation to this syndrome is only in increasing rats´ TCDD-resistance by a mechanism not related to biliverdin accumulation. If L-E rats would stay alive a longer time after acutely toxic dose of TCDD, as high biliverdin levels would be detected in them as are detected in line B rats.
- According to *Choice 2,* although biliverdin levels increase in response to TCDD in both line B and L-E rats, the observed difference in the magnitude of effect (level of hepatic biliverdin) is significant. This means that even if L-E rats would stay alive longer after a high dose of TCDD, biliverdin levels would never become as high as in line B rats. What follows from *Choice 2* is that B^{hw} allele would be a necessary factor for substantial biliverdin accumulation, whereas B^{wt} would limit biliverdin accumulation to relatively low levels. Hence, B^{hw} allele would make the rat more susceptible to biliverdin accumulation than B^{wt} allele but simultaneously more resistant to TCDD-induced acute lethality. Although hypothetical, this is mechanistically an intriguing option, as a possible role for BVR-A in TCDD toxicity can be suggested. Namely, it has been shown that inhibition of BVR-A increases cellular glucose intake (Lerner Marmarosh et al. 2005). If the function of BVR-A in line B would be abnormal, it might lead to accumulation of biliverdin, and concurrently to increased cellular glucose intake and thus better TCDD resistance, assuming that problems in cellular glucose intake would be crucial to TCDD's acute lethality. It is known that TCDD has effects on cellular glucose uptake and metabolism (Enan et al. 1992; Liu and Matsumura 1995, 2006; Olsen et al. 1994; Tonack et al. 2007; Viluksela et al. 1999), however, the significance of these effects in TCDD-induced acute lethality is unresolved.

6.2.2 Does oxidative stress play a role in biliverdin accumulation?

According to an early hypothesis, TCDD-induced oxidative stress might be an important player in TCDD-induced biliverdin accumulation, as oxidative stress is known to induce HO-1 (Section 2.3.3 and references therein) and thereby increase heme degradation. On the other hand, it was pondered whether the accumulated biliverdin and its conjugates might be formed oxidatively from bilirubin and its conjugates in TCDD-exposed rats. Quite surprisingly, TCDD did not induce hepatic mRNA expression of HO-1 in any studied rat strain, however, it clearly induced protein expression of HO-1, although only after the dose 100 µg/kg or more (Table 11; **IV**, **V**). In spleen, TCDD even dramatically decreased the expression of HO-1 mRNA (Table 11; **IV**). Therefore, while TCDD-induced oxidative stress may explain part of the observed HO-1 induction, increased hepatic free heme concentration may be even more important effector, as discussed in Section 6.1.2. In concordance with this, splenic decrease in HO-1 mRNA expression could be

explained by a quantitative swift of heme degradation from spleen to liver – for an unknown but TCDD-related reason.

What comes to the second hypothesis, that biliverdin and its conjugates might be formed oxidatively from bilirubin and its conjugates, the results of correlation analysis do not support this option (Figure 8; **V**). As explained in Section 6.1.3, biliverdin conjugates seem to be formed as secondary products of biliverdin. This conclusion is supported by literature, as chemical oxidation of bilirubin mostly leads to degradation products other than biliverdin (McDonagh 2010c). On the other hand, presence of hepatic bilirubin oxidase has been shown in rats, mice and humans (Abu-Bakar et al. 2011, 2012; Cardenas-Vazguez et al. 1986), and the reaction catalyzed by it apparently leads to formation of biliverdin. It is indeed possible that this reaction takes place in the livers of TCDD-exposed rats, however, it does not seem to play a major role in TCDD-induced biliverdin accumulation for the following two reasons. Firstly, correlation between biliverdin and bilirubin was poor (Figure 8; **V**). Secondly, biliverdin conjugates rather than free compound were the main accumulating form of biliverdin (**V**).

Third possibility of oxidative stress as a cause for biliverdin accumulation is related to the observations of Barone et al. (2011b) that in human brain, oxidative or nitrosative stress-induced modifications decrease the activity of BVR-A. This is an intriguing observation, however, we did not observe decreased hepatic BVR-A activity *in vitro* in rats whose hepatic biliverdin content was largely increased (**V**). As the oxidative stress-induced modifications mostly seem to be covalent (Barone et al. 2011b), they hardly are reversible without enzymatic action, for which reason *in vivo* inhibition of enzymatic activity should be observable in *in vitro* measurements in study settings used in **IV** and **V**. Therefore, this possibility for oxidative stress as the fundamental cause of biliverdin accumulation in TCDD-exposed rats does not seem likely either. Only at the late stage of the biliverdin accumulation syndrome, when the livers were swollen and leaked black fluid, clear decrease in BVR-A activity was observed *in vitro* (**IV**), but this does not explain biliverdin accumulation at earlier stages of the syndrome, when macroscopic signs of biliverdin accumulation were very mild or absent, but the HPLC analysis showed significantly elevated levels of biliverdin and its conjugates (**V**).

In conclusion, the current knowledge on the cellular effects of TCDD-induced oxidative stress does not provide ultimate explanations for biliverdin accumulation. Problems or insufficiency in the function of BVR-A are anyhow possible, especially those reversible in nature, which option was discussed in previous section (6.2.1).

6.2.3 Possible linkage of biliverdin accumulation and hepatic peliosis

In line B rats, a concurrent TCDD-induced finding with biliverdin accumulation was dilatation of hepatic sinusoids which eventually reached hepatic peliosis –like state. In this condition, liver parenchyma is substituted with blood-filled cavities (Tzirogiannis et al. 2004). TCDD-induced sinusoidal distension/hepatic peliosis does not require wild-type AHR as it was detected also in line A and H/W rats, however, these strains showed milder effects than line B rats. After our findings (**I**), TCDD-induced sinusoidal distension has been described in mice and rhesus monkeys during repeated or long-term exposures (Chang et al. 2005; Korenaga et al. 2007). In rats and mice, TCDD-induced sinusoidal distension has been found to start at the centrilobular area of hepatic tissue, which is also the area of AHR localization in liver (**III**; Chang et al. 2005; Lindros et al. 1997). There was no clear potency difference between TCDD and HxCDD in the ability to cause sinusoidal distension, in fact, HxCDD induced the worst cases (**III**). Altogether, sinusoidal distension may be quite a common effect of dioxins, if the administrated dose is relatively high and/or duration of exposure subacute, subchronic or chronic.

In humans, hepatic peliosis has been detected in context of various infections, chronic illnesses, malignancies and drugs, for example, human immunodeficiency virus (HIV) infection, hepatocellular carcinoma, tuberculosis, anabolic steroids, oral contraceptives, corticosteroids and many other (Bashir et al. 2012; Perkocha et al. 1990). However, it is an uncommon finding and its pathogenesis is uncertain (Bashir et al. 2012; Gushiken 2000). Hypothesis for the pathogenesis of hepatic peliosis includes breakdown of the sinusoidal borders / sinusoidal epithelial damage, hepatic outflow obstruction and dilatation of the central vein of the hepatic lobule (Bashir et al. 2012; Gushiken 2000). Interestingly, thrombocytopenia has been shown to induce hepatic peliosis (Sullivan et al. 2010) and thrombocytopenia is one of the effects of dioxins in rats (Pohjanvirta and Tuomisto et al. 1994; Viluksela et al. 1998). The essentiality of peliotic findings in regard to biliverdin accumulation comes from the increased amount of blood in liver due to this condition, which might contribute to the increased hepatic and/or serum levels of biliverdin and bilirubin detected in TCDD-treated rats, as it is plausible that increased amount of blood in liver would lead to increased hepatic heme degradation and subsequent elevation of biliverdin and bilirubin levels. On the other hand, TCDD increases serum bilirubin levels with lower doses and/or sooner than what probably is required for sinusoidal distension (**III**, **V**; Simanainen et al. 2003), suggesting that the TCDD-induced heme degradation starts before sinusoidal distension can be detected in histological examination. In fact, equimolar amounts of carbon monoxide, an established vasorelaxant (Wu and Wang 2005), is formed during heme degradation (Figure 7). This prompts to speculate, whether the probable increased production of carbon monoxide might contribute to TCDD-induced hepatic peliosis.

6.3 HxCDD-specific mechanism of dioxin toxicity

The results of Study **III** suggest that two different mechanisms of PCDD lethality and toxicity exist. The other is the classical AHR-mediated mechanism, where both *AHR* and gene *B* affect sensitivity to responses such as mortality, body weight loss and biliverdin accumulation. Further, TEFs mainly apply. In contrast, the other mechanism seems to be specifically related to HxCDD and possibly to another higher chlorinated PCDD, 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (Pohjanvirta et al. 1995). The typical endpoints of the HxCDD-specific mechanism are mortality, rapid body weight loss, fatty degeneration of the liver, and gastrointestinal hemorrhage. The lack of differences between rat strains/lines in these responses suggests that the *AHR* and gene *B* do not play a role here. The HxCDD-specific mechanism is not very sensitive, as the effects started to appear only when the dose was 600 (fatty liver) – 2000 µg/kg (other effects) or more. Further studies are needed to clarify the HxCDD-mechanism at the molecular level. One of its main manifestations is liver toxicity: fatty liver and hepatic peliosis. Thus, in addition to the AHR-mediated mechanism, HxCDD seems to impair the function of hepatocytes via another mechanism not used by TCDD. Another possibility is that TCDD indeed uses the HxCDD-specific mechanism, but with such small potency that the responses are always masked by the AHR-mediated effects. The responses mediated by the HxCDD-mechanism are probably modified and confounded by the AHR-mediated effects.

6.4 Implications for dioxin toxicology, risk assessment and heme metabolism

Implications for dioxin toxicology

The results of this thesis strongly suggest that **TCDD increases hepatic heme degradation in rats**, which is a novel finding. The observed threshold for this effect was 10 µg/kg TCDD in line B rats, as elevation of unconjugated serum bilirubin was observed with this dose (**V**). As discussed above, increased heme degradation might be a consequence of increased amount of free heme in liver, in addition, oxidative stress might play a role. On the other hand, splenic degradation of heme seems to be decreased (**IV**). Clarifying the pathogenesis of decreased splenic and increased hepatic heme degradation might be interesting in regard to toxic mechanisms of TCDD. On the other hand, all products of heme degradation – carbon monoxide, iron and biliverdin/bilirubin – are biologically active and might thus either potentiate or alleviate the toxicity of TCDD.

Other important implications of this study are those related to **gene *B*** and **the ultimate cause of biliverdin accumulation**. Unfortunately, the identity of gene *B* still remains unknown, however, the results of Study **III** support previous views

(Simanainen et al. 2003; Tuomisto et al. 1999b) that gene *B* is most likely part of the AHR signaling cascade. It remains unclear, whether B^{hw} allele increases TCDD-resistance independently of biliverdin metabolism, or whether B^{hw} allele – in addition to increasing TCDD resistance – is a prerequisite for substantial accumulation of biliverdin and its conjugates.

The ultimate cause of biliverdin accumulation is related to increased heme degradation due to TCDD exposure and, most probably, concurrent insufficient action of BVR-A on biliverdin in rats expressing wild-type AHR (**V**). In addition to this connection, both BVR-A and TCDD have complicated connections to protein kinase activities (Barone et al. 2014; Carlson and Perdew 2002; Ebner et al. 1993; Gibbs et al. 2012) and glucose transport (Lerner-Marmarosh et al. 2005; Olsen et al. 1994; Tonack et al. 2007), which might be crucial to TCDD-induced mortality and other toxicity. Therefore, in order to reveal downstream pathways of dioxin toxicity beyond AHR activation, studies concerning the various functions of BVR-A in relation to dioxin toxicity might be worthwhile. Even a speculative view could be constructed on how possible abnormalities in the function of BVR-A in line B rats might explain both increased TCDD-resistance and biliverdin accumulation (Section 6.2.1, *Choice 2*) – it would be very interesting if someone could prove this view right or wrong in the future.

Implications for dioxin risk assessment

This study does not have direct effects on dioxin risk assessment since all the effects were observed after moderately or very high doses of TCDD or HxCDD, to which humans do not get exposed in their everyday life. However, results provide indirect impacts on risk assessment. Most importantly, TCDD-induced biliverdin accumulation was most pronounced in semi-TCDD-resistant rat strains such as line B, absent in resistant strains such as line A, and very mild in sensitive L-E rats. Therefore, although biliverdin accumulation clearly belongs to Type II dioxin endpoints, it is an especially complex response, as most other Type II responses are most pronounced in sensitive rat strains such as L-E. Thus, biliverdin accumulation serves as a reminder that **differences in genes other than AHR may also have major influence on the manifestations of dioxin toxicity**. This should be kept in mind when carrying out dioxin risk assessments.

The present results on acute toxicity of HxCDD (**III**) suggest caution in regard to TEF-concept, as the **AHR-mediated effects may not cover all toxic effects of PCDDs** and **unexpected effects are possible** due to e.g. genetic variation or after high doses. However, the results do not warrant change in the settled TEF value of HxCDD (0.1), since the AHR does not seem to be involved in HxCDD-specific

toxicity, and the HxCDD-specific toxic effects appeared only after very high doses, to which humans are unlikely to be exposed.

Implications for heme metabolism

Results of this thesis suggest that **efficient hepatic conjugation of biliverdin in rats is possible** (**V**). Conjugation of biliverdin has been suggested to occur in mammals (Garay et al. 1965), but definite proof is lacking in literature. Possibly biliverdin has other potential fates in the body in addition to reduction to bilirubin.

Technical implications

TCDD was found to modify the expression level of several housekeeping genes (**II**). Therefore, **the housekeeping gene used for normalization of qRT-PCR data in toxicological dioxin studies should be carefully validated in advance.** Among the 18 housekeeping genes studied, the expression levels of *GAPDH* and *Pgk 1* were most refractory to changes due to TCDD exposure (**II**), therefore, they are good first-line candidates for normalization of gene expression data in dioxin studies.

7 Conclusions

1. In semi-TCDD-resistant line B rats, TCDD causes a novel type of liver toxicity characterized by hepatic accumulation of green pigment. According to HPLC-MS/MS analysis, the accumulating pigment consists of the heme degradation product biliverdin and its conjugates, mainly biliverdin monoglucuronide. Biliverdin conjugates are most likely secondary products of biliverdin. An associated finding to TCDD-induced biliverdin accumulation was sinusoidal distension eventually progressing to hepatic peliosis. In contrast to biliverdin accumulation, also TCDD-resistant rats are prone to sinusoidal distension.

2. At least one AHR^{wt} allele is required for biliverdin accumulation. TCDD-resistant rat strains such as line A and H/W expressing only mutated AHR seem to be completely protected from the syndrome, whereas a slight increase in hepatic biliverdin was observed in TCDD-sensitive L-E rats due to TCDD exposure. Biliverdin accumulation belongs to Type II dioxin endpoints, which are sensitive to alterations in the transactivation domain of AHR.

3. In line B rats, the lowest observed adverse effect level (LOAEL) for hepatic biliverdin accumulation is 100 µg/kg TCDD after subacute exposure (28 days), which is somewhat more than the observed LOAEL for wasting (30 µg/kg TCDD) and significantly more than the LOAEL for increased serum unconjugated bilirubin (10 µg/kg TCDD).

4. The principal cause for TCDD-induced increase in serum bilirubin, a well-known effect in many rat strains, is increased hepatic heme degradation, not decreased biliary excretion of bilirubin. The probable reason for TCDD-induced biliverdin accumulation in line B and L-E rats is increased heme degradation coupled with non-quantitative conversion of biliverdin to bilirubin. The molecular cause for the non-quantitative conversion of biliverdin to bilirubin remains obscure, however, it is possible that TCDD exposure leads to insufficient catalytic potential of BVR-A in line B rats versus, for example, in line A rats, relative to the actual need for BVR-A activity during increased heme degradation.

5. Results support the role of gene *B* as part of the AHR signaling cascade. In regard to biliverdin accumulation, two possible roles for B^{hw} allele exist: either B^{hw} is unrelated to biliverdin metabolism but enables detection of the syndrome via increased TCDD-resistance of the rat, or B^{hw} allele simultaneously increases rat´s TCDD-resistance and is necessary for substantial accumulation of biliverdin.

6. In addition to biliverdin, protein kinase activities and glucose transport are aspects connecting BVR-A and TCDD. Therefore, studies in these areas might give further insight into mechanisms of TCDD toxicity, including the biliverdin accumulation syndrome.

7. HxCDD did not induce biliverdin accumulation but it induced severe hepatic peliosis. There seem to be two different mechanisms of PCDD toxicity and lethality. One is the classical AHR-mediated mechanism where TEFs apply. The other is characteristic to HxCDD, whereas if TCDD ignites this mechanism at all, it is a weak inducer. Typical endpoints of HxCDD-specific mechanism are liver toxicity, body weight loss, gastrointestinal hemorrhage and mortality. HxCDD-specific toxic effects appear only after high doses.

8. The expression level of several housekeeping genes is modified by dioxin exposure.

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9 References

Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev. 2008; 60(1):79-127.

Abu-Bakar A, Arthur DM, Aganovic S, Ng JC, Lang MA. Inducible bilirubin oxidase: a novel function for the mouse cytochrome P450 2A5. Toxicol Appl Pharmacol. 2011; 257(1):14-22.

Abu-Bakar A, Arthur DM, Wikman AS, Rahnasto M, Juvonen RO, Vepsäläinen J, Raunio H, Ng JC, Lang MA. Metabolism of bilirubin by human cytochrome P450 2A6. Toxicol Appl Pharmacol. 2012; 261(1):50-58.

Ahlfors CE. Competitive interaction of biliverdin and bilirubin only at the primary bilirubin binding site on human albumin. Anal Biochem. 1981; 110(2):295-307.

Ahmad Z, Salim M, Maines MD. Human biliverdin reductase is a leucine zipper-like DNA-binding protein and functions in transcriptional activation of heme oxygenase-1 by oxidative stress. J Biol Chem. 2002; 277(11):9226-9232.

Akhtar FZ, Garabrant DH, Ketchum NS, Michalek JE. Cancer in US Air Force veterans of the Vietnam War. J Occup Environ Med. 2004; 46(2):123-136.

Alaluusua S, Calderara P, Gerthoux PM, Lukinmaa P-L, Kovero O, Needham L, Patterson DG, Tuomisto J, Mocarelli P. Developmental dental aberrations after the dioxin accident in Seveso. Environ Health Perspect. 2004; 112(13):1313-1318.

Alaluusua S, Lukinmaa P-L, Pohjanvirta R, Unkila M, Tuomisto J. Exposure to 2,3,7,8-tetrachlorodibenzo-*para*-dioxin leads to defective dentin formation and pulpal perforation in rat incisor tooth. Toxicology. 1993; 81(1):1-13.

Alaluusua S, Lukinmaa P-L, Torppa J, Tuomisto J, Vartiainen T. Developing teeth as biomarker of dioxin exposure. Lancet. 1999; 353(9148):206.

Alaluusua S, Lukinmaa P-L, Vartiainen T, Partanen M, Torppa J, Tuomisto J. Polychlorinated dibenzo-*p*-dioxins and dibenzofurans via mother´s milk may cause developmental defects in the child´s teeth. Environ Toxicol Pharmacol. 1996; 1(3):193-197.

Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des. 2003; 9(30):2499-2511.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem. 1999; 274(37):26071-26078.

Alonso-Magdalena P, Quesada I, Nadal A. Endocrine disruptors in the etiology of type 2 diabetes mellitus. Nat Rev Endocrinol. 2011; 7(6):346-353.

Alsharif NZ, Lawson T, Stohs SJ. Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is mediated by the aryl hydrocarbon (Ah) receptor complex. Toxicology. 1994; 92(1-3):39-51.

An Y, Bekesova S, Edwards N, Goldman R. Peptides in low molecular weight fraction of serum

associated with hepatocellular carcinoma. Dis Markers. 2010; 29(1):11-20.

Baccarelli A, Giacomini SM, Corbetta C, Landi MT, Bonzini M, Consonni D, Grillo P, Patterson DG, Pesatori AC, Bertazzi PA. Neonatal thyroid function in Seveso 25 years after maternal exposure to dioxin. PLoS Med. 2008; 5(7):e161.

Barañano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. Proc Natl Acad Sci U S A. 2002; 99(25):16093-16098.

Barone E, Di Domenico F, Cenini G, Sultana R, Cini C, Preziosi P, Perluigi M, Mancuso C, Butterfield DA. Biliverdin reductase-A protein levels and activity in the brains of subjects with Alzheimer disease and mild cognitive impairment. Biochim Biophys Acta. 2011a; 1812(4):480-487.

Barone E, Di Domenico F, Cenini G, Sultana R, Coccia R, Preziosi P, Perluigi M, Mancuso C, Butterfield DA. Oxidative and nitrosative modifications of biliverdin reductase-A in the brain of subjects with Alzheimer's disease and amnestic mild cognitive impairment. J Alzheimers Dis 2011b; 25(4):623-633.

Barone E, Di Domenico F, Mancuso C, Butterfield DA. The Janus face of the heme oxygenase/biliverdin reductase system in Alzheimer disease: It's time for reconciliation. Neurobiol Dis. 2014; 62:144-159.

Barone E, Mancuso C, Di Domenico F, Sultana R, Murphy MP, Head E, Butterfield DA. Biliverdin reductase-A: a novel drug target for atorvastatin in a dog pre-clinical model of Alzheimer disease. J Neurochem. 2012; 120(1):135-146.

Bashir O, Gaillard F et al. Hepatic peliosis. 2012. Available: http://radiopaedia.org/articles/hepatic-peliosis

Basu NK, Ciotti M, Hwang MS, Kole L, Mitra PS, Cho JW, Owens IS. Differential and special properties of the major human *UGT1*-encoded gastrointestinal UDP-glucuronosyltransferases enhance potential to control chemical uptake. J Biol Chem. 2004; 279(2):1429-1441.

Beatty PW, Vaughn WK, Neal RA. Effect of alteration of rat hepatic mixed-function oxidase (MFO) activity on the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicol Appl Pharmacol. 1978; 45(2):513-519.

Bell DR, Clode S, Fan MQ, Fernandes A, Foster PM, Jiang T, Loizou G, MacNicoll A, Miller BG, Rose M, Tran L, White S. Interpretation of studies on the developmental reproductive toxicology of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in male offspring. Food Chem Toxicol. 2010; 48(6):1439-1447.

Bertazzi PA, Bernucci I, Brambilla G, Consonni D, Pesatori AC. The Seveso studies on early and long-term effects of dioxin exposure: a review. Environ Health Perspect. 1998; 106(Suppl 2):625-633.

Bertazzi PA, Consonni D, Bachetti S, Rubagotti M, Baccarelli A, Zocchetti C, Pesatori AC. Health effects of dioxin exposure: a 20-year mortality study. Am J Epidemiol. 2001; 153(11):1031-1044.

Birnbaum LS. Developmental effects of dioxins and related endocrine disrupting chemicals. Toxicol Lett. 1995; 82-83:743-750.

Birnbaum LS, Cummings AM. Dioxins and endometriosis: a plausible hypothesis. Environ Health Perspect. 2002; 110(1):15-21.

Birnbaum LS, Fenton SE. Cancer and developmental exposure to endocrine disruptors. Environ Health Perspect. 2003; 111(4):389-394.

Birnbaum LS, Tuomisto J. Non-carcinogenic effects of TCDD in animals. Food Addit Contam. 2000; 17(4):275-288.

Blauer G, Wagnière G. Conformation of bilirubin and biliverdin in their complexes with serum albumin. J Am Chem Soc. 1975; 97(7):1949-1954.

Bock KW. Regulation of bilirubin clearance by ligand-activated transcription factors of the endo- and xenobiotic metabolism system. Front Pharmacol. 2011a; 2:82.

Bock KW. From differential induction of UDP-glucuronosyltransferases in rat liver to characterization of responsible ligand-activated transcription factors, and their multilevel crosstalk in humans. Biochem Pharmacol. 2011b; 82(1):9-16.

Bock KW, Köhle C. The mammalian aryl hydrocarbon (Ah) receptor: from mediator of dioxin toxicity toward physiological functions in skin and liver. Biol Chem. 2009; 390(12):1225-1235.

Bock KW, Köhle C. Contributions of the Ah receptor to bilirubin homeostasis and its antioxidative and atheroprotective functions. Biol Chem. 2010; 391(6):645-653.

Bonkovsky HL, Wood SG, Howell SK, Sinclair PR, Lincoln B, Healey JF, Sinclair JF. High-performance liquid chromatographic separation and quantitation of tetrapyrroles from biological materials. Anal Biochem. 1986; 155(1):56-64.

Boutros PC, Yan R, Moffat ID, Pohjanvirta R, Okey AB. Transcriptomic responses to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in liver: comparison of rat and mouse. BMC Genomics. 2008; 9:419.

Boutros PC, Yao CQ, Watson JD, Wu AH, Moffat ID, Prokopec SD, Smith AB, Okey AB, Pohjanvirta R. Hepatic transcriptomic responses to TCDD in dioxin-sensitive and dioxin-resistant rats during the onset of toxicity. Toxicol Appl Pharmacol. 2011; 251(2):119-129.

Boverhof DR, Burgoon LD, Tashiro C, Chittim B, Harkema JR, Jump DB, Zacharewski TR. Temporal and dose-dependent hepatic gene expression patterns in mice provide new insights into TCDD-mediated hepatotoxicity. Toxicol Sci. 2005; 85(2):1048-1063.

Boverhof DR, Burgoon LD, Tashiro C, Sharratt B, Chittim B, Harkema JR, Mendrick DL, Zacharewski TR. Comparative toxicogenomic analysis of the hepatotoxic effects of TCDD in Sprague Dawley rats and C57BL/6 mice. Toxicol Sci. 2006; 94(2):398-416.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.

Braggins PE, Trakshel GM, Kutty RK, Maines MD. Characterization of two heme oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun. 1986; 141(2):528-533.

Brewster DW, Uraih LC, Birnbaum LS. The acute toxicity of 2,3,4,7,8-pentachlorodibenzofuran (4PeCDF) in the male Fischer rat. Fundam Appl Toxicol. 1988; 11(2):236-249.

Brower JO, Lightner DA, McDonagh AF. Aromatic congeners of bilirubin: synthesis, stereochemistry, glucuronidation and hepatic transport. Tetrahedron. 2001; 57(37):7813-7827.

Bulmer AC, Ried K, Blanchfield JT, Wagner KH. The anti-mutagenic properties of bile pigments. Mutat Res. 2008; 658(1-2):28-41.

Bulmer AC, Ried K, Coombes JS, Blanchfield JT, Toth I, Wagner KH. The anti-mutagenic and antioxidant effects of bile pigments in the Ames Salmonella test. Mutat Res. 2007; 629(2):122-132.

Buu-Hoi NP, Chanh PH, Sesque G, Azum-Gelade MC, Saint-Ruf G. Enzymatic functions as targets of the toxicity of "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). Naturwissenschaften. 1972; 59(4):173-174.

Cantoni L, Salmona M, Rizzardini M. Porphyrogenic effect of chronic treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female rats. Dose – effect relationship following urinary excretion of porphyrins. Toxicol Appl Pharmacol. 1981; 57(2):156-163.

Cardenas-Vazquez R, Yokosuka O, Billing BH. Enzymic oxidation of unconjugated bilirubin by rat liver. Biochem J. 1986; 236(3):625-633.

Carlson DB, Perdew GH. A dynamic role for the Ah receptor in cell signaling? Insights from a diverse group of Ah receptor interacting proteins. J Biochem Mol Toxicol. 2002; 16(6):317-325.

Chang JW, Ou HY, Chen HL, Guo HR, Liao PC, Lee CC. Interrelationship between exposure to PCDD/Fs and hypertension in metabolic syndrome in Taiwanese living near a highly contaminated area. Chemosphere. 2010; 81(8):1027-1032.

Chang H, Wang YJ, Chang LW, Lin P. A histochemical and pathological study on the interrelationship between TCDD-induced AhR expression, AhR activation, and hepatotoxicity in mice. J Toxicol Environ Health A. 2005; 68(17-18):1567-1579.

Choi AM, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol. 1996; 15(1):9-19.

Ciftci O, Ozdemir I. Protective effects of quercetin and chrysin against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced oxidative stress, body wasting and altered cytokine productions in rats. Immunopharmacol Immunotoxicol. 2011; 33(3):504-508.

Cohen AN, Kapitulnik J, Ostrow JD, Webster CC. Effect of combined treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and phototherapy on bilirubin metabolism in the jaundiced Gunn rat. Hepatology. 1986; 6(3):490-494.

Converso DP, Taillé C, Carreras MC, Jaitovich A, Poderoso JJ, Boczkowski J. HO-1 is located in liver mitochondria and modulates mitochondrial heme content and metabolism. FASEB J. 2006; 20(8):1236-1238.

Couture LA, Elwell MR, Birnbaum LS. Dioxin-like effects observed in male rats following exposure to octachlorodibenzo-p-dioxin (OCDD) during a 13-week study. Toxicol Appl Pharmacol. 1988; 93(1):31-46.

Covaci A, Voorspoels S, Schepens P, Jorens P, Blust R, Neels H. The Belgian PCB/dioxin crisis-8 years later An overview. Environ Toxicol Pharmacol. 2008; 25(2):164-170.

Cranmer M, Louie S, Kennedy RH, Kern PA, Fonseca VA. Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is associated with hyperinsulinemia and insulin resistance. Toxicol Sci. 2000; 56(2):431-436.

Croutch CR, Lebofsky M, Schramm K-W, Terranova PF, Rozman KK. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin (HxCDD) alter body weight by decreasing insulin-like growth factor I (IGF-I) signaling. Toxicol Sci. 2005; 85(1):560-571.

Davies R, Clothier B, Robinson SW, Edwards RE, Greaves P, Luo J, Gant TW, Chernova T, Smith AG. Essential role of the AH receptor in the dysfunction of heme metabolism induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Chem. Res. Toxicol. 2008; 21(2):330-340.

Dennery PA, McDonagh AF, Spitz DR, Rodgers PA, Stevenson DK. Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia. Free Radic Biol Med. 1995; 19(4):395-404.

Doré S, Takahashi M, Ferris CD, Hester LD, Guastella D, Snyder SH. Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. Proc Natl Acad Sci U S A. 1999; 96(5):2445-2450.

Downey DC. Porphyria and chemicals. Med Hypotheses. 1999; 53(2):166-171.

Ebner K, Matsumura F, Enan E, Olsen H. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) alters pancreatic membrane tyrosine phosphorylation following acute treatment. J Biochem Toxicol. 1993; 8(2):71-81.

Elferink CJ, Whitlock JP. Dioxin-dependent, DNA sequence-specific binding of a multiprotein complex containing the Ah receptor. Receptor. 1994; 4(3):157-173.

Enan E, Liu PC, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes reduction of glucose transporting activities in the plasma membranes of adipose tissue and pancreas from the guinea pig. J Biol Chem. 1992; 267(28):19785-19791.

Eskenazi B, Mocarelli P, Warner M, Samuels S, Vercellini P, Olive D, Needham LL, Patterson DG, Brambilla P, Gavoni N, Casalini S, Panazza S, Turner W, Gerthoux PM. Serum dioxin concentrations and endometriosis: a cohort study in Seveso, Italy. Environ Health Perspect. 2002; 110(7):629-634.

Eskenazi B, Warner M, Marks AR, Samuels S, Needham L, Brambilla P, Mocarelli P. Serum dioxin concentrations and time to pregnancy. Epidemiology. 2010; 21(2):224-231.

Eskew JD, Vanacore RM, Sung L, Morales PJ, Smith A. Cellular protection mechanisms against extracellular heme. Heme-hemopexin, but not free heme, activates the N-terminal c-jun kinase. J Biol Chem. 1999; 274(2):638-648.

EU (European Union) Standing Committee on the Food Chain and Animal Health, Statement 2011. Available: http://ec.europa.eu/food/food/chemicalsafety/contaminants/20110222_statement_final_en.pdf

Ewing JF, Maines MD. Rapid induction of heme oxygenase 1 mRNA and protein by hyperthermia in rat brain: heme oxygenase 2 is not a heat shock protein. Proc Natl Acad Sci U S A. 1991; 88(12):5364-5368.

Falchuk KH, Contin JM, Dziedzic TS, Feng Z, French TC, Heffron GJ, Montorzi M. A role for biliverdin IXα in dorsal axis development of *Xenopus laevis* embryos. Proc Natl Acad Sci U S A. 2002; 99(1):251-256.

Fang J, Akaike T, Maeda H. Antiapoptotic role of heme oxygenase (HO) and the potential of HO as a target in anticancer treatment. Apoptosis. 2004; 9(1):27-35.

Fang LS, Bada JL. The blue-green blood plasma of marine fish. Comp Biochem Physiol B. 1990; 97(1):37-45.

Feeley M, Brouwer A. Health risks to infants from exposure to PCBs, PCDDs and PCDFs. Food Addit Contam. 2000; 17(4):325-333.

FELASA: Rehbinder C, Baneux P, Forbes D, van Herck H, Nicklas W, Rugaya Z, Winkler G. FELASA recommendations for the health monitoring of mouse, rat, hamster, gerbil, guinea pig and rabbit experimental units. Report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Animal Health accepted by the FELASA Board of Management, November 1995. Lab Anim. 1996 30(3):193-208.

FELASA: Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, Illgen-Wilcke B; FELASA (Federation of European Laboratory Animal Science Associations Working Group on Health Monitoring of Rodent and Rabbit Colonies). Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim. 2002; 36(1):20-42.

Fenech FF, Bannister WH, Grech JL. Hepatitis with biliverdinaemia in association with indomethacin therapy. Br Med J. 1967; 3(5558):155-156.

Fletcher N, Wahlström D, Lundberg R, Nilsson CB, Nilsson KC, Stockling K, Hellmold H, Håkansson H. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis, and bile transport in rat liver: a microarray study. Toxicol Appl Pharmacol. 2005; 207(1):1-24.

Florczyk U, Golda S, Zieba A, Cisowski J, Jozkowicz A, Dulak J. Overexpression of biliverdin reductase enhances resistance to chemotherapeutics. Cancer Lett. 2011; 300(1):40-47.

Florczyk UM, Jozkowicz A, Dulak J. Biliverdin reductase: new features of an old enzyme and its potential therapeutic significance. Pharmacol Rep. 2008; 60(1):38-48.

Foresti R, Green CJ, Motterlini R. Generation of bile pigments by haem oxygenase: a refined cellular strategy in response to stressful insults. Biochem Soc Symp. 2004; 71:177-192.

Franklin E, Browne S, Hayes J, Boland C, Dunne A, Elliot G, Mantle TJ. Activation of biliverdin-IXα reductase by inorganic phosphate and related anions. Biochem J. 2007; 405(1):61-67.

Furuyama K, Kaneko K, Vargas PD. Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. Tohoku J Exp Med. 2007; 213(1):1-16.

Gambone CJ, Hutcheson JM, Gabriel JL, Beard RL, Chandraratna RA, Soprano KJ, Soprano DR. Unique property of some synthetic retinoids: activation of the aryl hydrocarbon receptor pathway. Mol Pharmacol. 2002; 61(2):334-342.

Garay ER, Noir B, Royer M. Biliverdin pigments in green biles. Biochim Biophys Acta. 1965; 100:411-417.

Geusau A, Abraham K, Geissler K, Sator MO, Stingl G, Tschachler E. Severe 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) intoxication: clinical and laboratory effects. Environ Health Perspect. 2001; 109(8):865-869.

Gibbs PE, Maines MD. Biliverdin inhibits activation of NF-κB: reversal of inhibition by human biliverdin reductase. Int J Cancer. 2007; 121(11):2567-2574.

Gibbs PE, Miralem T, Lerner-Marmarosh N, Tudor C, Maines MD. Formation of a ternary complex of human biliverdin reductase-protein kinase Cδ-ERK2 protein is essential for ERK2-mediated activation of Elk1 protein, NF-κB and inducible nitric-oxidase synthase (iNOS). J Biol Chem. 2012; 287(2):1066-1079.

Gibbs PE, Miralem T, Maines MD. Characterization of the human biliverdin reductase gene structure and regulatory elements: promoter activity is enhanced by hypoxia and suppressed by TNF-α-activated NF-κB. FASEB J. 2010; 24(9):3239-3254.

Gibbs PE, Tudor C, Maines MD. Biliverdin reductase: more than a namesake - the reductase, its peptide fragments, and biliverdin regulate activity of the three classes of protein kinase C. Front Pharmacol. 2012; 3:31.

Goldstein JA, Hickman P, Bergman H, Vos JG. Hepatic porphyria induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the mouse. Res Commun Chem Pathol Pharmacol. 1973; 6(3):919-928.

Goldstein JA, Linko P, Bergman H. Induction of porphyria in the rat by chronic versus acute exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Biochem Pharmacol. 1982; 31(8):1607-1613.

Goodman DG, Sauer RM. Hepatotoxicity and carcinogenicity in female Sprague-Dawley rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): a pathology working group reevaluation. Regul Toxicol Pharmacol. 1992; 15, 245-252.

Gorski JR, Weber LW, Rozman K. Tissue-specific alterations of de novo fatty acid synthesis in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. Arch Toxicol. 1988; 62(2-3):146-151.

Gorski JR, Weber LW, Rozman K. Reduced gluconeogenesis in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. Arch Toxicol. 1990; 64(1):66-71.

Goven D, Boutten A, Leçon-Malas V, Marchal-Sommé J, Soler P, Boczkowski J, Bonay M. Induction of heme oxygenase-1, biliverdin reductase and H-ferritin in lung macrophage in smokers with primary spontaneous pneumothorax: role of HIF-1α. PLoS One. 2010; 5(5):e10886.

Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol. 2010; 50:323-354.

Gray LE, Ostby JS, Kelce WR. A dose-response analysis of the reproductive effects of a single

gestational dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in male Long Evans Hooded rat offspring. Toxicol Appl Pharmacol. 1997; 146(1):11-20.

Greenberg AJ, Bossenmaier I, Schwartz S. Green jaundice. A study of serum biliverdin, mesobiliverdin and other green pigments. Am J Dig Dis. 1971; 16(10):873-880.

Guberman AS, Scassa ME, Giono LE, Varone CL, Cánepa ET. Inhibitory effect of AP-1 complex on 5-aminolevulinate synthase gene expression through sequestration of cAMP-response element protein (CRE)-binding protein (CBP) coactivator. J Biol Chem. 2003; 278(4):2317-2326.

Gushiken FC. Peliosis hepatis after treatment with 2-chloro-3'-deoxyadenosine. South Med J. 2000; 93(6):625-626.

Guyton AC. Red blood cells, anemia and polycythemia. In: Guyton, AC (ed.), Textbook of Medical Physiology, 7th ed., WB Saunders Company, Philadelphia, 1986; 42-50.

Gåfvels M, Holmström P, Somell A, Sjövall F, Svensson JO, Ståhle L, Broomé U, Stål P. A novel mutation in the biliverdin reductase-A gene combined with liver cirrhosis results in hyperbiliverdinaemia (green jaundice). Liver Int. 2009; 29(7):1116-1124.

Haarmann-Stemmann T, Abel J. Influence of HIF-1α and Nrf2 signaling on AHR-mediated gene expression, toxicity, and biological functions. In: Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012; 109-126.

Hallikainen A, Airaksinen R, Rantakokko P, Koponen J, Mannio J, Vuorinen P, et al. Environmental pollutants in Baltic fish and other domestic fish: PCDD/F, PCB, PBDE, PFC and OT compounds. Finnish Food Safety Authority Evira. Report No. 2/2011.

Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE. Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. Arch Biochem Biophys. 1991; 289(2):387-392.

Hassan MQ, Stohs SJ, Murray WJ. Inhibition of TCDD-induced lipid peroxidation, glutathione peroxidase activity and toxicity by BHA and glutathione. Bull Environ Contam Toxicol. 1985; 34(6):787-796.

Hassoun EA, Al-Ghafri M, Abushaban A. The role of antioxidant enzymes in TCDD-induced oxidative stress in various brain regions of rats after subchronic exposure. Free Radic Biol Med. 2003; 35(9):1028-1036.

Himes JA, Cornelius CE. Biliverdin and bilirubin excretion in the turkey. Cornell Vet. 1975; 65(3):374-379.

Huang TJ, Maines MD. Bromobenzene-mediated alteration in activity and electrophoretic pattern of biliverdin reductase variants in rat kidney. Mol Pharmacol. 1990; 37(1):25-29.

Huang TJ, Trakshel GM, Maines MD. Detection of 10 variants of biliverdin reductase in rat liver by two-dimensional gel electrophoresis. J Biol Chem. 1989a; 264(14):7844-7849.

Huang TJ, Trakshel GM, Maines MD. Multiple forms of biliverdin reductase: modification of the pattern of expression in rat liver by bromobenzene. Arch Biochem Biophys. 1989b; 270(2):513-520.

Huang TJ, Trakshel GM, Maines MD. Microheterogeneity of biliverdin reductase in rat liver and spleen: selective suppression of enzyme variants in liver by bromobenzene. Arch Biochem Biophys. 1989c; 274(2):617-625.

Huffman C, Chillag S, Paulman L, McMahon C. It's not easy bein' green. Am J Med. 2009; 122(9):820-822.

Huuskonen H, Unkila M, Pohjanvirta R, Tuomisto J. Developmental toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the most TCDD-resistant and -susceptible rat strains. Toxicol Appl Pharmacol. 1994; 124(2):174-180.

Hwang SW. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the biliary excretion of indocyanine green in rat. Environ Health Perspect. 1973; 5:227-231.

IARC. IARC Monographs on the evaluation of carcinogenic risks to humans. Polychlorinated dibenzo-*para*-dioxins and polychlorinated dibenzofurans, 1997; 69:1–666.

Idriss NK, Blann AD, Lip GY. Hemoxygenase-1 in cardiovascular disease. J Am Coll Cardiol. 2008; 52(12):971-978.

Inamdar NM, Ahn YI, Alam J. The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. Biochem Biophys Res Commun. 1996; 221(3):570-576.

Jandal JM. Studies on biliverdin in Awassi sheep milk. Small Ruminant Research. 1995; 18(3):273-275.

Jansen PL, Bittar EE. Bilirubin metabolism. In: Bittar EE (ed). The Liver in Biology and Disease. Principles of Medical Biology, Elsevier Ltd., 2004; 15:257-289.

Jansen T, Daiber A. Direct antioxidant properties of bilirubin and biliverdin. Is there a role for biliverdin reductase? Front Pharmacol. 2012; 3:30.

Kamisako T, Kobayashi Y, Takeuchi K, Ishihara T, Higuchi K, Tanaka Y, Gabazza EC, Adachi Y. Recent advances in bilirubin metabolism research: the molecular mechanism of hepatocyte bilirubin transport and its clinical relevance. J Gastroenterol. 2000; 35(9):659-664.

Kapitulnik J. Bilirubin: an endogenous product of heme degradation with both cytotoxic and cytoprotective properties. Mol Pharmacol. 2004; 66(4):773-779.

Kapitulnik J, Gonzalez FJ. Marked endogenous activation of the *CYP1A1* and *CYP1A2* genes in the congenitally jaundiced Gunn rat. Mol Pharmacol. 1993; 43(5):722-725.

Kapitulnik J, Maines MD. Pleiotropic functions of biliverdin reductase: cellular signaling and generation of cytoprotective and cytotoxic bilirubin. Trends Pharmacol Sci. 2009; 30(3):129-137.

Kattainen H, Tuukkanen J, Simanainen U, Tuomisto JT, Kovero O, Lukinmaa P-L, Alaluusua S, Tuomisto J, Viluksela M. *In utero*/lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure impairs molar tooth development in rats. Toxicol Appl Pharmacol. 2001; 174(3):216-224.

Kawano Y, Nishiumi S, Tanaka S, Nobutani K, Miki A, Yano Y, Seo Y, Kutsumi H, Ashida H, Azuma T, Yoshida M. Activation of the aryl hydrocarbon receptor induces hepatic steatosis

via the upregulation of fatty acid transport. Arch Biochem Biophys. 2010; 504(2):221-227.

Kelling CK, Christian BJ, Inhorn SL, Peterson RE. Hypophagia-induced weight loss in mice, rats, and guinea pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Fundam Appl Toxicol. 1985; 5(4):700-712.

Kikuchi A, Yamaya M, Suzuki S, Yasuda H, Kubo H, Nakayama K, Handa M, Sasaki T, Shibahara S, Sekizawa K, Sasaki H. Association of susceptibility to the development of lung adenocarcinoma with the heme oxygenase-1 gene promoter polymorphism. Hum Genet. 2005; 116(5):354-360.

Kim HP, Wang X, Galbiati F, Ryter SW, Choi AM. Caveolae compartmentalization of heme oxygenase-1 in endothelial cells. FASEB J. 2004; 18(10):1080-1089.

Kiviranta H, Ovaskainen M-L, Vartiainen T. Market basket study on dietary intake of PCDD/Fs, PCBs, and PBDEs in Finland. Environ Int. 2004; 30(7):923-932.

Knerr S, Schrenk D. Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in experimental models. Mol Nutr Food Res. 2006; 50(10):897-907.

Kociba RJ, Keeler PA, Park CN, Gehring PJ. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): results of a 13-week oral toxicity study in rats. Toxicol Appl Pharmacol. 1976; 35(3):553-574.

Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA, Humiston CG. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. Toxicol Appl Pharmacol. 1978; 46(2):279-303.

Korenaga T, Fukusato T, Ohta M, Asaoka K, Murata N, Arima A, Kubota S. Long-term effects of subcutaneously injected 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the liver of rhesus monkeys. Chemosphere. 2007; 67(9):S399-404.

Korkalainen M, Lindén J, Tuomisto J, Pohjanvirta R. Effect of TCDD on mRNA expression of genes encoding bHLH/PAS proteins in rat hypothalamus. Toxicology. 2005; 208(1):1-11.

Korkalainen M, Tuomisto J, Pohjanvirta R. Identification of novel splice variants of ARNT and ARNT2 in the rat. Biochem Biophys Res Commun. 2003; 303(4):1095-1100.

Korkalainen M, Tuomisto J, Pohjanvirta R. Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) of aryl hydrocarbon receptor repressor in a TCDD-sensitive and a TCDD-resistant rat strain. Biochem Biophys Res Commun. 2004; 315(1):123-131.

Krasner J, Juchau MR, Niswander KR, Yaffe SJ. Theoretical considerations regarding the presence of biliverdin in amniotic fluid. Am J Obstet Gynecol. 1971; 109(1):159-165.

Krasner J, Spencer RA, Yaffe SJ. Isolation of biliverdin from amniotic fluid. Clin Chim Acta. 1969; 23(3):437-439.

Kravets A, Hu Z, Miralem T, Torno MD, Maines MD. Biliverdin reductase, a novel regulator for induction of activating transcription factor-2 and heme oxygenase-1. J Biol Chem. 2004; 279(19):19916-19923.

Kreuzer PE, Csanády GA, Baur C, Kessler W, Päpke O, Greim H, Filser JG. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and congeners in infants. A toxicokinetic model of human lifetime body burden by TCDD with special emphasis on its uptake by nutrition. Arch Toxicol. 1997; 71(6):383-400.

Krysiak-Baltyn K, Toppari J, Skakkebaek NE, Jensen TS, Virtanen HE, Schramm KW, Shen H, Vartiainen T, Kiviranta H, Taboureau O, Brunak S, Main KM. Country-specific chemical signatures of persistent environmental compounds in breast milk. Int J Androl. 2010; 33(2):270-278.

Kumar MA, Dastur NN, Ganguli NC. Biliverdin reductase-like activity in buffalo mammary tissues. Experientia. 1983; 39(9):1010-1011.

Kutty RK, Maines MD. Purification and characterization of biliverdin reductase from rat liver. J Biol Chem. 1981; 256(8):3956-3962.

Kutty RK, Maines MD. Biliverdin reductase: characterization in the rat kidney and the inhibition of activity by mercuric chloride. Biochem Pharmacol. 1983; 32(13):2095-2102.

Köhle C, Bock KW. Activation of coupled Ah receptor and Nrf2 gene batteries by dietary phytochemicals in relation to chemoprevention. Biochem Pharmacol. 2006; 72(7):795-805.

Lafarge-Frayssinet C, Morel-Chany E, Trincal G, Frayssinet C. Promoting effect of biliverdin for liver cells initiated by aflatoxin B1. Cancer Lett. 1983; 18(3):277-282.

Larson EA, Evans GT, Watson CJ. A study of the serum biliverdin concentration in various types of jaundice. J Lab Clin Med. 1947; 32(5):481-488.

Lascano Alcoser VH, Velthuis AG, Hoogenboom LA, van der Fels-Klerx HJ. Financial impact of a dioxin incident in the Dutch dairy chain. J Food Prot. 2011; 74(6):967-979.

Latchoumycandane C, Chitra KC, Mathur PP. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. Arch Toxicol. 2003; 77(5):280-284.

Lee DH, Lee IK, Song K, Steffes M, Toscano W, Baker BA, Jacobs DR Jr. A strong dose-response relation between serum concentrations of persistent organic pollutants and diabetes: results from the National Health and Examination Survey 1999-2002. Diabetes Care. 2006; 29(7):1638-1644.

Lee JH, Wada T, Febbraio M, He J, Matsubara T, Lee MJ, Gonzalez FJ, Xie W. A novel role for the dioxin receptor in fatty acid metabolism and hepatic steatosis. Gastroenterology. 2010; 139(2):653-663.

Lehmann E, El-Tantawy WH, Ocker M, Bartenschlager R, Lohmann V, Hashemolhosseini S, Tiegs G, Sass G. The heme oxygenase 1 product biliverdin interferes with hepatitis C virus replication by increasing antiviral interferon response. Hepatology. 2010; 51(2):398-404.

Lengyel G, Veres Z, Szabó P, Vereczkey L, Jemnitz K. Canalicular and sinusoidal disposition of bilirubin mono- and diglucuronides in sandwich-cultured human and rat primary hepatocytes. Drug Metab Dispos. 2005; 33(9):1355-1360.

Lensu S, Tuomisto JT, Tuomisto J, Viluksela M, Niittynen M, Pohjanvirta R. Immediate and highly sensitive aversion response to a novel

food item linked to AH receptor stimulation. Toxicol Lett. 2011; 203(3):252-257.

Lerner-Marmarosh N, Shen J, Torno MD, Kravets A, Hu Z, Maines MD. Human biliverdin reductase: a member of the insulin receptor substrate family with serine/threonine/tyrosine kinase activity. Proc Natl Acad Sci U S A. 2005; 102(20):7109-7114.

Liem AK, Fürst P, Rappe C. Exposure of populations to dioxins and related compounds. Food Addit Contam. 2000; 17(4):241-259.

Lin GL, Himes JA, Cornelius CE. Bilirubin and biliverdin excretion by the chicken. Am J Physiol. 1974; 226(4):881-885.

Lindén J, Lensu S, Tuomisto J, Pohjanvirta R. Dioxins, the aryl hydrocarbon receptor and the central regulation of energy balance. Front Neuroendocrinol. 2010; 31(4):452-478.

Lindros KO, Oinonen T, Johansson I, Ingelman-Sundberg M. Selective centrilobular expression of the aryl hydrocarbon receptor in rat liver. J Pharmacol Exp Ther. 1997; 280(1):506-511.

Liu PC, Matsumura F. Differential effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the "adipose-type" and "brain-type" glucose transporters in mice. Mol Pharmacol. 1995; 47(1):65-73.

Liu PC, Matsumura F. TCDD suppresses insulin-responsive glucose transporter (GLUT-4) gene expression through C/EBP nuclear transcription factors in 3T3-L1 adipocytes. J Biochem Mol Toxicol. 2006; 20(2):79-87.

Loonen H, van de Guchte C, Parsons JR, de Voogt P, Govers HA. Ecological hazard assessment of dioxins: hazards to organisms at different levels of aquatic food webs (fish-eating birds and mammals, fish and invertebrates). Sci Total Environ. 1996; 182(1-3):93-103.

Ma Q. Overview of AHR functional domains and the classical AHR signaling pathway: induction of drug metabolizing enzymes. In: Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012; 35-45.

Mably TA, Bjerke DL, Moore RW, Gendron-Fitzpatrick A, Peterson RE. *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 3. Effects on spermatogenesis and reproductive capability. Toxicol Appl Pharmacol. 1992a; 114(1):118-126.

Mably TA, Moore RW, Goy RW, Peterson RE. *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 2. Effects on sexual behavior and the regulation of luteinizing hormone secretion in adulthood. Toxicol Appl Pharmacol. 1992b; 114(1):108-117.

Mably TA, Moore RW, Peterson RE. *In utero* and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. 1. Effects on androgenic status. Toxicol Appl Pharmacol. 1992c; 114(1):97-107.

Maghzal GJ, Leck MC, Collinson E, Li C, Stocker R. Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. J Biol Chem. 2009; 284(43):29251-29259.

Maines MD. New insights into biliverdin reductase functions: linking heme metabolism to cell signaling. Physiology. 2005; 20(6):382-389.

Maines MD, Ewing JF, Huang TJ, Panahian N. Nuclear localization of biliverdin reductase in the rat kidney: response to nephrotoxins that induce heme oxygenase-1. J Pharmacol Exp Ther. 2001; 296(3):1091-1097.

Maines MD, Mayer RD, Erturk E, Huang TJ, Disantagnese A. The oxidoreductase, biliverdin reductase, is induced in human renal carcinoma – pH and cofactor-specific increase in activity. J Urol. 1999; 162(4):1467-1472.

Mann PC. Selected lesions of dioxin in laboratory rodents. Toxicol Pathol. 1997; 25(1):72-79.

Matsui I, Otani S, Morisawa S. Induction of ornithine decarboxylase by biliverdin in rat liver. Life Sci. 1979; 24(24):2231-2236.

Matsumura F. Nongenomic route of action of TCDD: identity, characteristics, and toxicological significance. In: Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012; 197-215.

Mayani A, Barel S, Soback S, Almagor M. Dioxin concentrations in women with endometriosis. Hum Reprod. 1997; 12(2):373-375.

McCoubrey WK, Cooklis MA, Maines MD. The structure, organization and differential expression of the rat gene encoding biliverdin reductase. Gene. 1995; 160(2):235-240.

McDonagh AF. Turning green to gold. Nat Struct Biol. 2001; 8(3):198-200.

McDonagh AF. Controversies in bilirubin biochemistry and their clinical relevance. Semin Fetal Neonatal Med. 2010a; 15(3):141-147.

McDonagh AF. Green jaundice revisited. Am J Med. 2010b; 123(9):e23.

McDonagh AF. The biliverdin-bilirubin antioxidant cycle of cellular protection: Missing a wheel? Free Radic Biol Med. 2010c; 49(5):814-820.

McDonagh AF, Palma LA, Schmid R. Reduction of biliverdin and placental transfer of bilirubin and biliverdin in the pregnant guinea pig. Biochem J. 1981; 194(1):273-282.

Miettinen H. The effects of TCDD on the development of teeth and cortical bone in rats: implications for risk assessment. Publications of the National Public Health Institute, A10/2006.

Miettinen HM, Alaluusua S, Tuomisto J, Viluksela M. Effect of *in utero* and lactational 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on rat molar development: the role of exposure time. Toxicol Appl Pharmacol. 2002; 184(1):57-66.

Milbrath MO, Wenger Y, Chang CW, Emond C, Garabrant D, Gillespie BW, Jolliet O. Apparent half-lives of dioxins, furans, and polychlorinated biphenyls as a function of age, body fat, smoking status, and breast-feeding. Environ Health Perspect. 2009; 117(3):417-425.

Miralem T, Hu Z, Torno MD, Lelli KM, Maines MD. Small interference RNA-mediated gene silencing of human biliverdin reductase, but not that of heme oxygenase-1, attenuates arsenite-mediated induction of the oxygenase and increases apoptosis in 293A kidney cells. J Biol Chem. 2005; 280(17):17084-17092.

Mocarelli P, Gerthoux PM, Ferrari E, Patterson DG, Kieszak SM, Brambilla P, Vincoli N, Signorini S, Tramacere P, Carreri V, Sampson EJ, Turner WE, Needham LL. Paternal concentrations of

dioxin and sex ratio of offspring. Lancet. 2000; 355(9218):1858-1863.

Mocarelli P, Gerthoux PM, Needham LL, Patterson DG, Limonta G, Falbo R, Signorini S, Bertona M, Crespi C, Sarto C, Scott PK, Turner WE, Brambilla P. Perinatal exposure to low doses of dioxin can permanently impair human semen quality. Environ Health Perspect. 2011; 119(5):713-718.

Mocarelli P, Gerthoux PM, Patterson DG, Milani S, Limonta G, Bertona M, Signorini S, Tramacere P, Colombo L, Crespi C, Brambilla P, Sarto C, Carreri V, Sampson EJ, Turner WE, Needham LL. Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. Environ Health Perspect. 2008; 116(1):70-77.

Mohammadpour H, Murray WJ, Stohs SJ. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-induced lipid peroxidation in genetically responsive and non-responsive mice. Arch Environ Contam Toxicol. 1988; 17(5):645-650.

Montorzi M, Dziedzic TS, Falchuk KH. Biliverdin during *Xenopus laevis* oogenesis and early embryogenesis. Biochemistry. 2002; 41(31):10115-10122.

Mori H, Otake T, Morimoto M, Ueba N, Kunita N, Nakagami T, Yamasaki N, Taji S. *In vitro* anti-human immunodeficiency virus type 1 activity of biliverdin, a bile pigment. Jpn J Cancer Res. 1991; 82(7):755-757.

Mueller C, Zhou W, VanMeter A, Heiby M, Magaki S, Ross MM, Espina V, Schrag M, Dickson C, Liotta LA, Kirsch WM. The heme degradation pathway is a promising serum biomarker source for the early detection of Alzheimer's disease. J Alzheimer's Dis. 2010; 19(3):1081-1091.

Muñoz ME, González J, Esteller A. Bile pigment formation and excretion in the rabbit. Comp Biochem Physiol A Comp Physiol. 1986; 85(1):67-71.

Murphy BJ, Laderoute KR, Short SM, Sutherland RM. The identification of heme oxygenase as a major hypoxic stress protein in Chinese hamster ovary cells. Br J Cancer. 1991; 64(1):69-73.

Muzi G, Gorski JR, Rozman K. Mode of metabolism is altered in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. Toxicol Lett. 1989; 47(1):77-86.

Nakagami T, Taji S, Takahashi M, Yamanishi K. Antiviral activity of a bile pigment, biliverdin, against human herpesvirus 6 (HHV-6) *in vitro*. Microbiol Immunol. 1992; 36(4):381-390.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem Pharmacol. 2000; 59(1):65-85.

Nguyen LP, Bradfield CA The search for endogenous activators of the aryl hydrocarbon receptor. Chem Res Toxicol. 2008; 21(1):102-116.

Niittynen M, Mantle T, Tuomisto JT, Tuomisto J, Pohjanvirta R. Cloning and sequencing of the biliverdin IXα reductase gene from a "black liver" syndrome rat. Abstracts of the Heme oxygenase: Regulation, Functions & Clinical Application, Uppsala, Sweden, Sep 28th-Oct 3rd, 2003.

Nishimura N, Miyabara Y, Suzuki JS, Sato M, Aoki Y, Satoh M, Yonemoto J, Tohyama C. Induction of metallothionein in the livers of female Sprague-Dawley rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Life Sci. 2001; 69(11):1291-1303.

Noir BA, Garay ER, Royer M. Separation and properties of conjugated biliverdin. Biochim Biophys Acta. 1965; 100:403-410.

Novelli M, Piaggi S, De Tata V. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced impairment of glucose-stimulated insulin secretion in isolated rat pancreatic islets. Toxicol Lett. 2005; 156(2):307-314.

Nuhn P, Künzli BM, Hennig R, Mitkus T, Ramanauskas T, Nobiling R, Meuer SC, Friess H, Berberat PO. Heme oxygenase-1 and its metabolites affect pancreatic tumor growth *in vivo*. Mol Cancer. 2009; 8:37.

Nytofte NS, Serrano MA, Monte MJ, Gonzalez-Sanchez E, Tumer Z, Ladefoged K, Briz O, Marin JJ. A homozygous nonsense mutation (c.214C→A) in the biliverdin reductase alpha gene (*BLVRA*) results in accumulation of biliverdin during episodes of cholestasis. J Med Genet. 2011; 48(4):219-225.

Oakes GH, Bend JR. Early steps in bilirubin-mediated apoptosis in murine hepatoma (Hepa 1c1c7) cells are characterized by aryl hydrocarbon receptor-independent oxidative stress and activation of the mitochondrial pathway. J Biochem Mol Toxicol. 2005; 19(4):244-255.

Okazaki K, Nishimura H, Arizono H, Nishimura N, Suzuki Y. Biliverdin initiates the liver regeneration in the rat – a hypothesis. Biochem Biophys Res Commun. 1978; 81(2):512-520.

Olsen H, Enan E, Matsumura F. Regulation of glucose transport in the NIH 3T3 L1 preadipocyte cell line by TCDD. Environ Health Perspect. 1994; 102(5):454-458.

Papiez MA, Dybala M, Sowa-Kucma M, Krzysciak W, Taha H, Jozkowicz A, Nowak G. Evaluation of oxidative status and depression-like responses in Brown Norway rats with acute myeloid leukemia. Prog Neuropsychopharmacol Biol Psychiatry. 2009; 33(4):596-604.

Pelclová D, Urban P, Preiss J, Lukás E, Fenclová Z, Navrátil T, Dubská Z, Senholdová Z. Adverse health effects in humans exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Rev Environ Health. 2006; 21(2):119-138.

Pereira PJ, Macedo-Ribeiro S, Párraga A, Pérez-Luque R, Cunningham O, Darcy K, Mantle TJ, Coll M. Structure of human biliverdin IXβ reductase, an early fetal bilirubin IXβ producing enzyme. Nat Struct Biol. 2001; 8(3):215-220.

Perkocha LA, Geaghan SM, Yen TS, Nishimura SL, Chan SP, Garcia-Kennedy R, Honda G, Stoloff AC, Klein HZ, Goldman RL, Van Meter S, Ferrell LD, LeBoit PE. Clinical and pathological features of bacillary peliosis hepatis in association with human immunodeficiency virus infection. N Engl J Med. 1990; 323(23):1581-1586.

Pesatori AC, Consonni D, Bachetti S, Zocchetti C, Bonzini M, Baccarelli A, Bertazzi PA. Short- and long-term morbidity and mortality in the population exposed to dioxin after the "Seveso accident". Ind Health. 2003; 41(3):127-138.

Pesatori AC, Consonni D, Rubagotti M, Grillo P, Bertazzi PA. Cancer incidence in the population exposed to dioxin after the "Seveso accident":

twenty years of follow-up. Environ Health. 2009; 8:39.

Phelan D, Winter GM, Rogers WJ, Lam JC, Denison MS. Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. Arch Biochem Biophys. 1998; 357(1):155-163.

Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012.

Pohjanvirta R. TCDD resistance is inherited as an autosomal dominant trait in the rat. Toxicol Lett. 1990; 50(1):49-56.

Pohjanvirta R, Korkalainen M, McGuire J, Simanainen U, Juvonen R, Tuomisto JT, Unkila M, Viluksela M, Bergman J, Poellinger L, Tuomisto J. Comparison of acute toxicities of indolo[3,2-*b*]carbazole (ICZ) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in TCDD-sensitive rats. Food Chem Toxicol. 2002; 40(7):1023-1032.

Pohjanvirta R, Korkalainen M, Moffat ID, Boutros PC, Okey AB. Role of the AHR and its structure in TCDD toxicity. In: Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012; 181-196.

Pohjanvirta R, Kulju T, Morselt AF, Tuominen R, Juvonen R, Rozman K, Männistö P, Collan Y, Sainio E-L, Tuomisto J. Target tissue morphology and serum biochemistry following 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in a TCDD-susceptible and a TCDD-resistant rat strain. Fundam Appl Toxicol. 1989; 12(4):698-712.

Pohjanvirta R, Sankari S, Kulju T, Naukkarinen A, Ylinen M, Tuomisto J. Studies on the role of lipid peroxidation in the acute toxicity of TCDD in rats. Pharmacol Toxicol. 1990; 66(5):399-408.

Pohjanvirta R, Tuomisto J. Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals: effects, mechanisms, and animal models. Pharmacol Rev. 1994; 46(4):483-549.

Pohjanvirta R, Unkila M, Lindén J, Tuomisto JT, Tuomisto J. Toxic equivalency factors do not predict the acute toxicities of dioxins in rats. Eur J Pharmacol. 1995; 293(4):341-353.

Pohjanvirta R, Unkila M, Tuomisto J. Comparative acute lethality of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin in the most TCDD-susceptible and the most TCDD-resistant rat strain. Pharmacol Toxicol. 1993; 73(1):52-56.

Pohjanvirta R, Viluksela M, Tuomisto JT, Unkila M, Karasinska J, Franc M-A, Holowenko M, Giannone JV, Harper PA, Tuomisto J, Okey AB. Physicochemical differences in the AH receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. Toxicol Appl Pharmacol. 1999; 155(1):82-95.

Pohjanvirta R, Wong JM, Li W, Harper PA, Tuomisto J, Okey AB. Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant rat strain. Mol Pharmacol. 1998; 54(1): 86-93.

Poland A, Glover E. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: a potent inducer of δ-aminolevulinic acid synthetase. Science. 1973; 179(4072):476-477.

Poland A, Knutson JC. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic

hydrocarbons: examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol. 1982; 22:517-554.

Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood. 1997; 89(1):1-25.

Ponka P. Overview of cellular iron metabolism: the crucial role of heme oxygenase in recycling heme iron. Abstracts of the Heme oxygenase: Regulation, Functions & Clinical Application, Uppsala, Sweden, Sep 28th- Oct 3rd, 2003.

Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci U S A. 1997; 94(20):10925-10930.

Prichard JS. Biliverdin appearing in a case of malnutrition. Br J Clin Pract. 1972; 26(10):481-482.

Puga A, Ma C, Marlowe JL. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem Pharmacol. 2009; 77(4):713-722.

Puga A, Maier A, Medvedovic M. The transcriptional signature of dioxin in human hepatoma HepG2 cells. Biochem Pharmacol. 2000; 60(8):1129-1142.

Päpke O. PCDD/PCDF: human background data for Germany, a 10-year experience. Environ Health Perspect. 1998; 106 (Suppl 2):723-731.

Radhakrishnan N, Yadav SP, Sachdeva A, Pruthi PK, Sawhney S, Piplani T, Wada T, Yachie A. Human heme oxygenase-1 deficiency presenting with hemolysis, nephritis, and asplenia. J Pediatr Hematol Oncol. 2011; 33(1):74-78.

Reichard JF, Dalton TP, Shertzer HG, Puga A. Induction of oxidative stress responses by dioxin and other ligands of the aryl hydrocarbon receptor. Dose Response. 2005; 3(3):306-331.

Remillard RB, Bunce NJ. Linking dioxins to diabetes: epidemiology and biologic plausibility. Environ Health Perspect. 2002; 110(9):853-858.

Rennert A, Wittsiepe J, Kasper-Sonnenberg M, Binder G, Fürst P, Cramer C, Krämer U, Wilhelm M. Prenatal and early life exposure to polychlorinated dibenzo-*p*-dioxins, dibenzo-furans and biphenyls may influence dehydroepiandrosterone sulfate levels at prepubertal age: results from the Duisburg birth cohort study. J Toxicol Environ Health A. 2012; 75(19-20):1232-1240.

Rigney E, Mantle TJ. The reaction mechanism of bovine kidney biliverdin reductase. Biochim Biophys Acta. 1988; 957(2):237-242.

Rigney E, Mantle TJ, Dickinson FM. The kinetics of ox kidney biliverdin reductase in the pre-steady state. Evidence that the dissociation of bilirubin is the rate-determining step. Biochem J. 1989; 259(3):709-713.

Rizzardini M, Carelli M, Cabello Porras MR, Cantoni L. Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by *N*-acetylcysteine. Biochem J. 1994; 304(Pt 2):477-483.

Rose JQ, Ramsey JC, Wentzler TH, Hummel RA, Gehring PJ. The fate of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin following single and repeated oral doses to the rat. Toxicol Appl Pharmacol. 1976; 36(2):209-226.

Ryter SW. Bile pigments in pulmonary and vascular disease. Front Pharmacol. 2012; 3:39.

Safe SH. Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Annu Rev Pharmacol Toxicol. 1986; 26:371-399.

Safe S. Limitations of the toxic equivalency factor approach for risk assessment of TCDD and related compounds. Teratog Carcinog Mutagen. 1997-1998; 17(4-5):285-304.

Salim M, Brown-Kipphut BA, Maines MD. Human biliverdin reductase is autophosphorylated, and phosphorylation is required for bilirubin formation. J Biol Chem. 2001; 276(14):10929-10934.

Sassa S. Why heme needs to be degraded to iron, biliverdin IXα, and carbon monoxide? Antioxid Redox Signal. 2004; 6(5):819-824.

Sassa S, Granick S. Induction of δ-aminolevulinic acid synthetase in chick embryo liver cells in culture. Proc Natl Acad Sci U S A. 1970; 67(2):517-522.

Saurat J-H, Kaya G, Saxer-Sekulic N, Pardo B, Becker M, Fontao L, Mottu F, Carraux P, Pham X-C, Barde C, Fontao F, Zennegg M, Schmid P, Schaad O, Descombes P, Sorg O. The cutaneous lesions of dioxin exposure: lessons from the poisoning of Victor Yushchenko. Toxicol Sci. 2012; 125(1):310-317.

Savouret J-F, Antenos M, Quesne M, Xu J, Milgrom E, Casper RF. 7-ketocholesterol is an endogenous modulator for the arylhydrocarbon receptor. J Biol Chem. 2001; 276(5):3054-3059.

Sedlak TW, Snyder SH. Cycling the wagons for biliverdin reductase. J Biol Chem. 2009; 284(46):le11.

Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B, Denison MS. Activation of the Ah receptor signaling pathway by prostaglandins. J Biochem Mol Toxicol. 2001; 15(4):187-196.

Senft AP, Dalton TP, Nebert DW, Genter MB, Hutchinson RJ, Shertzer HG. Dioxin increases reactive oxygen production in mouse liver mitochondria. Toxicol Appl Pharmacol. 2002; 178(1):15-21.

Shertzer HG, Nebert DW, Puga A, Ary M, Sonntag D, Dixon K, Robinson LJ, Cianciolo E, Dalton TP. Dioxin causes a sustained oxidative stress response in the mouse. Biochem Biophys Res Commun. 1998; 253(1):44-48.

Shibahara S. The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism. Tohoku J Exp Med. 2003; 200(4):167-186.

Shibahara S, Kitamuro T, Takahashi K. Heme degradation and human disease: diversity is the soul of life. Antioxid Redox Signal. 2002; 4(4):593-602.

Shibahara S, Nakayama M, Kitamuro T, Udono-Fujimori R, Takahashi K. Repression of heme oxygenase-1 expression as a defense strategy in humans. Exp Biol Med. 2003; 228(5):472-473.

Shishido N, Nakayama K, Nakamura M. Porphyrin-induced photooxidation of conjugated bilirubin. Free Radic Res. 2003; 37(10):1061-1067.

Simanainen U. Dioxin resistance alleles and developmental stage at exposure as determinants

of sensitivity to dioxin-induced short-term toxicity and male reproductive effects. Publications of the National Public Health Institute, A8/2004.

Simanainen U, Tuomisto JT, Pohjanvirta R, Syrjälä P, Tuomisto J, Viluksela M. Postnatal development of resistance to short-term high-dose toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in TCDD-resistant and -semiresistant rats. Toxicol Appl Pharmacol. 2004; 196(1):11-19.

Simanainen U, Tuomisto JT, Tuomisto J, Viluksela M. Structure-activity relationships and dose responses of polychlorinated dibenzo-*p*-dioxins for short-term effects in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-resistant and -sensitive rat strains. Toxicol Appl Pharmacol. 2002; 181(1):38-47.

Simanainen U, Tuomisto JT, Tuomisto J, Viluksela M. Dose-response analysis of short-term effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in three differentially susceptible rat lines. Toxicol Appl Pharmacol. 2003; 187(2):128-136.

Sinal CJ, Bend JR. Aryl hydrocarbon receptor-dependent induction of Cyp1a1 by bilirubin in mouse hepatoma Hepa 1c1c7 cells. Mol Pharmacol. 1997; 52(4):590-599.

Singleton JW, Laster L. Biliverdin reductase of guinea pig liver. J Biol Chem. 1965; 240(12):4780-4789.

Siow RC, Ishii T, Sato H, Taketani S, Leake DS, Sweiry JH, Pearson JD, Bannai S, Mann GE. Induction of the antioxidant stress proteins heme oxygenase-1 and MSP23 by stress agents and oxidised LDL in cultured vascular smooth muscle cells. FEBS Lett. 1995; 368(2):239-242.

Smith AG, Clothier B, Carthew P, Childs NL, Sinclair PR, Nebert DW, Dalton TP. Protection of the *Cyp1a2(-/-)* null mouse against uroporphyria and hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol Appl Pharmacol. 2001; 173(2):89-98.

Smith AG, Elder GH. Complex gene-chemical interactions: hepatic uroporphyria as a paradigm. Chem Res Toxicol. 2010; 23(4):712-723.

Steenland K, Bertazzi P, Baccarelli A, Kogevinas M. Dioxin revisited: developments since the 1997 IARC classification of dioxin as a human carcinogen. Environ Health Perspect. 2004; 112(13):1265-1268.

Stocker R. Antioxidant activities of bile pigments. Antioxid Redox Signal. 2004; 6(5):841-849.

Stocker R, Maghzal G. Reply to Sedlak and Snyder: The little bighorn of the biliverdin reductase amplification cycle. J Biol Chem. 2009; 284(46):le12.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science. 1987; 235(4792):1043-1046.

Stohs SJ. Oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Free Radic Biol Med. 1990; 9(1):79-90.

Stohs SJ, Shara MA, Alsharif NZ, Wahba ZZ, Al-Bayati ZA. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced oxidative stress in female rats. Toxicol Appl Pharmacol. 1990; 106(1):126-135.

Sullivan BP, Wang R, Tawfik O, Luyendyk JP. Protective and damaging effects of platelets in acute cholestatic liver injury revealed by

depletion and inhibition strategies. Toxicol Sci. 2010; 115(1):286-294.

Swanson H. Dioxin response elements and regulation of gene transcription. In: Pohjanvirta R (ed.) The AH receptor in biology and toxicology. John Wiley & Sons, Inc. Hoboken, New Jersey, 2012; 81-91.

Sweeney MH, Mocarelli P. Human health effects after exposure to 2,3,7,8-TCDD. Food Addit Contam. 2000; 17(4):303-316.

Takeda A, Onodera H, Sugimoto A, Itoyama Y, Kogure K, Shibahara S. Increased expression of heme oxygenase mRNA in rat brain following transient forebrain ischemia. Brain Res. 1994; 666(1):120-124.

Tenhunen R, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. Proc Natl Acad Sci U S A. 1968; 61(2):748-755.

Thigpen JE, Faith RE, McConnell EE, Moore JA.Increased susceptibility to bacterial infection as a sequela of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Infect Immun. 1975; 12(6):1319-1324.

Tickner TR, Gutteridge JM. A simple colorimetric method for the estimation of plasma biliverdin. Clin Chim Acta. 1978; 85(2):125-129.

Tofilon PJ, Piper WN. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-mediated depression of rat testicular heme synthesis and microsomal cytochrome P-450. Biochem Pharmacol. 1982; 31(22):3663-3666.

Togawa H, Shinkai S, Mizutani T. Induction of human UGT1A1 by bilirubin through AhR dependent pathway. Drug Metab Lett. 2008; 2(4):231-237.

Tonack S, Kind K, Thompson JG, Wobus AM, Fischer B, Santos AN. Dioxin affects glucose transport via the arylhydrocarbon receptor signal cascade in pluripotent embryonic carcinoma cells. Endocrinology. 2007; 148(12):5902-5912.

Tudor C, Lerner-Marmarosh N, Engelborghs Y, Gibbs PE, Maines MD. Biliverdin reductase is a transporter of haem into the nucleus and is essential for regulation of *HO-1* gene expression by haematin. Biochem J. 2008; 413(3):405-416.

Tuomisto JT, Pohjanvirta R, Unkila M, Tuomisto J. TCDD-induced anorexia and wasting syndrome in rats: effects of diet-induced obesity and nutrition. Pharmacol Biochem Behav. 1999a; 62(4):735-742.

Tuomisto J, Tuomisto JT. Is the fear of dioxin cancer more harmful than dioxin? Toxicol Lett. 2012; 210(3):338-344.

Tuomisto JT, Viluksela M, Pohjanvirta R, Tuomisto J. The AH receptor and a novel gene determine acute toxic responses to TCDD: segregation of the resistant alleles to different rat lines. Toxicol Appl Pharmacol. 1999b; 155(1):71-81.

Tuomisto J, Vartiainen T, Tuomisto J. Synopsis on dioxins and PCBs. National Institute for Health and Welfare. Report No. 14/2011.

Turunen AW, Verkasalo PK, Kiviranta H, Pukkala E, Jula A, Männistö S, Räsänen R, Marniemi J, Vartiainen T. Mortality in a cohort with high fish consumption. Int J Epidemiol. 2008; 37(5):1008-1017.

Tzirogiannis KN, Panoutsopoulos GI, Demonakou MD, Hereti RI, Alexandropoulou KN, Mykoniatis MG. Effect of hepatic stimulator substance (HSS) on cadmium-induced acute

hepatotoxicity in the rat liver. Dig Dis Sci. 2004; 49(6):1019-1028.

Unkila M, Pohjanvirta R, Honkakoski P, Törrönen R, Tuomisto J. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induced ethoxyresorufin-*O*-deethylase (EROD) and aldehyde dehydrogenase ($ALDH_3$) activities in the brain and liver. A comparison between the most TCDD-susceptible and the most TCDD-resistant rat strain. Biochem Pharmacol. 1993; 46(4):651-659.

Unkila M, Pohjanvirta R, MacDonald E, Tuomisto JT, Tuomisto J. Dose response and time course of alterations in tryptophan metabolism by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the most TCDD-susceptible and the most TCDD-resistant rat strain: relationship with TCDD lethality. Toxicol Appl Pharmacol. 1994; 128(2):280-292.

Uno S, Dalton TP, Sinclair PR, Gorman N, Wang B, Smith AG, Miller ML, Shertzer HG, Nebert DW. *Cyp1a1(-/-)* male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyria. Toxicol Appl Pharmacol. 2004; 196(3):410-421.

Urban P, Pelclová D, Lukás E, Kupka K, Preiss J, Fenclová Z, Smerhovský Z. Neurological and neurophysiological examinations on workers with chronic poisoning by 2,3,7,8-TCDD: follow-up 35 years after exposure. Eur J Neurol. 2007; 14(2):213-218.

U.S. EPA. An inventory of sources and environmental releases of dioxin-like compounds in the U.S. for the years 1987, 1995, and 2000 (final, Nov 2006). U.S. Environmental Protection Agency, Washington, DC, EPA/600/P-03/002F. Available: http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=159286

U.S. EPA (U.S. Environmental Protection Agency). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) IRIS assessment summary (2012). Available: http://www.epa.gov/iris/subst/1024.htm

Van den Berg M, Birnbaum L, Bosveld AT, Brunström B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect. 1998; 106(12):775-792.

Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Håkansson H, Hanberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE. The 2005 World Health Organization re-evaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci. 2006; 93(2):223-241.

Van den Berg M, Peterson RE, Schrenk D. Human risk assessment and TEFs. Food Addit Contam. 2000; 17(4):347-358.

Viluksela M, Bager Y, Tuomisto JT, Scheu G, Unkila M, Pohjanvirta R, Flodström S, Kosma VM, Mäki-Paakkanen J, Vartiainen T, Klimm C, Schramm KW, Wärngård L, Tuomisto J. Liver tumor-promoting activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in TCDD-sensitive and TCDD-resistant rat strains. Cancer Res. 2000; 60(24):6911-6920.

Viluksela M, Stahl BU, Birnbaum LS, Schramm KW, Kettrup A, Rozman KK. Subchronic/chronic toxicity of a mixture of four chlorinated dibenzo-*p*-dioxins in rats. I. Design, general observations, hematology, and liver concentrations. Toxicol Appl Pharmacol. 1998; 151(1):57-69.

Viluksela M, Unkila M, Pohjanvirta R, Tuomisto JT, Stahl BU, Rozman KK, Tuomisto J. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on liver phosphoenolpyruvate carboxykinase (PEPCK) activity, glucose homeostasis and plasma amino acid concentrations in the most TCDD-susceptible and the most TCDD-resistant rat strains. Arch Toxicol. 1999; 73(6):323-336.

Wardle EN, Williams R. Raised serum biliverdin in hepatic necrosis. Biochem Med. 1981; 26(1):8-11.

Warner M, Mocarelli P, Brambilla P, Wesselink A, Samuels S, Signorini S, Eskenazi B. Diabetes, metabolic syndrome, and obesity in relation to serum dioxin concentrations: the Seveso women's health study. Environ Health Perspect. 2013; 121(8):906-911.

Warner M, Mocarelli P, Samuels S, Needham L, Brambilla P, Eskenazi B. Dioxin exposure and cancer risk in the Seveso Women's Health Study. Environ Health Perspect. 2011; 119(12):1700-1705.

Weber LW, Lebofsky M, Greim H, Rozman K. Key enzymes of gluconeogenesis are dose-dependently reduced in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. Arch Toxicol. 1991a; 65(2):119-123.

Weber LW, Lebofsky M, Stahl BU, Gorski JR, Muzi G, Rozman K. Reduced activities of key enzymes of gluconeogenesis as possible cause of acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. Toxicology. 1991b; 66(2):133-144.

Wegiel B, Baty CJ, Gallo D, Csizmadia E, Scott JR, Akhavan A, Chin BY, Kaczmarek E, Alam J, Bach FH, Zuckerbraun BS, Otterbein LE. Cell surface biliverdin reductase mediates biliverdin-induced anti-inflammatory effects via phosphatidylinositol 3-kinase and Akt. J Biol Chem. 2009; 284(32):21369-21378.

Wegiel B, Gallo D, Csizmadia E, Roger T, Kaczmarek E, Harris C, Zuckerbraun BS, Otterbein LE. Biliverdin inhibits Toll-like receptor-4 (TLR4) expression through nitric oxide-dependent nuclear translocation of biliverdin reductase. Proc Natl Acad Sci U S A. 2011; 108(46):18849-18854.

WHO (World Health Organization), 2000. Consultation on assessment of the health risk of dioxins; re-evaluation of the tolerable daily intake (TDI): executive summary. Food Addit Contam. 2000; 17(4):223-240.

WHO/IPCS, 2002. Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2, World Health Organisation, Geneva, pp.180. Available: http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/

WHO (World Health Organization), 2009. Persistent organic pollutants in human milk. European Environment and Health Information System ENHIS, Fact sheet 4.3. Available: http://www.euro.who.int/__data/assets/pdf_file/0003/97032/4.3.-Persistant-Organic-Pollutantsm-EDITED_layouted_V2.pdf

WHO (World Health Organization), 2010. Dioxins and their effects on human health. Fact sheet No

225. Available: http://www.who.int/mediacentre/factsheets/fs225/en/

Wong EW, Cheng CY. Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol Sci. 2011; 32(5):290-299.

Woods JS. Studies of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on mammalian hepatic δ-aminolevulinic acid synthetase. Environ Health Perspect. 1973; 5:221-225.

Wu TW. Delta bilirubin: the fourth fraction of bile pigments in human serum. Israel J Chem. 1983; 23(2):241-247.

Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol Rev. 2005; 57(4):585-630.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest. 1999; 103(1):129-135.

Yamada N, Yamaya M, Okinaga S, Nakayama K, Sekizawa K, Shibahara S, Sasaki H. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet. 2000; 66(1):187-195.

Yamamoto M, Hayashi N, Kikuchi G. Evidence for the transcriptional inhibition by heme of the synthesis of δ-aminolevulinate synthase in rat liver. Biochem Biophys Res Commun. 1982; 105(3):985-990.

Yamamoto M, Hayashi N, Kikuchi G. Translational inhibition by heme of the synthesis of hepatic δ-aminolevulinate synthase in a cell-free system. Biochem Biophys Res Commun. 1983; 115(1):225-231.

Yamamoto M, Kure S, Engel JD, Hiraga K. Structure, turnover, and heme-mediated suppression of the level of mRNA encoding rat liver δ-aminolevulinate synthase. J Biol Chem. 1988; 263(31):15973-15979.

Yamashita K, McDaid J, Öllinger R, Tsui TY, Berberat PO, Usheva A, Csizmadia E, Smith RN, Soares MP, Bach FH. Biliverdin, a natural product of heme catabolism, induces tolerance to cardiac allografts. FASEB J. 2004; 18(6):765-767.

Yamauchi K, Hayashi N, Kikuchi G. Translocation of δ-aminolevulinate synthase from the cytosol to the mitochondria and its regulation by hemin in the rat liver. J Biol Chem. 1980; 255(4):1746-1751.

Yang KH, Croft WA, Peterson RE. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on plasma disappearance and biliary excretion of foreign compounds in rats. Toxicol Appl Pharmacol. 1977; 40(3):485-496.

Yang KH, Yoo BS, Choe SY. Effects of halogenated dibenzo-p-dioxins on plasma disappearance and biliary excretion of ouabain in rats. Toxicol Lett. 1983; 15(2-3):259-264.

Yasuda H, Okinaga S, Yamaya M, Ohrui T, Higuchi M, Shinkawa M, Itabashi S, Nakayama K, Asada M, Kikuchi A, Shibahara S, Sasaki H. Association of susceptibility to the development of pneumonia in the older Japanese population with haem oxygenase-1 gene promoter polymorphism. J Med Genet. 2006; 43(4):e17.

Yasuda K, Yatomi Y, Matsushima T, Yoneyama A, Higashihara M, Kurokawa K, Nakahara K. A case of POEMS syndrome with thrombocytopenia and biliverdinaemia. Br J Haematol. 1994; 86(2):389-390.

Yeager RL, Reisman SA, Aleksunes LM, Klaassen CD. Introducing the "TCDD-inducible AhR-Nrf2 gene battery". Toxicol Sci. 2009; 111(2):238-246.

Yueh MF, Huang YH, Hiller A, Chen S, Nguyen N, Tukey RH. Involvement of the xenobiotic response element (XRE) in Ah receptor-mediated induction of human UDP-glucuronosyltransferase 1A1. J Biol Chem. 2003; 278(17):15001-15006.

Zelenka J, Muchova L, Zelenkova M, Vanova K, Vreman HJ, Wong RJ, Vitek L. Intracellular accumulation of bilirubin as a defense mechanism against increased oxidative stress. Biochimie. 2012; 94(8):1821-1827.

Zhu Z, Wilson AT, Luxon BA, Brown KE, Mathahs MM, Bandyopadhyay S, McCaffrey AP, Schmidt WN. Biliverdin inhibits hepatitis C virus nonstructural 3/4A protease activity: mechanism for the antiviral effects of heme oxygenase? Hepatology. 2010; 52(6):1897-1905.

Zinkl JG, Vos JG, Moore JA, Gupta BN. Hematologic and clinical chemistry effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals. Environ Health Perspect. 1973; 5:111-118.

Öllinger R, Pratschke J. Role of heme oxygenase-1 in transplantation. Transpl Int. 2010; 23(11):1071-1081.