STUDIES OF THE EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) ON THE RETINOID SYSTEM USING RETINOID PATHWAY KNOCKOUT MICE

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Introduction

Retinoid (vitamin A) signaling carries out vital hormonal tasks invoved in embryonal development, cell growth and differentiation, epithelia maintenance and metabolism. The signaling pathway comprises the binding of different forms of retinoic acid (RA) to various isoforms of RA receptors, RAR and RXR, affecting the transcriptional activity of hundreds of genes[1,2]. The RAR and RXR isoforms (α, β, γ) act as homo- or heterodimers in different combinations within the RAR/RXR subfamily or with other hormone and orphan receptor subfamilies. The different binding proteins for retinoids; cellular retinol binding protein (CRBP), cellular RA binding protein (CRABP), and serum retinol binding protein (RBP), affect the uptake and processing of retinoids in the cells and hence the bioavailability of RA in the cell. As both deficiency and excess of vitamin A are deleterious to the animal, the retinoid system is strictly regulated to avoid fluctuations in intracellular content of signaling RA metabolites.

Dioxins are highly toxic food contaminants, known to interfere with retinoid turnover and metabolism.[3] The molecular events causing this interference are not known, but most likely involve the Ah-receptor (AhR). Modulations of retinoid signaling could have detrimental effects on several hormonal systems. In the present study we have made use of transgenic (knockout) mice to investigate the role of retinoid receptors and binding proteins in AhR-mediated toxicity as well as normal retinoid processing. The advantage of using knockout mice in this task is that we will be able to compare the profile of biochemical and toxic effects, which may or may not be mediated by dioxin-induced retinoid disruption, with the possible abnormalities induced by the selective mutations. The absence (or attenuation) of a specific toxic response in a knockout strain will provide evidence that the gene that has been knocked out plays a role in this response. In the same way, the absence of differences between exposed wild type and knockout mice will rule out the knocked out gene as being involved in the toxicity. As model compound for AhR-mediated toxicity in this study, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was used. In addition to its use in toxicology, TCDD may also be looked upon as a tool to understand the significance of retinoid receptors and binding proteins in the control of normal retinoid homeostasis.

Study design and methods

We performed four separate studies in transgenic (knockout) and wild type (WT) mice of a C57Bl6 and 129/SV mixed background. The WT controls were of the same C57Bl6 - 129/SV ratio as the knockout strains.

1) Two-, four-, and eight-week-old male and female mice lacking retinoic acid receptor α1, 2, and 3 (RARαtotal), RARβ2, RARγ2, or cellular retinoic acid binding proteins types I and II (CRABP I/II double knockout) were compared with their corresponding WT control for body weight gain, retinoid storage capacity (total hepatic retinoid content), and hepatic retinoic acid (RA) profile.

2) Six to fourteen-week-old male mice lacking RARαtotal, RARβtotal, RXRβtotal, RXRγtotal, and their corresponding WTs were exposed to a single i.p. dose of 0 or 50 μg TCDD/kg bodyweight. An RXRα heterozygous knockout strain was also included. Twenty-eight days after exposure, cytochrome P4501A (CYP1A) activity, hepatic retinol and total retinoid content, and RA metabolites in liver, were assayed.

3) Eight-week-old male mice lacking CRABP I and II, or all three binding proteins (CRBP I / CRABP I / CRABP II), and the corresponding WT were exposed to a single oral dose of 0 or 50 μg TCDD/kg bodyweight and assayed for CYP1A activity, hepatic retinol and total retinoid content, and RA metabolites in selected organs 7 or 28 days after exposure.

4) 22-week-old male and female CRABP I/II and RARβ2 knockouts and their corresponding WT were exposed to a single oral dose of 0, 50, or 250 μg TCDD/kg bodyweight, and assayed for CYP1A acitivity, hepatic retinoid content, and hepatic retinol esterification activity.

CYP1A activity:

Study 2) The formation of resorufin from 7-ethoxyresorufin in liver microsomes was detected fluorimetrically (λex 530 nm, λem 590 nm) using 2.5-100 μg/ml protein in 96 wells plates and a fluorospectrophotometric plate reader.[5]

Study 3) and 4) The formation of resorufin from 7-ethoxy-resorufin by liver homogenates was detected fluorimetrically (λex 522 nm, λem 586 nm) using 20-200 μg/ml protein at 37°C.[6,7]

Retinoid analyses by HPLC:

Study 1), 2) and 3): Retinoids were analyzed according to Schmidt and Nau (manuscript in preparation). Briefly, hepatic retinoids were extracted from tissues homogenized in water by a single liquid-liquid extraction with isopropanol. Polar and apolar retinoids were separated via solid-phase-extraction using an aminopropyl phase. Polar retinoids were analyzed on a narrow-bore RP18 column with UV detection at 340 nm using a binary gradient composed of methanol and water. Apolar retinoids were separated using a ternary gradient composed of acetonitrile, chloroform, and methanol on a normal-bore RP18 column with UV detection at 325 nm.

Study 4): Briefly, hepatic retinoids from tissues homogenized in water were extracted using diisopropyl ether and separated on a Nucleosil C18 5μ HPLC column using an ethanol: water gradient elution. Retinol, retinyl acetate (internal standard), retinyl palmitate, and retinyl stearate were detected with a JASCO 821-FP fluorescence detector (λex. 325 nm, λem. 475 nm).[4,8]

Retinol esterification assay:

Liver homogenates, 20% in 0.2 M sucrose, were assayed for retinol esterification using 500 µg protein and 5 minutes of incubation. An enzyme-saturating concentration of free [^{3}H]retinol was used as substrate. Phenylmethylsulfonyl fluoride was used as an LRAT inhibitor to separate retinol esterification catalyzed by LRAT from that by acyl CoA:retinol acyltransferase (ARAT). Following extraction with n-hexane and separation by alumina oxide chromatography, radioactivity from the retinyl ester eluate was counted in a liquid scintillation counter. Results were corrected for nonenzymatic contribution of ^{3}H.[8]

Results and discussion

Hepatic retinoid storage capacity from 2 to 8 weeks of age in the different mutants did not differ significantly from WT controls. For all experiments with TCDD, typical dioxin-like responses (decreased body and thymus weights, increased liver weight, decreased hepatic retinyl ester stores, and induced hepatic CYP1A activity) were observed in all strains and sexes, but no drastic differences from WT could be identified for these parameters in any mutant. As measured in study 4, neither hepatic lecithin:retinol acyltransferase (LRAT) activity nor acyl CoA:retinol acyltransferase (ARAT) activity displayed any differences in the knockouts compared to WT controls. Whereas none of the receptor knockouts or the CRABP I/II double knockout displayed any consistent differences compared to their corresponding WT, the CRBP I / CRABP I / CRABP II triple knockout exhibited marked differences from its WT concerning retinoid contents both with and without TCDD exposure. Unexposed triple knockouts showed significantly decreased hepatic all-trans-RA, retinol, and retinyl ester content compared to WT, whereas hydroxylated RA metabolites were elevated. After exposure to TCDD, the loss of retinyl esters from the liver was more drastic in the triple binding protein knockout (-88%) than in the WT (-43%). With this data at hand, we speculate that the lack of CRBP I directs vitamin A towards a less controlled catabolism, leading to reduced levels of transcriptionally active RA and subsequently increased mobilization of retinoids from liver stores. We have previously shown that the activities of CYP1A as well as CYP2B, both of which may have activities towards retinoids, are increased in the adult CRBP I knockout mouse.[9] Without the intracellular binding protein CRBP I holding its lipophilic ligands in the cytosol, retinol and retinal could be more accessible to metabolism by the cytochrome P450 enzymes. TCDD would increase this unspecific metabolism/catabolism by inducing the CYP enzymes and perhaps others as well, like UDP-glucuronosyltransferases (UGTs) and glutathione S-transferases (GSTs).

In all mice including WTs, hepatic levels of all-*trans* RA were unchanged by TCDD. This is an interesting difference from the rat, where we have previously shown a sensitive and rapid increase of all-*trans* RA in response to TCDD.[4,10] This difference may show importance for TCDD toxicity and may contribute to the several-fold difference in TCDD-caused lethality between rat and mouse.

Taken together, the data in this study suggest that CRBP I plays an important role in retinoid storage and retinoic acid homeostasis, as well as in TCDD-induced retinoid disruption. The possible consequences for dioxin toxicity are however still unestablished and are currently under investigation. The lack of major alterations of retinoid processing in the receptor mutants is likely to be due to the vast redundancy in the retinoid receptor signaling system. It remains to clarify the role of the two retinoic acid binding proteins CRABP I and II, as the absence of these proteins did not have an effect on either a normal or a TCDD-challenged retinoid system. The results of this study contribute to an increased knowledge of the role of retinoid-specific genes in the context of retinoid homeostasis as well as dioxin-retinoid interactions.

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