The importance of thyroid hormone sulfation during fetal development

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The importance of thyroid hormone sulfation during fetal development

Het belang van schildklierhormoonsulfatering tijdens de foetale ontwikkeling

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Contents

List of abbreviations

AhR	arylhydrocarbon receptor (dioxin receptor)
ARS	arylsulfatase
bovSULT	bovine sulfotransferase
BPA	bisphenol A
BrAc	N-bromoacetyl
canSULT	canine sulfotransferase
CYP	cytochrome P450
D1	type I iodothyronine deiodinase
D2	type II iodothyronine deiodinase
D3	type III iodothyronine deiodinase
Da	Dalton
DHEAS	dehydroepiandrosterone sulfate
DIT	3,5-diiodotyrosine
DTT	dithiothreitol
E1	estrone
E2	estradiol
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
FT3	free T3
FT4	free T4
gpiSULT	guinea pig sulfotransferase
HPLC	high performance liquid chromatography
hSULT	human sulfotransferase
IC_{50}	concentration causing 50% inhibition
IRD	inner ring deiodination
MIT	3-monoiodotyrosine
monSULT	monkey sulfotransferase
mouSULT	mouse sulfotransferase
M_r	relative molecular mass
ORD	outer ring deiodination
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PCB	polychlorinated biphenyl

PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PHAH	polyhalogenated aromatic hydrocarbon
PHAH-OH	hydroxylated polyhalogenated aromatic hydrocarbon
PHDE	polyhalogenated diphenylether
PTU	6-N-propyl-2-thiouracil
rSULT	rat sulfotransferase
rT3	reverse triiodothyronine (3,3',5'-triiodothyronine)
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T0	thyronine
T1	monoiodothyronine
T2	diiodothyronine
T3	3,3',5-triiodothyronine
T4	thyroxine (3,3',5,5'-tetraiodothyronine)
TBG	thyroxine-binding globulin
TBPA	thyroxine-binding prealbumin (transthyretin)
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	thyroid hormone receptor
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone (thyrotropin)
TTR	transthyretin (thyroxine-binding prealbumin)
UDP	uridine diphosphate
UGP	UDP-glucuronyltransferase

Chapter 1

General introduction

Outline of the introduction

Normal fetal development requires the presence of thyroid hormone. Disruption of any of the processes regulating the bioavailability of thyroid hormone may contribute to congenital anomalies. This thesis is focussed a) on the importance of thyroid hormone sulfation during fetal development, and b) on the potential sulfation-disrupting effects of environmental chemicals such as hydroxylated polychlorinated biphenyls (PCBs), because of potential pathogenetic consequences of disturbed thyroid hormone sulfation for the development of organs, such as lungs and brain.

In this general introduction, first some information is given on the development of fetal thyroid status, and the importance of thyroid hormone for the development of organs such as the brain is discussed. Secondly, thyroid hormone synthesis, transport and metabolism, which are all processes regulating thyroid hormone bioavailability, are reviewed. Additionally, the role of sulfation in thyroid hormone metabolism, especially during fetal development, is addressed. Furthermore, some general information on PCBs and other polyhalogenated aromatic hydrocarbons is given, and their potential estrogen and thyroid hormone-disrupting effects are discussed. Finally, the outline of this thesis is presented.

Thyroid hormone and fetal development

The biological activity of thyroid hormone is largely mediated by specific nuclear T3 receptors in target tissues. The T3 receptors belong to the nuclear hormone receptor superfamily that also include steroid, retinoic acid and vitamin D3 receptors, and contain a central DNA-binding domain and a C-terminal ligand-binding domain (1-5). Several thyroid hormone receptor (TR) isoforms exist, among which TRα1, α2, TRβ1, β2 and the recently identified TRβ3 (6), which are expression products from the TRα and TRβ genes. Whereas TRα2 does not bind T3, TRα1, β1, β2 and β3 act as functional receptors. The thyroid hormone receptors bind to the DNA as homodimers or as heterodimers in association with the retinoid X receptor; the dimers regulate

gene-expression via binding to thyroid hormone response elements present in the promoter region of target genes (1-5). Expression patterns for the different receptor isoforms differ. While TRα1 is widely expressed from early embryonic stages, TRβ1 is present later in development, basically in the same tissues as TRα1. TRβ2 is predominantly expressed in the developing retina and inner ear, and in the pituitary gland and hypothalamus (3-5); this TR-isoform plays an important role in the feedback regulation of the hypothalamic-pituitary-thyroid axis (7,8). The ontogenic expression pattern of TRβ3 remains to be investigated.

Thyroid hormone is essential for normal development of organs such as the brain (9). Severe iodine deficiency during the first half of pregnancy is clearly related to neurological cretinism (10). Moreover, also undiagnosed maternal hypothyroidism has been suggested to adversely affect neurological development of the child (11,12). The T4 and T3 that are present in the fetus before the onset of fetal thyroid function are of maternal origin; if pregnant rats are thyroidectomized, no T4 and T3 is detected in the offspring before the onset of fetal thyroid function (13), and when early in pregnancy dams are injected with labeled T4 and T3, the labeled thyroid hormones are found in the fetal compartment (14,15). In rats, fetal thyroid function starts at gestational day 17.5-18; at this time a sudden increase in tissue and plasma T4 and T3 pools is found (16). In humans, secondary to the development of fetal hypothalamic-pituitary function, active thyroidal secretion starts at week 18-22 (17-19). However, also after the onset of fetal thyroid function transfer of thyroid hormones from mother to fetus continues. Thus, fetal thyroid status remains dependent on the thyroid status of the mother until birth (20).

T3 has been demonstrated in extracts from fetal human brain of 9-10 weeks gestational age (21,22). By midgestation, fetal brain T3 level reaches about 30% of the T3 level in the adult human brain. This fetal brain T3 level is much higher than the fetal serum T3 level at this stage, which may indicate that brain type II deiodinase (D2), which is important for the local conversion of T4 to T3, is already important in the human fetal brain (23). Nuclear TRs are present in human brain by week 10, and steadily increase until week 16 (21). Also in other human fetal tissues such as the lung, T4, T3 and the nuclear TR are already found early in gestation (22). Serum T4 and free T4 levels steadily increase during development, until adult levels are reached at the end of gestation; whereas normal adult serum T3 amounts to 2 nM and normal adult serum rT3 to 0.25 nM (17-19), serum T3 is low (~0.5 nM), and serum rT3 is high (2-4 nM) in the human fetus, indicating that the ratio of outer ring deiodination versus inner ring deiodination is low in the fetus compared to the adult, as will be explained in the next paragraph.

Thyroid hormone synthesis, transport and metabolism

Thyroid hormones are synthesized in the follicular cells of the thyroid gland. The follicular cells take iodide up from the blood, and after thyroid peroxidase-catalyzed oxidation at the apical membrane the iodine is bound to tyrosyl residues of thyroglobulin, yielding monoiodotyrosyl (MIT) and diiodotyrosyl (DIT) residues. Subsequently, T4 is formed by thyroid peroxidase-catalyzed coupling of two DIT residues within thyroglobulin, and T3 by coupling of a MIT and a DIT residue. Following endocytosis of the thyroglobulin molecule from the follicular lumen, the molecule is hydrolyzed by lysosomal hydrolases, liberating thyroid hormones, which are then secreted into the blood stream (24). Normally, T4 is the main product of the thyroid gland. Besides T4, also 20% of the daily amount of T3 is synthesized in the thyroid gland. The remaining 80% of plasma T3 is formed in tissues such as liver and kidney by outer ring deiodination of the prohormone T4 (25,26). Thyroid hormone synthesis is under positive control of hypophyseal TSH (thyroid stimulating hormone) or thyrotropin (27,28). In turn, TSH release is stimulated by hypothalamic TRH (thyrotropin releasing hormone) (28,29). Negative feedback of this regulation takes place via inhibition of TRH and TSH secretion by thyroid hormones (28,30,31).

Iodothyronines circulate in blood plasma bound to the plasma transport proteins thyroxine-binding globulin (TBG), transthyretin (TTR), formerly known as thyroxine-binding prealbumin (TBPA), and albumin. In humans, TBG, TTR and albumin carry about 75%, 15% and 10%, respectively, of plasma T4 and T3 (26,32-34). Of these transport proteins, TTR may play an active role in tissue thyroid hormone supply (35). Since normal adult rats lack TBG, TTR is the main plasma transport protein in rat serum (34).

In normal human serum free T4 (FT4) and free T3 (FT3) comprise only about 0.02 and 0.2% of total T4 and T3 levels, respectively (26,32-34). The free fraction determines the amount of thyroid hormone taken up by cells. Although the hydrophobic thyroid hormones were originally believed to pass the cell membrane by passive diffusion (36), evidence has accumulated that cellular uptake of T4 and T3 into different organs is a saturable and energy-dependent process (reviewed by Hennemann et al. (37)). Plasma membrane transporters may be different in different organs. For instance, in the liver T4 and T3 are transported into the cell by different carrier systems (38-40), whereas in the pituitary and the heart only one transport mechanism has been identified, in the pituitary for both T4 and T3 (41-43) and in the heart only for T3 (44). Recently, several organic anion transporters and L-type amino acid transporters have been shown to facilitate the cellular uptake of thyroid hormone (45-51). The physiological relevance of these transporters, their tissue distribution, and the mechanisms by which they regulate the bioavailability of thyroid hormone remain to be established.

Besides thyroidal hormone secretion and the exchange of thyroid hormone between tissue and plasma, the bioavailability of T3 depends on the intracellular iodothyronine metabolism. T4 is converted by outer ring deiodination (ORD) to the receptor-active T3, or by inner ring deiodination to the metabolite rT3. (25). T3 and rT3 are further deiodinated, by IRD and ORD, respectively, to 3,3'-T2 (Fig. 1). Deiodination is catalyzed by a family of three iodothyronine deiodinases (D1-3), which all have the amino acid selenocysteine (Sec) in their catalytic center (52,53).

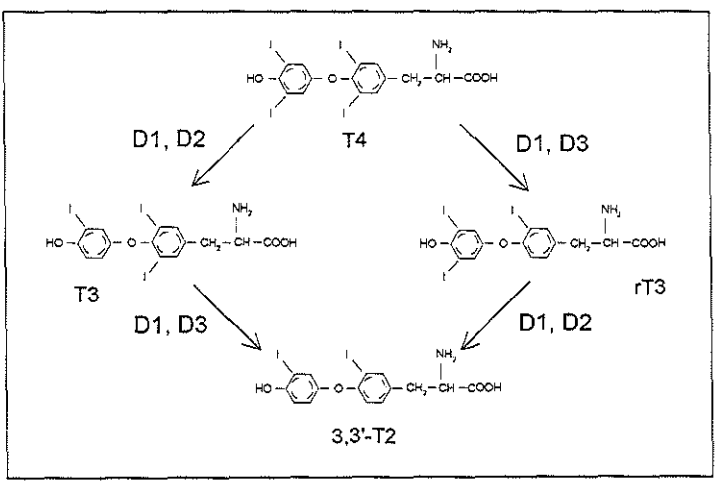

Fig. 1. Stepwise deiodination of thyroxine (T4).

D1 catalyzes both ORD and IRD, and is expressed in liver, kidney and thyroid (52-54). D1 is the main site for plasma T3 production and plasma rT3 clearance in adults, but its expression is low in fetal tissues (19,55). Hepatic and renal D1 gene expression is increased in hyperthyroidism and decreased in hypothyroidism (52,53). Recent studies have revealed the presence of two functional T3 response elements in the D1 gene promoter (56,57).

D2 has only ORD activity and, thus, converts T4 solely to active T3. It is mainly expressed in brain, pituitary and brown adipose tissue (BAT), but D2 mRNA is also found in the human heart and skeletal muscle (58-60). The physiological role of D2 lies in the local production of T3 in these tissues. Its activity shows tissue-specific, development stage-dependent patterns, generally increasing in the late fetal period and peaking in the neonatal period (19). D2 activity in the different tissues is down-regulated in hyperthyroidism and up-regulated in hypothyroidism. Although D2 mRNA levels may also be decreased by T3, the negative control of D2 activity by thyroid state is predominantly a post-translational mechanism involving substrate (T4, rT3)-induced enzyme inactivation (52,53,61).

D3 has only IRD activity. It is predominantly localized in brain, placenta, pregnant uterus and fetal tissues (55,62-68), and is important for the inactivation of tissue and plasma T3, as well as for the production of plasma rT3. Brain D3 activity is up-regulated in hyperthyroidism and down-regulated in hypothyroidism (19,69). D3 activity shows tissue-specific, development stage-dependent profiles, i.e., rat brain D3 activity is higher in the fetus than in the adult, rat skin D3 peaks in the neonatal period, and human liver D3 activity is high in the fetus and becomes undetectable after birth (19,55,69). The high levels of D3 in the placenta have been demonstrated to limit transplacental passage of T4 and T3 from mother to fetus (70,71). The very high D3 levels that have recently been found in the rat uterus indicate that also the uterus is involved in the regulation of fetal thyroid state (64).

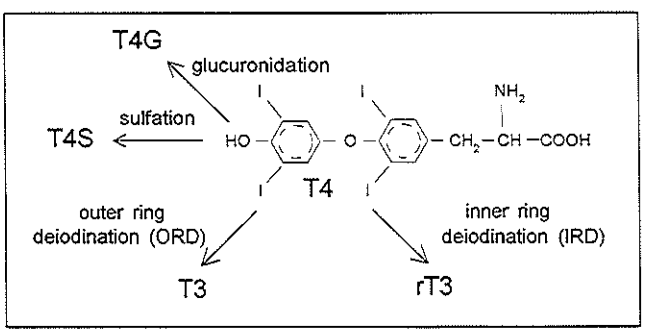

Fig. 2. Pathways of thyroid hormone metabolism.

Besides deiodination, glucuronidation and sulfation are important pathways in thyroid hormone metabolism (Fig. 2) (72,73). These conjugation reactions facilitate the urinary and biliary excretion of iodothyronines by increasing their water-solubility (72,73). However, excretion of iodothyronine glucuronides is only partial, since, subsequent to their secretion in the bile, at least part of the iodothyronines are reabsorbed after hydrolysis by β-glucuronidases present in the intestine (73). Glucuronidation is catalyzed by multispecific uridine diphosphate-glucuronyltransferases (UGTs), which are located in the endoplasmic reticulum of tissues such as the liver. UGTs catalyze the transfer of glucuronic acid from the cofactor uridine diphosphate-glucuronic acid to the hydroxyl group of iodothyronines. Based on sequence homology, two families of UGTs have been identified (74,75). So far, the bilirubin UGT1A1, and phenol UGT1A9 are known to be involved in the glucuronidation of T4 in humans (74-76). Whereas in the rat androsterone UGT seems largely responsible for the glucuronidation of T3, no human UGT preferring 3,3',5-triiodothyronine over T4 has been found yet (75-77). Since the enzymes catalyzing the glucuronidation of thyroid hormone mostly develop around or after birth, they do not play an important role in thyroid hormone metabolism in the developing fetus (78).

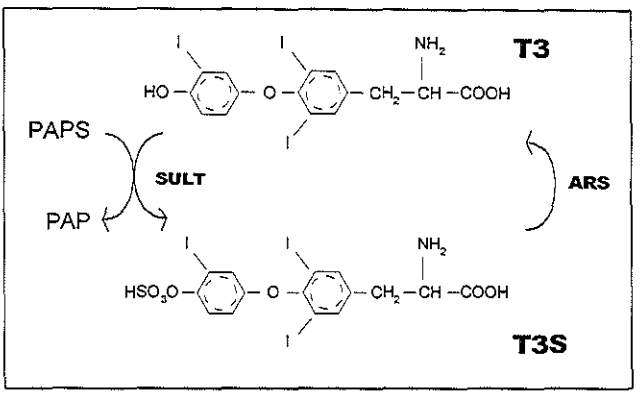

Fig. 3. Formation and hydrolysis of triiodothyronine sulfate (T3S), catalysed by sulfotransferases (SULT) and arylsulfatase (ARS) respectively. 3'-Phosphoadenosine-5'-phosphosulfate (PAPS) is used as sulfate donor.

Like glucuronidation, sulfation is an important detoxification mechanism for exogenous chemicals (79,80). Apart from this, sulfation is involved in the regulation of the biological activity of endogenous compounds such as steroids and thyroid hormone (80,81). Sulfotransferases catalyze the sulfation of the hydroxyl group of the compounds, using 3'-phosphoadenosine-5'-phoshosulfate (PAPS) as the universal sulfate donor (Fig. 3) (82). This cofactor PAPS is synthesized from 2 ATP molecules and inorganic sulfate. All cytosolic sulfotransferases are members of a large gene superfamily (Fig. 4) (79,81,83-85). They are located in different tissues such as liver, kidney and brain, have a molecular weight of about 32 kDa, and exist predominantly as homodimers (86). Before sulfotransferase cDNAs were cloned, three different human cytosolic sulfotransferases were defined on the basis of biochemical characteristics: a thermostable phenol sulfotransferase that catalyzes the sulfation of 4-nitrophenol and other phenolic compounds in the micromolar range (later called hSULT1A1), a thermolabile phenol sulfotransferase that prefers dopamine as substrate (later called hSULT1A3), and a hydroxysteroid sulfotransferase that catalyzes the sulfation of cholesterol, bile acids and 3β-hydroxysteroids such as DHEA (later called hSULT2A1) (81,83-85). In addition to hSULT1A1, 1A3 and 2A1 (87-94), eight other human sulfotransferases have been identified by cDNA and gene cloning strategies: hSULT1A2, 1B1, 1C2 and 1C4, 1E1, 2B1a, 2B1b and 4A1 (95-105). The sulfotransferases, which show overlapping substrate specificities, are classified on the basis of amino acid homology. The phylogenetic tree depicted in Figure 4 shows the homologies between the various mammalian sulfotransferases. Except for hSULT2B1a and 2B1b, which are splice variants of the same gene (104), all sulfotransferases are encoded by different genes. So far, functionally relevant polymorphisms have been found for hSULT1A1 and 1A2 (96,106-109); in the hSULT2A1 gene polymorphisms have been detected which could not be associated with enzyme activity levels (110).

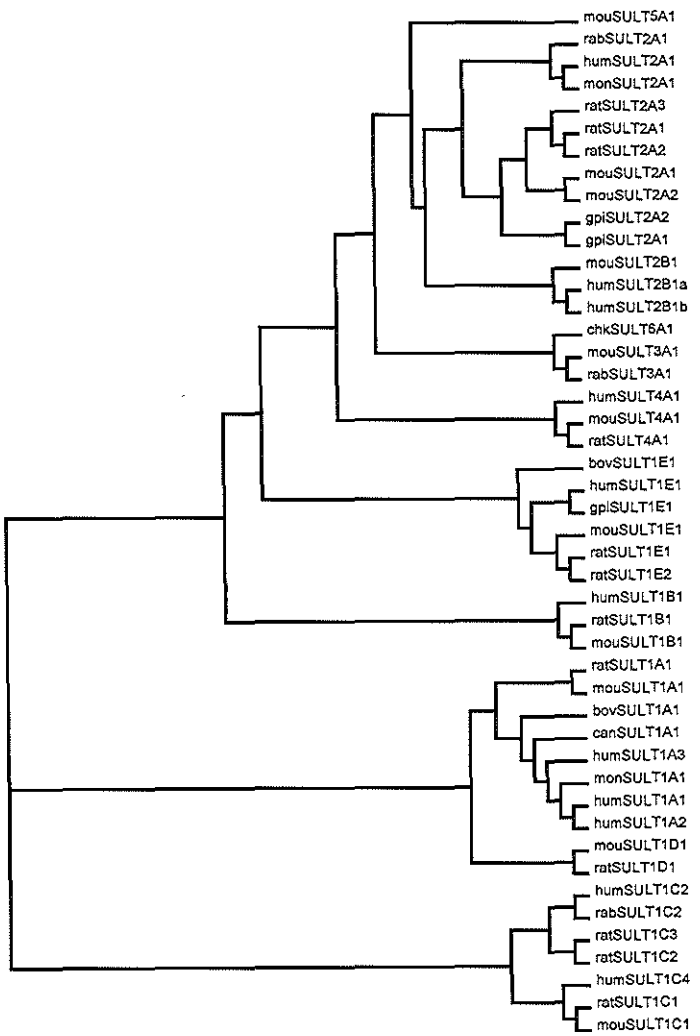

Fig. 4. Phylogenetic tree of mammalian cytosolic sulfotransferases (courtesy of Dr. M.W.H. Coughtrie). All human SULT1 enzymes catalyze iodothyronine sulfation, in the rat only SULT1B1 and 1C1 are known to catalyze iodothyronine sulfation. Abbreviations: bov = bovine; can = canine; chk = chicken; gpi = guinea pig; hum = human; mon = monkey; mou = mouse; rab = rabbit.

Importance of sulfation in thyroid hormone metabolism

The role of sulfation in thyroid hormone metabolism is fascinating. Figure 5 shows the interaction between the deiodination and sulfation pathways. Neither D2 nor D3 are capable of catalyzing the deiodination of sulfated iodothyronines. However, sulfation strongly facilitates the inner ring deiodination of T4 and T3 (inactivation) by D1, but blocks the outer ring deiodination of T4 (activation) by this enzyme (111-113). The outer ring deiodination of rT3 by D1 is not affected by sulfation (111-113). Under normal conditions, therefore, the main function of sulfation is to induce the irreversible degradation of thyroid hormone. As a result of the very rapid inner ring deiodination of T4S and T3S and outer ring deiodination of rT3S, the plasma concentrations of these sulfated iodothyronines are very low in the normal adult (114-118). However, iodothyronine sulfates accumulate in serum and bile when deiodination by D1 is prevented. For instance, increased thyroid hormone sulfate levels were observed in rats that were fed a selenium-deficient diet or were administered inhibitors of D1 such as propylthiouracil or iopanoic acid (119-121). Most likely, the high iodothyronine sulfate levels found in patients with non-thyroidal illness are also caused by impaired D1 activity, although reduced iodothyronine (sulfate) uptake in liver and kidney may play an additional role (115-118,122).

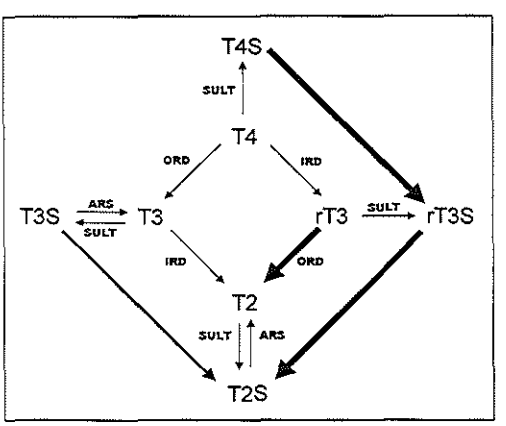

Fig. 5. Interaction between deiodination and sulfation pathways (courtesy of Dr. M.W.H. Coughtrie). Sulfation strongly facilitates the IRD of T4 and T3 by D1, but blocks the ORD of T4 by D1.

The high concentrations of the different iodothyronine sulfates, T4S, T3S, rT3S and 3,3'-T2S, that are found in human fetal serum and amniotic fluid (115-118,123) were also believed to be due to low hepatic D1 expression until after birth. However, although D1 activity in rat fetal liver is low, increasing just before birth (65-68), significant D1 activity is already present in the fetal human liver in the second trimester (55). Little is known about the ontogeny of hepatic thyroid hormone transporters in the developing fetus; absent or low expression of these transporters would be an alternative explanation for the high iodothyronine sulfate levels in the human fetus.

It has been suggested that the sulfates in the fetal circulation represent a reservoir of inactive thyroid hormone, from which active hormone is recovered when required, by the action of sulfatases present in the different tissues (112,123,125). Although iodothyronine sulfate hydrolysis has been observed in tissues such as liver, kidney and brain (126), and several sulfatases have been identified (127), it is still not known which sulfatases are involved in iodothyronine sulfate hydrolysis, and how these sulfatases are regulated. To delineate the role of sulfation/desulfation in the regulation of bioactive thyroid hormone during fetal development, it is of crucial importance to determine the contribution of the different sulfotransferases and sulfatases to iodothyronine sulfation/desulfation in the fetus, and to enhance our understanding of the mechanisms involved in the regulation of the expression of these enzymes.

Polyhalogenated aromatic hydrocarbons and endocrine disruption

The existence of chemicals in our environment that may disrupt endocrine systems and affect developmental and other physiological processes in humans and wildlife has received much attention in recent years. These so called 'endocrine disrupters', among which polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (for general structures see Fig. 6), could affect the sex steroid systems as well as the thyroid hormone system in different ways. PCBs, PCDDs and PCDFs consist of two halogenated benzene rings, and comprise 209, 75 and 135 possible congeners, respectively. Whereas PCDDs and PCDFs are rigid planar structures, the benzene rings of the PCBs usually assume non-planar positions (128).

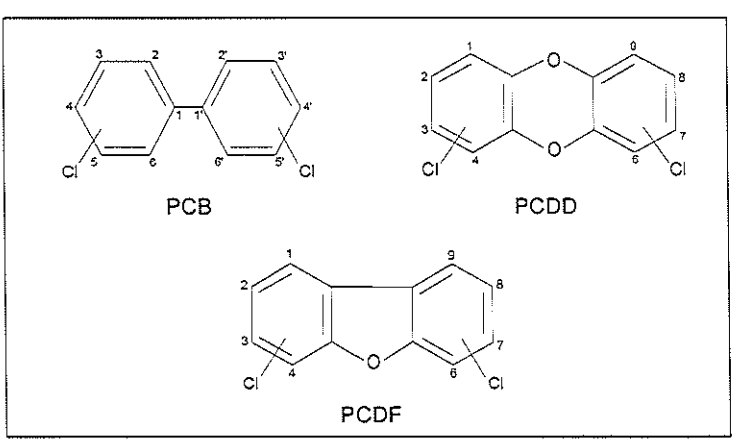

Fig. 6. General structures of polychlorinated biphenyls (PCB), polychlorinated dibenzo-*p*-dioxins (PCDD), and polychlorinated dibenzofurans (PCDF).

PCBs were used as plasticizers, as flame retardants and as hydrolytic fluids in transformers and capacitors, PCDDs and PCDFs are formed during organochlorine synthesis and combustion processes. Emissions of these compounds have been reduced for several years now, but the PHAHs are resistant to breakdown and therefore still widely present in the environment. Cytochrome P450-catalyzed hydroxylation is the main route of PHAH metabolism; the hydroxylated metabolites can be further metabolized by sulfotransferase and UDP-glucuronyltransferase-catalyzed conjugation reactions (129). Many of the effects of PCDDs, PCDFs, non-ortho and mono-ortho PCBs occur via the arylhydrocarbon receptor (AhR) pathway. Via binding to specific DNA sequences, so called dioxin response elements (DREs), the ligand-AhR complex affects transcription of genes containing functional DREs in their promoter region (130). Di- to tetra-ortho PCBs and hydroxylated PHAH metabolites exert their effects through non-AhR pathways.

Disruption of the estrogen- or androgen system is generally believed to be involved in abnormalities in sexual development, gonadal functions, and reproduction (131-133). Exposure of rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during development causes changes in gonadal development, reduced sperm counts, and feminization of male offspring (134,135). Administration of the PCB mixture Aroclor 1254 to rats results in delayed puberty, reduced fertility and reduced prostate, testicular and uterine weights (134,136). In humans, during the last 50 years a decline in semen quality and sperm count and a rise in male reproductive tract abnormalities have been observed in some developed countries, which is suspected to be due to exposure to environmental chemicals (137,138). Furthermore, breast cancer, prostate cancer and testicular cancer incidence have been suggested to be related to PHAH exposure, however, hard evidence for the correlation between PHAH exposure and endocrine tumors is still lacking (131,138-141). The mechanisms involved in disruption of the estrogen system remain unclear. At least in part, planar PHAHs are believed to act via the AhR pathway. Additionally, estrogenic as well as anti-estrogenic effects have been suggested to be induced by binding of hydroxylated PHAHs to the estrogen receptors, although binding affinities are low compared to the natural substrate E2 (142-144). Cytochrome P450 enzymes involved in estrogen metabolism, may also be affected by dioxin and dioxin-like PCBs. For instance, catechol estrogen formation is decreased in hepatic microsomes obtained from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated rats, and the aromatase (CYP19)-catalyzed conversion of testosterone to estradiol is inhibited by various PHAHs (145-147). Additionally, 3,3',4,4',5-pentachlorobiphenyl increases the 7α-hydroxylation, but reduces the 2α-, 6β- and 16α-hydroxylation and 5α-reduction of progesterone and testosterone in rat liver microsomes (148).

Thyroid hormone disruption by PHAHs has been suggested to be related to impaired neurological development. In pregnant women exposed to background levels of PHAHs, a negative correlation was observed between PHAH levels and plasma T3 and T4 levels in newborns and their neurologic development (149-153). Also in experimental animals exposed to PHAHs, plasma T4 levels are reduced and neurological abnormalities have been observed (154-157). As it is still not clear whether the reduced T4 levels contribute to the impaired brain development, this possible relationship needs further study. PHAHs interfere with the thyroid system at various levels: they affect thyroid gland morphology, thyroid hormone receptor binding, the thyroid hormone plasma transport system, and thyroid hormone metabolism (for detailed reviews see 154,156,157). In *in vitro* studies PHAHs and their hydroxylated metabolites inhibit the binding of T3 to the nuclear thyroid hormone receptors (158,159). In addition, hydroxylated metabolites potently inhibit the binding of T4 to the plasma transport protein TTR in rats as well as in humans (154,160). Regarding thyroid hormone metabolism, PHAHs strongly induce UDP-glucuronyltransferase activities in the rat, thus increasing the hepatic clearance of thyroid hormone (154,156,157), while hydroxylated PHAH metabolites inhibit D1 activity (161) and iodothyronine sulfotransferase activity (162). As we expect that iodothyronine sulfotransferases are important in the protection of fetal tissues from excessive thyroid hormone, we speculate that this inhibition of iodothyronine sulfotransferase activity could result in inappropriate levels of active T3, leading to precocious organ development.

Congenital diaphragmatic hernia is an anomaly characterized by a diaphragmatic defect, pulmonary hypoplasia, pulmonary hypertension and eventually surfactant deficiency (163,164). The mechanisms underlying the etiology of congenital diaphragmatic hernia remain unclear (different theories reviewed in 165). To study its pathogenesis, an animal model is frequently used, in which the administration of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) to pregnant rats during mid-gestation induces congenital diaphragmatic hernia in the offspring (166). Nitrofen is a halogenated diphenylether, i.e. a PHAH showing an even greater resemblance to thyroid hormone than the PCBs, PCDDs and PCDFs. Therefore, it may be speculated that nitrofen or its metabolites interfere with the thyroid hormone system in a manner similar to other PHAH(-OHs), and that the interference of nitrofen (metabolites) with the thyroid hormone system may contribute to the abnormal lung development associated with nitrofen-induced congenital diaphragmatic hernia.

Outline of the thesis

Strictly regulated thyroid hormone bioavailability is of crucial importance for normal fetal development. Thyroid hormone metabolism plays a key role in this regulation. The aims of the study described in this thesis were to test the hypotheses that sulfation is a reversible pathway for the inactivation of thyroid hormone during fetal development, and that inactivation of thyroid hormone sulfation by environmental chemicals leads to excessive levels of active T3, resulting in precocious differentiation of different organs.

To assess the importance of thyroid hormone sulfation in thyroid hormone metabolism during fetal development, we studied the ontogeny of the different iodothyronine-metabolizing enzymes in the developing rat (Chapter 2), we identified and characterized the different rat iodothyronine sulfotransferases (Chapter 3), and we characterized the iodothyronine sulfatase activities in human and rat liver and placenta (Chapter 4). In contrast to the situation in humans, reversible iodothyronine sulfation appeared not to be important in thyroid hormone metabolism in the rat. We also characterized the human iodothyronine sulfotransferases (Chapters 5 and 6), and identified the human estrogen sulfotransferase hSULT1E1 as an efficient iodothyronine sulfotransferase (Chapter 6).

Polyhalogenated aromatic hydrocarbons are known for their endocrine-disrupting effects, which may lead to abnormal fetal development. Since estrogen sulfotransferase apparently catalyzes sulfation of both estrogens and iodothyronines (Chapter 6), effects of environmental chemicals on estrogen sulfotransferase could affect the estrogen as well as the thyroid hormone system. In the last part of the thesis, the effects of the various PHAH-OHs on estrogen sulfotransferase were studied (Chapters 7 and 8). In the discussion the results of the studies reported in this thesis are evaluated, and an outlook to future research is presented (Chapter 9).

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Chapter 2

Ontogeny of iodothyronine sulfotransferase, type I and type III deiodinase activities in the rat

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Abstract

Thyroid hormone-deiodinating pathways regulate the bioavailability of T3. In the human fetus low T3, high rT3 and high iodothyronine sulfate levels are found. The low T3 and high rT3 levels can be explained by high D3 levels in placenta and uterus, and low D1 and high D3 levels in the fetus compared to the adult. The high iodothyronine sulfate levels suggest an important role for sulfation in thyroid hormone inactivation. Whereas in the adult the sulfated iodothyronines are very rapidly degraded by D1, in the fetus iodothyronine sulfates form a pool of inactive thyroid hormone which may be reactivated by the action of sulfatases.

In the present study we determined iodothyronine sulfotransferase, sulfatase, D1 and D3 activities in different fetal, neonatal and maternal tissues. The tissues examined were liver, lung, kidney, intestine, brain, skin, heart and placenta. In most tissues D1 activity was low during fetal development. In the liver, however, considerable D1 activity was found from E19 onwards. In the intestine, significant D1 activity was found from E19 to E21, becoming undetectable again after birth. The pattern of D1 protein expression, determined by affinity-labeling with BrAcT3, resembled the pattern of D1 activity in the different tissues. D3 activity was found in placenta, in fetal and neonatal skin and intestine, and in fetal, neonatal and maternal brain. No significant D3 activity was found in fetal liver, which is in contrast to the situation in humans. Sulfotransferase activity was present in all tissues except heart and placenta. No significant sulfatase activity was found in any fetal or neonatal tissue.

In conclusion, iodothyronine deiodinase and sulfotransferase activities vary in a tissue-specific and development stage-dependent manner. D3 activity in placenta, uterus and fetal tissues protects the fetus from excessive T3. The considerable sulfotransferase activities in the fetus suggest an additional role for sulfation in the inactivation of thyroid hormone. However, the low sulfatase activities suggest that desulfation is not important in the reactivation of thyroid hormone in the fetal rat.

Introduction

The tissue-specific and development stage-dependent regulation of thyroid hormone bioactivity during fetal development is essential for normal organ development; disruption of thyroid hormone homeostasis may contribute to congenital anomalies. Deiodination is a key process in the regulation of thyroid hormone homeostasis. The prohormone T4 is converted by outer ring deiodination (ORD) to the receptor-active T3, or inactivated by inner ring deiodination (IRD) to rT3. T3 and rT3 are further deiodinated, by IRD and ORD respectively, to 3,3'-T2 (1). Deiodination is catalyzed by three deiodinases, which have been characterized as homologous transmembrane proteins containing a selenocysteine in the catalytic center. They require thiol as cofactor (2-4). Type I deiodinase (D1) performs both inner ring and outer ring deiodination. In adults, it is predominantly expressed in liver, kidney and thyroid, and is important for the production of plasma T3 and for the clearance of plasma rT3 (2). Type II deiodinase (D2) has only ORD activity. It is predominantly expressed in brain, pituitary and brown adipose tissue (BAT), in humans it is also expressed in the thyroid and perhaps in skeletal muscle and heart (3,5-7); its physiological role is the local production of T3 in these tissues (3). D3 has only IRD activity and is expressed in brain, skin, placenta, pregnant uterus and fetal tissues (8-16). D3 is important for the inactivation of tissue and plasma T3 as well as for the production of plasma rT3. The high levels of D3 in the placenta have been demonstrated to limit transplacental passage of maternal T4 and T3 to the fetus (17,18).

Besides deiodination, glucuronidation and sulfation are important pathways of thyroid hormone metabolism. Glucuronidation is catalyzed by uridinediphospate glucuronyl-transferases. It facilitates the biliary excretion of iodothyronines by increasing their water solubility. Glucuronyltransferase expression is low during fetal development (19,20). Another conjugation pathway is sulfation. Members of the phenol sulfotransferase family (SULT1) catalyze the transfer of a SO_4^{2-} from the universal sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to various exogenous and endogenous compounds, among which iodothyronines (21-25). Sulfation strongly facilitates the IRD (inactivation) of T4 and T3 by D1, but blocks the ORD (activation) of T4 by this enzyme. Under normal conditions, therefore, sulfation is the primary step in the irreversible degradation of thyroid hormone (26). However, when D1 activity is low, sulfation may represent a pathway of reversible thyroid hormone inactivation, depending on activity of arylsulfatases (26-28). This situation may exist in the human fetus, where high levels of iodothyronine sulfates have been detected in fetal serum and amniotic fluid (18,29).

The ontogeny of the different deiodinases in the rat and in humans has been studied before

(10-16), but little is known about the role of sulfotransferases in thyroid hormone metabolism during fetal development. In the present study we determined the ontogeny of sulfotransferase, D1 and D3 activity in different tissues of the developing rat, to elucidate the importance of the different metabolic pathways in the regulation of thyroid hormone homeostasis during fetal development.

Materials and Methods

Materials

Following approval from the Institutional Animal Care and Use Committee of the Erasmus University Medical Center Rotterdam, female Sprague Dawley rats were purchased from Harlan Olac (Oxon, England). The day the vaginal plug was observed was designated as embryonic day 0 (E0). At different gestational ages, after cesarian section (E14-E21) or spontaneous birth (N0 and N1) placenta, fetal and maternal liver, kidney, lung, heart, intestine and brain and fetal skin were dissected for the preparation of homogenates. Tissues were washed in 0.9% NaCl, frozen in liquid N_2 and stored at –80 C until homogenization. Because of the small size, the tissues from different fetuses or newborns (8-15 pups) from the same litter, or from three different litters (E14), were pooled and homogenized on ice in 3-5 volumes 0.1 M phosphate (pH 7.2), 2 mM EDTA, 1 mM dithiothreitol (DTT), using a polytron (Kinematica, Lucerne, Switzerland) and a motor-driven potter (Ika Labortechnik, Staufen, Germany). The homogenates were stored at –80 C until further analysis. Protein concentrations were determined by the method of Bradford (30), using bovine serum albumin as standard.

[3',5'-^{125}I]T4 and [3'-^{125}I]T3 were obtained from Amersham (Amersham, UK); PAPS, protein molecular weight markers, DTT and 6-n-propyl-2-thiouracil (PTU) were purchased from Sigma (St. Louis, MO, USA); T4, rT3, T3 and 3,3'-T2 were purchased from Henning Berlin GmbH (Berlin, Germany); Sephadex LH-20 was obtained from Pharmacia (Woerden, The Netherlands); electrophoresis grade SDS-PAGE reagents were obtained from Bio-Rad (Richmond, IL); and Coomassie Brilliant Blue R-250 was purchased from Merck (Darmstadt, Germany). 3,[3'-^{125}I]T2 and [3',5'-^{125}I]rT3 were prepared by radioiodination of 3-T1 and 3,3'-T2, respectively, as previously described (31). N-bromoacetyl-[3'-^{125}I]T3 (BrAc[^{125}I]T3) was prepared as previously described (32). 3,3'-T2S and 3,[3'-^{125}I]T2S were prepared by reaction of unlabeled and ^{125}I-labeled 3,3'-T2 with chlorosulfonic acid in dimethylformamide. They were purified by LH-20 chromatography as previously described (33).

Sulfotransferase assays

Iodothyronine sulfotransferase activities were analyzed by incubation of usually 0.1 µM

3,3'-T2 and 10^5 cpm of the ^{125}I-labeled compound for 30 min at 37 C with the indicated amounts of tissue homogenate in the absence (blank) or presence of 50 µM PAPS in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA. The reactions were stopped by addition of 0.9 ml ice-cold 0.1 M NaOAc (pH 5.0). The samples were analyzed for 3,3'-T2S formation by applying them to Sephadex LH-20 minicolumns (1 ml bed volume), equilibrated in 0.1 M NaOAc (pH 5.0). Iodide, iodothyronine sulfates and unconjugated iodothyronines were subsequently eluted with 2 x 1 ml 0.1 M NaOAc (pH 5.0), 6 x 1 ml H_2O and 3 x 1 ml 0.1 M NaOH/ethanol (vol/vol 1:1) respectively. Enzymatic sulfation was corrected for background radioactivity detected in the blanks.

T2S sulfatase assays

T2S sulfatase activities were determined by incubation of 0.1 µM T2S and 10^5 cpm 3,[3'-^{125}I]T2S and 0.1 mM PTU (to block D1 activity) for 60 min at 37 C with 0.5 mg protein/ml in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA. The reactions were stopped by addition of 0.9 ml ice-cold 0.1 M NaOAc (pH 5.0). The mixtures were analyzed for 3,3'-T2 formation by chromatography on Sephadex LH-20 minicolumns as described above. Enzymatic T2S hydrolysis was corrected for background radioactivity detected in blanks without homogenate.

Type I deiodinase assays

D1 activities were measured by incubation of 0.1 µM [3',5'-^{125}I]rT3 for 60 minutes at 37 C with the indicated amounts of tissue homogenates in the absence or presence of 0.1 mM PTU and 0.1 µM rT3 in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, 10 mM DTT. Reactions were stopped by addition of 0.1 ml 5% bovine serum albumin. The protein-bound ^{125}I-labeled iodothyronines were precipitated by addition of 0.5 ml 10% trichloroacetic acid. After centrifugation, the supernatants were analyzed for $^{125}I^-$ production on Sephadex LH-20 minicolumns (bed volume 0.25 ml), equilibrated and eluted with 0.1 M HCl.

Type III deiodinase assays

D3 activities were measured by incubation of 1 nM or 1 µM [3'-^{125}I]T3 for 30 minutes at 37 C with the indicated amounts of tissue homogenates in the presence of 0.1 µM rT3 and 0.1 mM PTU (to block D1 activity) in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, 50 mM DTT. Reactions were stopped by addition of 0.1 ml ice-cold methanol. After centrifugation, 0.15 ml supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The proportion of acetonitrile was

increased linearly from 30% to 44% in 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Affinity-labeling

0.1 µCi BrAc[^{125}I]T3 was reacted for 15 min at 37 C with 0.1 mg protein in 75 µl 0.1 M phosphate (pH 7.2), 2 mM EDTA, 3 mM DTT. The reaction was stopped by addition of 25 µl SDS-PAGE loading buffer containing 10 mM DTT. Subsequently the samples were heated for 10 min at 80 C. Proteins were separated overnight by SDS-PAGE in a 16 cm 12% polyacrylamide gel. Gels were stained for 2 h with Coomassie Brilliant Blue R-250 in 45% methanol / 10% acetic acid and destained for 5 h with 45% methanol / 10% acetic acid, both at room temperature. Gels were dried at 80 C under vacuum, and autoradiographed at room temperature using BioMax MS-1 film (Eastman Kodak, Rochester, NY).

Results

Sulfotransferase, sulfatase, D1 and D3 activities were determined in rat tissues from fetuses and newborns from different development stages (E14-21 and N0 and 1). Enzyme activities were also determined in maternal rat tissue homogenates.

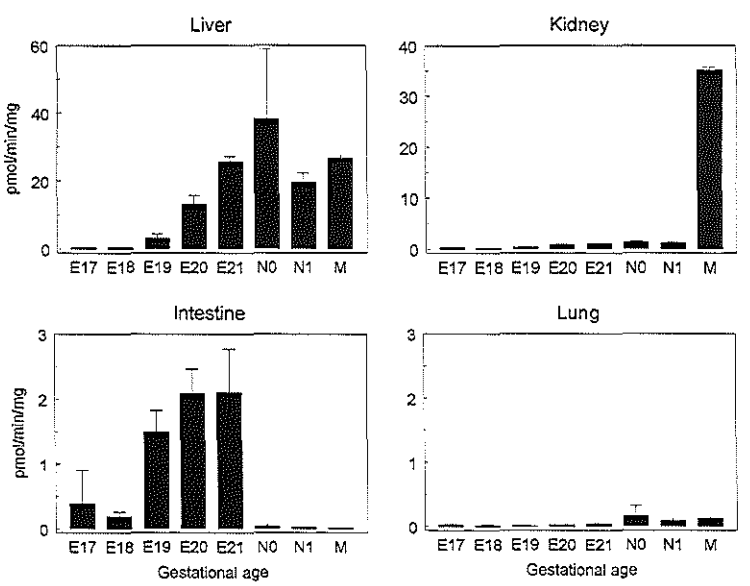

Fig. 1. D1 activities in fetal (E17-21), neonatal (N0-1) and maternal (M) rat liver, kidney, intestine or lung homogenate. Reaction conditions: 0.1 µM [3',5'-^{125}I]rT3, 10 mM DTT, 0.5 mg (fetal and neonatal tissue homogenate) or 0.1 mg (maternal liver and kidney homogenate) or 1 mg (maternal lung and intestine homogenate) protein/ml, 60 min incubation. Results are the means ± SD from 2 to 4 pools of tissue homogenate, except for E21 in kidney, which represents the mean of triplicate determinations on a single pool of tissue homogenates.

Figure 1 shows D1 activities in fetal, neonatal and maternal rat liver, kidney, intestine and lung. Clear development stage-dependent patterns were observed in the different tissues. In the liver, deiodinase activity is present from E19 onwards, reaching a maximum value (30 pmol/min/mg protein) at E21. The apparent increase in D1 activity at neonatal day 0 compared to other stages is not significant. D1 activities in the developing kidney remain low until after birth, while D1 activities in maternal kidney homogenates were as high as in maternal liver. D1 activity in rat lung is very low in the fetal, neonatal and in the adult stage. In the intestine a remarkable pattern was observed. While significant D1 activity is present from E18 to E21 (conversion rates increasing from around 0.2 to 2 pmol/min/mg protein), D1 activity becomes undetectable after birth. No significant D1 activities were found in placenta, skin, brain and heart homogenates at any age tested (data not shown). The kinetics of D1 activity were measured in fetal liver, kidney and intestine homogenate from E20. The apparent K_m values for liver, kidney and intestine were 0.17, 0.16 and 0.23 µM, respectively, and their V_{max} values approximately 14, 2 and 4 pmol/min/mg, respectively.

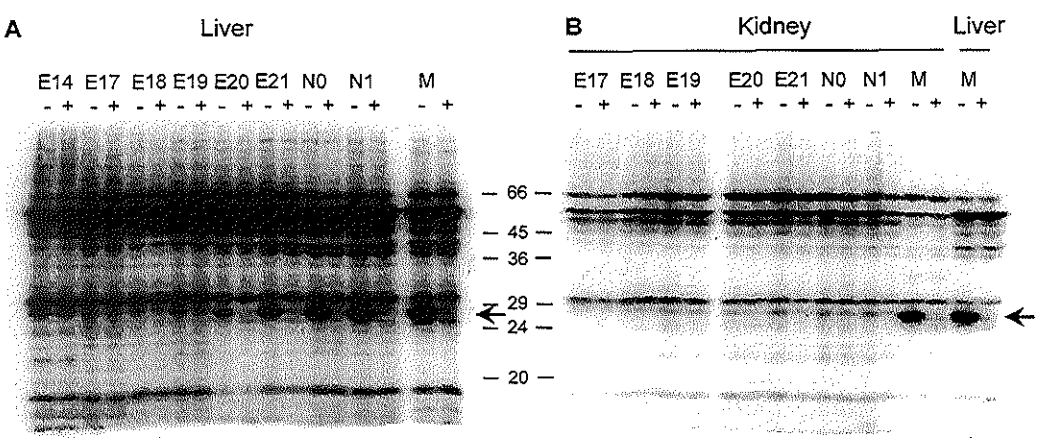

Fig. 2. Affinity-labeling of fetal (E14-21), neonatal (N0-1) and maternal (M) rat liver (A and B) or kidney (B) homogenate with BrAc[^{125}I]T3 in the absence (-) or presence (+) of 100 µM PTU and 10 µM rT3. Reaction conditions: 0.1 µCi BrAc[^{125}I]T3, 100 µg protein, 3 mM DTT, without (-) or with (+) 100 µM PTU and 10 µM rT3, 15 min incubation. The molecular weight of the marker proteins and the labeled D1 protein (*arrow*) are indicated.

Figure 2 shows the results of the affinity-labeling of fetal, neonatal and adult rat liver and kidney by BrAc[^{125}I]T3. As demonstrated previously, the 27 kD band represents the affinity-labeling of D1 (34). The D1 protein expression level, although semi-quantitative, correlates well with the enzyme activity patterns for the different tissues. When the liver homogenates were

incubated with BrAc[^{125}I]T3 in the presence of PTU and rT3, the labeling of the 27 kD band was completely inhibited. In the liver, D1 protein expression is found from E19 onwards, increasing until birth (Fig. 2A). The ontogenic profile of D1 protein expression in the intestine was less clear because of the low levels of expression (data not shown). In fetal and neonatal kidney (E20-N1) D1 protein expression is just above background; high D1 protein expression was found in the maternal kidney (Fig. 2B). We were not able to identify D3 protein and sulfotransferase expression by affinity-labeling with BrAc[^{125}I]T3.

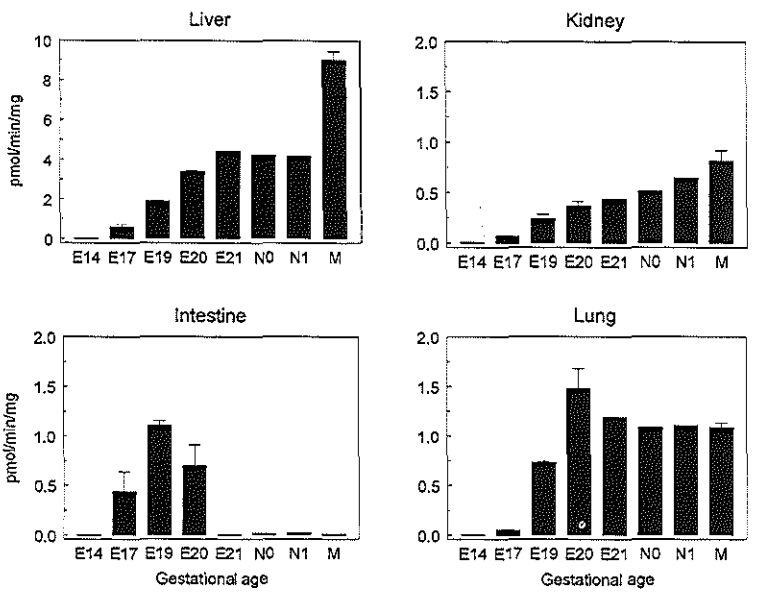

Fig. 3. Sulfotransferase activities in fetal (E14-21), neonatal (N0-1) and maternal (M) rat liver, kidney, intestine or lung homogenate. Reaction conditions: 1 µM 3,[3'-^{125}I]T2, 0.5 mg (fetal and neonatal tissue homogenate and maternal liver, kidney and lung homogenate) or 1 mg (maternal intestine homogenate) protein/ml, 50 µM PAPS, 30 min incubation. Results are the means ± SD from 2 to 3 pools of tissue homogenate, except for E14, E21, N0 and N1, which represent the means of triplicate determinations on a single pool.

Figure 3 presents the iodothyronine sulfotransferase activities in liver, kidney, intestine and lung. In the liver, sulfotransferase activity is detected from E17 onwards, increasing until E21. Maximum sulfation rates are reached after N1, the maternal sulfation rate of 9 pmol/min/mg protein being twice as high as the activity expressed around birth. Sulfotransferase activity in the kidney steadily increases from E17, and reaches maximum values just after birth. In the intestine sulfotransferase activity peaks around E19 (1.2 pmol/min/mg protein), and has become undetectable again at E21, whereas in the lung sulfotransferase activity increases from

E17 to reach a maximum sulfation rate of 1.4 pmol/min/mg at E20. The sulfation rate in the lung at E20 does not significantly differ from sulfation rates at later development stages.

Figure 4 shows the HPLC analysis of a representative D3 assay. Radiolabeled 3,3'-T2 was formed from [3'-^{125}I]T3 after incubation with brain homogenate from neonatal day 0 (Fig. 4A). In the presence of 1 µM unlabeled T3, conversion of labeled T3 to 3,3'-T2 was completely inhibited (Fig. 4B).

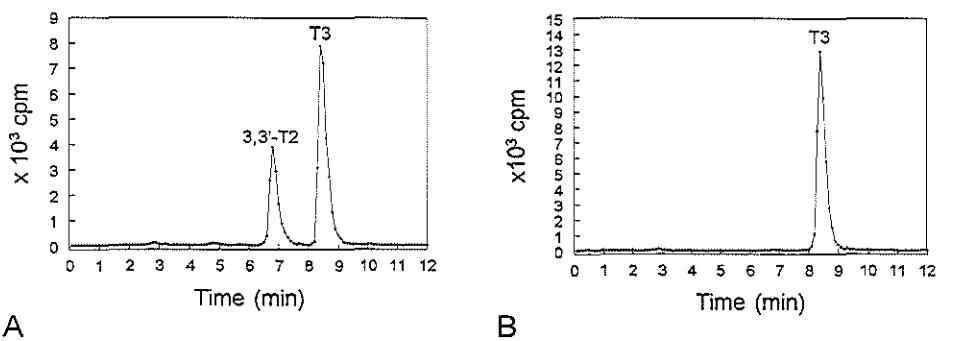

Fig. 4. HPLC analysis of the inner ring deiodination of [3'-^{125}I]T3 by neonatal rat brain homogenate in the absence (A) or presence (B) of 1 µM T3. Reaction conditions: 1 nM [3'-^{125}I]T3, 0.5 mg/ml neonatal (N0) rat brain homogenate, 0.1 µM rT3, 0.1 mM PTU, 50 mM DTT, without (A) or with (B) 1 µM T3, 30 min incubation.

High D3 activities were found in the placenta and the developing brain (Fig. 5). In the placenta, D3 activity is already maximal at E14, whereas in the brain maximal D3 deiodination activity is reached at E18. A maximum deiodination rate of 40 fmol/min/mg protein is detected from E18 until after birth, which is ~3-fold higher than the D3 activity in the maternal brain. Significant D3 activities were also found in fetal and neonatal skin and in intestine at all stages tested (Fig. 5). D3 activity in fetal rat liver, kidney and lung was very low (results not shown); no D3 activity was found in the fetal rat heart.

Figure 6 depicts sulfotransferase activities in the placenta, developing brain and skin. In the placenta no significant sulfotransferase activity was found at any development stage. In the brain, basal iodothyronine sulfation rates of ~250 fmol/min/mg protein were observed at all fetal and neonatal stages; in the maternal brain homogenates sulfation rate is >10-fold higher. In the skin, iodothyronine sulfotransferase activity was constant (500 fmol/min/mg protein) at all fetal and neonatal stages. No significant iodothyronine sulfation was detected in the fetal, neonatal or maternal heart.

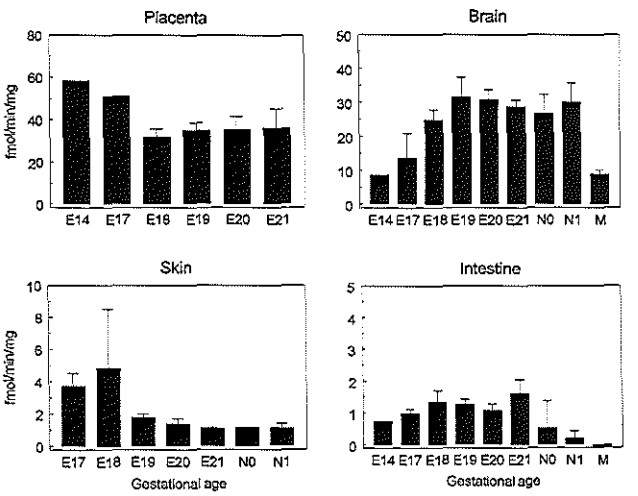

Fig. 5. D3 activities in fetal (E14-21), neonatal (N0-1) and maternal (M) rat placenta, brain, skin or intestine homogenate. Reaction conditions: 1 nM [3'-^{125}I]T3, 0.5 (fetal and neonatal tissue homogenate) or 1 mg (maternal tissue homogenate) protein/ml, 0.1 µM rT3, 0.1 mM PTU, 50 mM DTT, 30 (placenta, brain and skin) or 60 (intestine) min incubation. Results are the means ± SD from 2 to 4 pools of tissue homogenate, except for E14 and E17 in placenta and E14 in brain and intestine, which represent the means of triplicate determinations from a single pool.

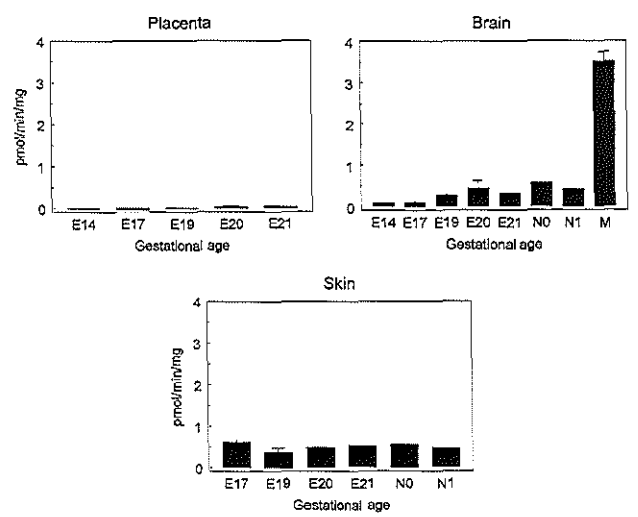

Fig. 6. Sulfotransferase activities in fetal (E14-21), neonatal (N0-1) and maternal (M) rat placenta, brain or skin homogenate. Reaction conditions: 1 µM 3,[3'-^{125}I]T2, 0.5 mg protein/ml, 50 µM PAPS, 30 min incubation. Results are the means ± SD from 2 to 3 pools of tissue homogenate, except for E14, E21, N0 and N1, which represent the means of triplicate determinations from a single pool.

T2S sulfatase activities were also tested in the different tissues. Whereas significant activities were found in maternal liver and kidney homogenate, sulfatase activities in fetal and neonatal tissue homogenates were negligible under the conditions used. As we have recently found that phosphate inhibits iodothyronine sulfatase activity (Chapter 4), our assay conditions which included the use of phosphate buffer were not optimal. However, also under optimal conditions (using 0.1 M Tris/HCl, pH 7.2) fetal sulfatase activities were low (i.e. <40 fmol/min/mg in fetal liver homogenate *vs* ~200 fmol/min/mg in maternal liver homogenate).

Discussion

Thyroid hormone is needed for organ development. Early in pregnancy the fetus is supplied by maternal thyroid hormone through transport across the placenta (29,35-38). The fetal thyroid starts to produce thyroid hormone after 18-22 weeks of gestation in humans, and at embryonic day 17.5-18 in rats (29,35,36). However, the substantial T4 levels in newborns with thyroid hormone agenesis or a total thyroid hormone synthesis defect suggest that also after the first trimester the maternal supply of thyroid hormone across the placenta remains an important source of fetal thyroid hormone (39).

Iodothyronine metabolism is key to the regulation of thyroid hormone bioavailability during fetal development. The low T3 and high rT3 levels in fetal serum (18,29,35,36) are explained by low D1 and high D3 activity in fetal tissues, placenta and uterus (10-16). D2 activity in tissues such as brain, placenta and uterus is probably important for local T3 but not for systemic T3 production (10,12,14,40). Whereas D3 catalyzes the irreversible inactivation of thyroid hormone, sulfation may play a role in the reversible thyroid hormone inactivation during fetal development. The iodothyronine sulfates that are abundantly present in the human fetal serum (18,41-43) are suggested to form a pool of inactive iodothyronines that can be reactivated by arylsulfatase-catalyzed desulfation (26-28). The aim of the present study was to delineate the role of the different thyroid hormone-metabolizing pathways during fetal development.

The pattern of D1 and D3 activities in the liver is different from that in humans. Whereas in human liver D1 activity is already present in the second trimester (16), we observed that in rat liver D1 activity rises just before birth. This is in agreement with previous findings (11,13). We confirmed the observation by Huang et al. and Galton et al. that no substantional D3 activity is present in the fetal rat liver (11,15), whereas significant hepatic D3 activity is present in the human fetus (16). Our findings, therefore, demonstrate that the rat is not the ideal model for the ontogeny of D1 and D3 expression in fetal human liver.

The remarkable pattern of ORD activity in the intestine is consistent with earlier

observations (11,15), and seems evolutionary conserved: the ontogenic profile of D1 activity in the rat intestine is similar to that of ORD D2 activity in the amphibian *Rana catesbeiana*. In the frog, ORD activity was highest during the thyroid hormone-dependent metamorphosis climax (44). In fetal rats there may be a peak in intestinal T3 level (at E21), as at that stage D1 activity is maximal whereas iodothyronine sulfotransferase activity has become undetectable. The reason for this putative T3 peak at E21 is unclear. Intestinal differentiation is subject to major changes at various stages of development, such as villi formation at day 17-19 (45). More so than in tissues such as liver and kidney, intestinal protein expression patterns determined by SDS-PAGE differ between development stages (data not shown). In the rat, intestinal maturation is not achieved until after weaning (45). The high D1 activity Bates et al. found at 10 days after birth may represent a second peak of D1 activity during intestinal development (14). D3 expression is similar at all developmental stages. Further studies are needed to determine in which parts of the intestine the different enzymes are expressed and to identify the factors regulating their expression.

Although D3 activity was undetectable in the fetal rat heart as a whole, it is still possible that D3 activity is present in specific parts of the fetal rat heart. In the fetal human heart, Richard et al. found low but significant D3 activity (Richard et al., unpublished observations). Furthermore, it is interesting to note that monocrotaline-induced heart failure due to obstruction of lung blood flow in rats leads to reexpression of various fetal genes, among which D3 (46, Simonides et al., unpublished observations). In this monocrotaline model D3 activity was highest in the right ventricle.

We found significant D3 expression in the fetal and neonatal skin. Literature data indicate that skin D3 increases after birth, reaching a maximum at two weeks after birth (14). As the rat is born immature, and placental and uterine D3 is not available to protect the newborn from excessive thyroid hormone, it would be interesting to also study D3 expression in tissues such as liver, heart and lung in the first three weeks after birth. Concerning brain D3, Ködding et al. found elevated D3 activities in various regions of the rat brain, including cerebellum, basal ganglia, brain stem and hypothalamus, immediately after birth (47). In the human brain the pattern of deiodinase expression of the fetus differs from that of the adult: while in the adult brain D3 activity is present in all brain regions except the cerebellum and pons (48), in the fetal cerebellum D3 activity is >2.5-fold higher than in any other region of the brain (49).

T4 and T3 are present in fetal rat serum before the fetal thyroid starts secretion at E17.5-18 (36). Whereas serum T4 steadily increases after the onset of fetal thyroid function until birth, i.e. from 0.5 to 4.6 ng/ml, serum T3 level shows only a modest increase from 0.05 to 0.09 ng/ml (13,36). The low rat fetal hepatic D1 activity and the high D3 activity in rat placenta, uterus and

fetal brain may contribute to this low fetal serum T3. It should be noted, however, that although the fetal serum T3 level is low compared to the adult, the difference between the fetal and adult T3 level may be smaller when free T3 levels are compared. Furthermore, the ratio of T3 *vs* T4 may not be lower in the fetus than in the adult.

Sulfation of iodothyronines has been demonstrated in human liver and kidney as well as with recombinant isoenzymes of the human SULT1 family, including SULT1A1, 1A3, 1B1, and 1E1 (22-24). In contrast to the activity of human SULT1A1, rat SULT1A1 does not catalyze the sulfation of these compounds (21). Both rat SULT1B1 and 1C1 as well as the above-mentioned human enzymes use 3,3'-T2 as the preferred substrate, although human estrogen sulfotransferase SULT1E1 equally prefers 3,3'-T2 and rT3. By immunoblot analysis, Richard et al. have recently shown that SULT1A1 is similarly expressed in human fetal and neonatal liver at levels roughly half of that in the adult liver. In contrast, SULT1A3 is present in the fetal liver, but becomes undetectable after birth (50). They also studied the ontogeny of iodothyronine sulfotransferase activity in the human fetal liver. Comparison of the 3,3'-T2 sulfation pattern with the SULT expression pattern led to the conclusion that SULT1A1 is predominantly involved in 3,3'-T2 sulfation (50). This conclusion is supported by our characterization of iodothyronine sulfation by human liver cytosol in comparison with that by the different recombinant SULT1 isoenzymes (23). In the rat, Hurd et al. found T3 sulfotransferase activity in various fetal tissues (51), but the ontogenic profile of iodothyronine sulfotransferase activity in different tissues has not been studied before. The considerable sulfotransferase activities that were found, which varied in a tissue-specific and development stage-dependent manner, suggest that sulfation plays a role in thyroid hormone inactivation in the developing fetal rat. Rat SULT1B1 and 1C1 expression are low at birth (52-54). Rat SULT1B1 expression starts to increase after two weeks, a maximum being reached in male as well as in female rats older than two months (52). Rat SULT1C1 expression remains low in females, but increases dramatically after 30 days in male rats (53). By northern blot analysis rat SULT1B1 and 1C1 mRNA were undetectable in fetal tissues, whereas the different mRNAs were identified in adult rat liver (results not shown). The undetectable rSULT1B1 and 1C1 expression before birth suggests that also other isoenzymes are involved in iodothyronine sulfation in the fetal rat.

In the placenta and developing brain, where insignificant sulfotransferase activities but high D3 activities were found, irreversible inactivation by D3 seems to be a predominant pathway of iodothyronine metabolism. In the liver, sulfotransferase activity is present from E17, when D1 activity is still absent. As explained above, when D1 activity is low, thyroid hormone sulfates may accumulate and form a reservoir of inactive thyroid hormone, from which active hormone may be liberated in a tissue-specific and development stage-dependent manner by the action of

arylsulfatases. So far, 6 members of the arylsulfatase family have been identified (ARSA–ARSF) in humans (55). We previously found that the lysosomal arylsulfatases ARSA and ARSB do not catalyze iodothyronine sulfate hydrolysis. However, we also demonstrated that the microsomal arylsulfatase ARSC, which is abundantly present in the human placenta, catalyzes the hydrolysis of iodothyronine sulfates in human placenta microsomes (Chapter 4). Although iodothyronine sulfatase activity has been detected in the microsomal fractions of different tissues in adult humans and rats (56, Chapter 4), very little is known about the regulation of these sulfatase activities. Santini et al. observed some T3S sulfatase activity in fetal liver and brain (18), Huang et al. found that T3S sulfatase activity in the liver progressively increases after birth until two months of age (57). In the present study we investigated the ontogeny of the iodothyronine sulfatase activities in different rat tissues. The negligible sulfatase activities we observed in fetal and neonatal rat tissues as well as the low T3S and T4S levels in fetal rat serum at E20 (Schuur et al., unpublished observations) suggest that sulfation of T3/T4 is not important as pathway of reversible thyroid hormone inactivation in the fetal rat.

In general, we can conclude from this study that, compared to the adult, D1 activity in the fetal liver and kidney are low and that D3 activity is high in placenta, pregnant uterus en fetal brain. Besides deiodination by D3, sulfation seems important to protect the fetus from excessive T3. However, the role of sulfation in the reversible inactivation of thyroid hormone during fetal development of rats remains unclear.

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Chapter 3

Characterization of rat iodothyronine sulfotransferases

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Abstract

Sulfation appears an important pathway for the reversible inactivation of thyroid hormone during fetal development. The rat is an often used animal model to study the regulation of fetal thyroid hormone status. The present study was done to determine which sulfotransferases are important for iodothyronine sulfation in the rat, using radioactive T4, T3, rT3, and 3,3'-T2 as substrates, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as cofactor, and rat liver, kidney and brain cytosol, and recombinant rat SULT1A1, 1B1, 1C1, 1E1, 2A1, 2A2 and 2A3 as enzymes. Recombinant rat SULT1A1, 1E1, 2A1, 2A2 and 2A3 failed to catalyze iodothyronine sulfation. For all tissue sulfotransferases and for rSULT1B1 and rSULT1C1, 3,3'-T2 was by far the preferred substrate. Apparent K_m values for 3,3'-T2 amounted to 1.9 μM in male liver, 4.4 μM in female liver, 0.76 μM in male kidney, 0.23 μM in male brain, 7.7 μM for SULT1B1, and 0.62 μM for SULT1C1, while apparent K_m values for PAPS showed less variation (2.0-6.9 μM). Sulfation of 3,3'-T2 was inhibited dose-dependently by other iodothyronines, with similar structure-activity relationships for most enzymes except for the sulfotransferase activity in rat brain. The apparent K_m values of 3,3'-T2 in liver cytosol were in between those determined for SULT1B1 and 1C1, supporting the importance of these enzymes for the sulfation of iodothyronines in rat liver, with a greater contribution of SULT1C1 in male than in female rat liver. The results further suggest that rSULT1C1 also contributes to iodothyronine sulfation in rat kidney, whereas other, yet unidentified forms appear more important for the sulfation of thyroid hormone in rat brain.

Introduction

Sulfation is a metabolic reaction which facilitates the excretion of endogenous and exogenous hydrophobic compounds in bile and urine, by increasing their water solubility (1-3). Biliary excretion of iodothyronines is also increased by sulfation. More importantly, however, sulfation appears to be a key step in the inactivation of thyroid hormone. The prohormone thyroxine (T4) is converted by outer ring deiodination (ORD) to the biologically active 3,3',5-triiodothyronine (T3), or by inner ring deiodination (IRD) to the inactive 3,3',5'-triiodothyronine (rT3) (4). By sulfation, T3 loses its affinity for the thyroid hormone receptors (5). Additionally, T3S is subject to accelerated degradation as sulfation facilitates the IRD of T3 by type I deiodinase (D1) (6,7). Sulfation also facilitates the inactivating IRD of T4 by D1, whereas the activating ORD of T4 by D1 is completely blocked by sulfation (6,7). Therefore, an important function of sulfation is to facilitate the irreversible degradation of thyroid hormone. Furthermore, under conditions in which the deiodinative clearance of sulfates is impaired, sulfation may be reversed by sulfatases. As T3S and T4S levels in the human fetal circulation are high (8-10), it has been speculated that sulfation is a mechanism to protect the fetus from excessive T3 and that sulfation/desulfation plays an important role in the regulation of thyroid hormone bioactivity during fetal development (11-13). The exact mechanism for the increased iodothyronine sulfate levels in the fetal circulation is unclear but the reversible nature of this inactivation step contrasts with the irreversible nature of D3-catalyzed IRD, which is also extensive during fetal development (14-18).

Sulfation is catalyzed by cytosolic sulfotransferases present in a wide range of tissues. The sulfotransferases transfer the sulfuryl group of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to usually OH groups of their substrates (3,19). All cytosolic sulfotransferases are members of a single gene superfamily termed SULT. A systematic nomenclature is in preparation, but not yet finalized. It is already widely used for human SULTs, but not for rat SULTs. Table 1 indicates the designations of the rat SULTs used in the present study together with synonymous names that have been used elsewhere. On the basis of amino acid sequence, two families of sulfotransferases have been identified in humans, the SULT1 family, which primarily represent phenol sulfotransferases, including hSULT1A1, 1A2, 1A3, 1B1, 1C2, 1C4 and 1E1 (20-33) and the SULT2 family, which usually prefer alcoholic substrates (including hydroxysteroids) (3,34-37). In the rat, the phenol sulfotransferases rSULT1A1, 1B1, 1C1, 1C2, 1C3, 1D1, 1E1 and 1E2 have been cloned (1,38-44), and the hydroxysteroid sulfotransferases rSULT2A1, 2A2 and 2A3 (45-47). For several human and rat phenol sulfotransferases allelic variants have been identified (2,3,48,49). Another important observation is that the sulfotransferases may not only

exist as homo- but also as heterodimers (50).

Alignment of the amino acid sequences of SULT enzymes revealed at least two areas of highly conserved amino acids (1,49,51,52). Recently the crystal structures of mouse SULT1E1, human SULT1A3 and human SULT2A1 have been elucidated (53-56). Based on these crystal structures, and on site-directed mutagenesis experiments, the N-terminal motif TYPKSGT and the conserved amino acids RKGXXGXXK near the C-terminal end are suggested to be involved in PAPS binding (57-60).

Sulfation of iodothyronines is catalyzed by phenol sulfotransferases. Recently, we identified hSULT1A1, 1A3, 1B1 and 1E1 as human iodothyronine sulfotransferases (61,62). Because the rat is the most frequently used animal model for *in vivo* studies of iodothyronine metabolism, we set out to characterize the sulfation of different iodothyronines by rat liver, kidney and brain cytosol, and by recombinant preparations of rSULT1A1, 1B1, 1C1, 1E1, 2A1, 2A2 and 2A3, to identify which sulfotransferases are important for iodothyronine sulfation in the rat.

Table 1. Designation of rat sulfotransferases

Designation used in this publication	Designation used in other publications	GenBank Accession Number (Protein)	Number of amino acids
SULT1A1	ST1A1, P-PST IV, AST-IV	CAA37065	291
SULT1B1	ST1B1	AAB31318	299
SULT1C1	ST1C1, HAST-I	A49098	304
SULT1C1var [2]	not published		304
SULT1C2 [1]	SULT1C2	CAB41460	296
SULT1C3 [1]	SULT1C2A	CAB41461	296
SULT1D1 [1]	not published	AAC99890	308
SULT1E1	ST1E2, rEST-1, rEST-3	AAA41128	295
SULT1E2 [1]	ST1E6, rEST-2, rEST-6	AAB33442	295
SULT2A1	ST2A1, ST-20/21	A34822	284
SULT2A2	ST2A2, ST-40/41, STa	BAA03632	284
SULT2A3	ST2A5, ST-60	BAA03634	284
SULT4A1[1]	rBR-STL	AAF61198	284

[1] Not investigated in the present study.

[2] The cDNA-deduced amino acid sequence differs from A49098 in three residues (S2A, T60A, S96P).

Materials and Methods

Materials

Male and female Wistar rat liver cytosols and male kidney and brain cytosols were obtained as previously described (63). Rat SULT1C1 cDNA (40) was kindly provided by Dr. Y. Yamazoe, and expressed in V79 cells as previously described (64). rSULT1A1 cDNA (65) was kindly provided by Dr. C.N. Falany, and expressed in *Salmonella typhimurium* (48). rSULT2A1 (ST-20), rSULT2A2 (ST-41) and rSULT2A3 (ST-60) were cloned and expressed in *S. typhimurium,* and rSULT2A1 was also expressed in V79 cells (64,66). rSULT1B1, a rSULT1C1 variant containing amino acid substitutions S2A, T60A and S96P, rSULT1E1 and rSULT2A3 were cloned by RT-PCR and expressed in *Salmonella typhimurium* (48). V79 and bacterial cell cytosols were prepared as previously described (48).

[3',5'-^{125}I]T4 and [3'-^{125}I]T3 were obtained from Amersham (Amersham, UK); T4, rT3, T3, 3,5-, 3,3'- and 3',5'-diiodothyronine (T2), 3- and 3'-iodothyronine (T1) and thyronine (T0) were purchased from Henning Berlin GmbH (Berlin, Germany); 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was obtained from Sigma (St. Louis, MO, USA); and Sephadex LH-20 were obtained from Pharmacia (Woerden, The Netherlands). 3,[3'-^{125}I]T2 and [3',5'-^{125}I]rT3 were prepared by radioiodination of 3-T1 and 3,3'-T2, respectively, as previously described (67).

Sulfotransferase assays

Iodothyronine sulfotransferase activities were analyzed by incubation of usually 0.1 or 1 μM T4, T3, rT3 or 3,3'-T2 and 10^5 cpm of the ^{125}I-labeled compound for 30 min at 37 C with the indicated amounts of liver, kidney or brain cytosol or recombinant sulfotransferase in the presence or absence (blank) of 50 μM PAPS in 0.2 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA. The reactions were stopped by addition of 0.8 ml 0.1 M HCl. The mixtures were analyzed for iodothyronine sulfate formation by chromatography on Sephadex LH-20 minicolumns as previously described (68). Enzymatic sulfation was corrected for background radioactivity detected in the blanks.

Results

Figure 1 shows the sulfation of 0.1 μM T4, T3, rT3 and 3,3'-T2 by male and female rat liver cytosol, male rat kidney and brain cytosol, rSULT1B1 and 1C1 in the presence of 50 μM PAPS. All enzyme preparations show a substrate preference for 3,3'-T2. Rates of 3,3'-T2 sulfation are >50-fold higher than those of T3 and rT3 sulfation; T4 is the poorest substrate for all enzyme preparations. rSULT1E1, 2A1, 2A2 and 2A3 did not catalyze iodothyronine sulfation (data not

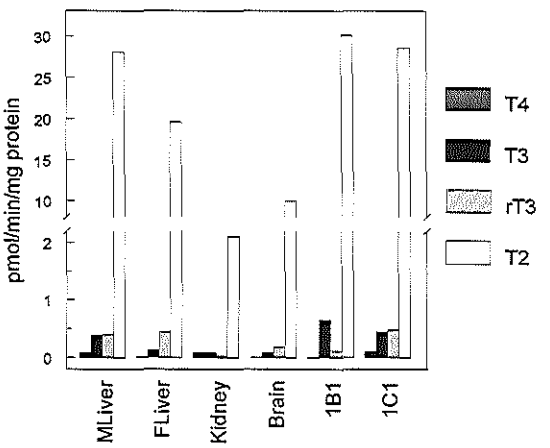

Fig. 1. Sulfation of iodothyronines by male and female rat liver cytosol, male rat kidney and brain cytosol, rSULT1B1 and rSULT1C1. Reaction conditions were 0.1 µM ^{125}I-labeled T4, T3, rT3 or 3,3'-T2, 0.1 mg protein/ml, 50 µM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

shown).

Figure 2 shows the sulfation of 3,3'-T2 by female rat liver, male rat liver, male kidney or brain cytosol as a function of the substrate concentration. Maximum sulfation rates were obtained at ~10 µM 3,3'-T2 in male and female rat liver cytosol, at ~2 µM in male rat kidney cytosol and at ~1 µM in male rat brain cytosol. Rat brain cytosol showed clear substrate inhibition for 3,3'-T2 at concentrations above 1 µM. K_m and V_{max} values for the different tissue cytosols were calculated from the linear double-reciprocal plots of sulfation rate *versus* 3,3'-T2 concentration and are presented in Table 2. V_{max} values decreased in the order male liver > female liver > brain > kidney. K_m values for T3 sulfation by the tissue cytosols, which were determined under the same conditions, were >50-fold higher than for the sulfation of 3,3'-T2 (data not shown).

Figure 3 depicts the sulfation of 3,3'-T2 by rSULT1B1 or rSULT1C1 as a function of the substrate concentration. For rSULT1C1 maximum sulfation rates were obtained at lower 3,3'-T2 concentrations than for rSULT1B1. The decrease in sulfation rate for rSULT1C1 at concentrations above 1 µM indicated substrate inhibition. The apparent K_m values calculated from the Lineweaver-Burk plots amounted to 7.7 µM for rSULT1B1 and 0.62 µM for rSULT1C1 (Table 2). As crude cytosols of rSULT1B1-expressing *Salmonella* cells and rSULT1C1-expressing V79 cells were tested, the V_{max} values for the different enzymes are not representative for their K_{cat} values.

Table 2. Kinetic parameters of rat iodothyronine sulfotransferases

Enzyme source	K_m (μM)	V_{max} (pmol/min/mg protein)
Substrate: 3,3'-T2		
Male Liver cytosol	1.85 ± 0.45	2042 ± 400
Female Liver cytosol	4.35 ± 0.59	1516 ± 214
Kidney cytosol	0.76 ± 0.05	15.6 ± 1.9
Brain cytosol	0.23 ± 0.01	32.0 ± 0.3
rSULT1B1 (*Salmonella*)	7.74 ± 1.46	6029 ± 1146
rSULT1C1 (V79 cells)	0.62 ± 0.16	251 ± 93
Substrate: T3		
rSULT1B1 (*Salmonella*)	142 ± 9	1156 ± 133
rSULT1C1 (V79 cells)	100 ± 6	50.8 ± 6.3

Data are presented as the means ± SD of 2-6 experiments. Incubations were done with 50 μM PAPS.

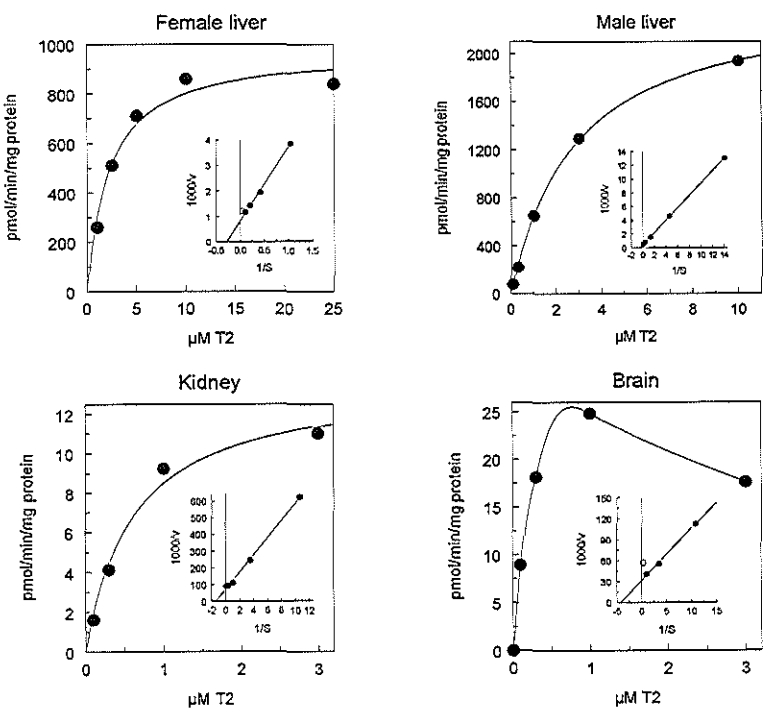

Fig. 2. Effects of substrate concentration on the sulfation of 3,3'-T2 by female or male rat liver cytosol, male kidney or brain cytosol. The *insets* show the double reciprocal plot. Reaction conditions were 0.1-25 μM 3,[3'-^{125}I]T2, 25 (male liver), 50 (female liver and male brain) or 250 (male kidney) μg protein/ml, 50 μM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

The kinetic parameters for T3 sulfation by the different isoenzymes are also presented in Table 2. Compared to 3,3'-T2, apparent K_m values for T3 were 20 to 150-fold higher. The apparent K_m value determined for 3,3'-T2 sulfation by the rSULT1C1 variant (5.8 µM) was 10-fold higher than for wild-type rSULT1C1.

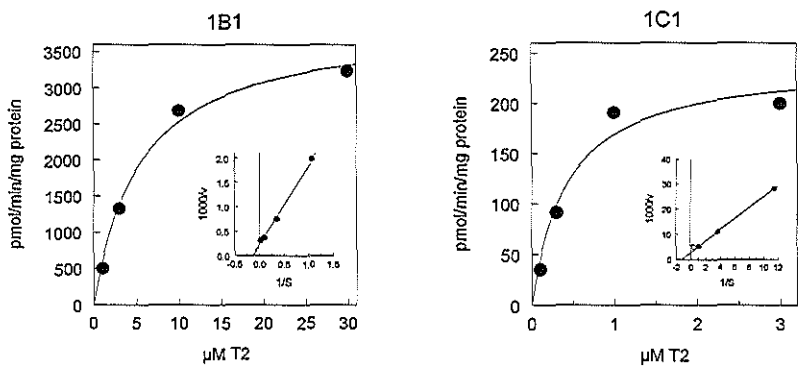

Fig. 3. Effects of substrate concentration on the sulfation of 3,3'-T2 by rSULT1B1 and rSULT1C1. The *insets* show the double reciprocal plot. Reaction conditions were 0.1-30 µM 3,[3'-^{125}I]T2, 10 (rSULT1B1) or 25 (rSULT1C1) µg protein/ml, 50 µM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

Figure 4 depicts the sulfation of 1 µM 3,3'-T2 by male rat liver cytosol at different PAPS concentrations (1-100 µM). Maximum sulfation rates were reached at PAPS concentrations ≥30 µM. Its apparent K_m value, calculated from the Lineweaver-Burk plot, was 4.7 µM. The K_m values for the other enzyme preparations were also in the low µM range, i.e. 3.8 µM for female rat liver, 2.2 µM for male rat kidney and 3.5 µM for brain cytosol, 2.0 µM for rSULT1B1 and 6.9 µM for rSULT1C1.

Figure 5 shows the effects of increasing concentrations (1–100 µM) of unlabeled iodothyronines on the sulfation of 3,[3'-^{125}I]T2 by male rat liver cytosol. 3,5-T2 had no effect; all other iodothyronines inhibited the sulfation of labeled 3,3'-T2 dose-dependently, in the order 3,3'-T2 ~ 3'-T1 > 3',5'-T2 > rT3 > T4 > T0 ~ 3-T1 ~ T3.

Figure 6 compares the effects of 10 µM unlabeled iodothyronines on the sulfation of 1 µM 3,[3'-^{125}I]T2 by male and female liver and male kidney and brain cytosol, rSULT1B1 and 1C1. 3,3'-T2 sulfation by rSULT1C1 was affected most by the different iodothyronines. Sulfation of 3,3'-T2 by female rat liver cytosol was inhibited less potently by the different analogs than 3,3'-T2 sulfation by male rat liver. The structure activity relationships for inhibition of T2 sulfation by analogs were similar for female and male liver, kidney, rSULT1B1 and 1C1. In general, iodothyronines without iodine substituent in the outer ring (T0, 3-T1, 3,5-T2) and those with two

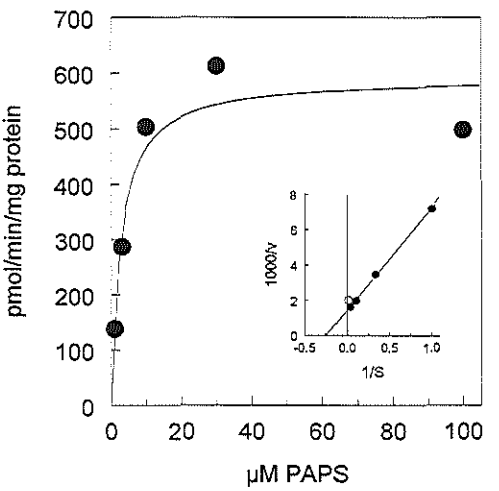

Fig. 4. Effect of PAPS concentration on the sulfation of 3,3'-T2 by male rat liver cytosol. The *inset* shows the double reciprocal plot. Reaction conditions were 1 µM 3,[3'-^{125}I]-T2, 1-100 µM PAPS, 20 µg protein/ml, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

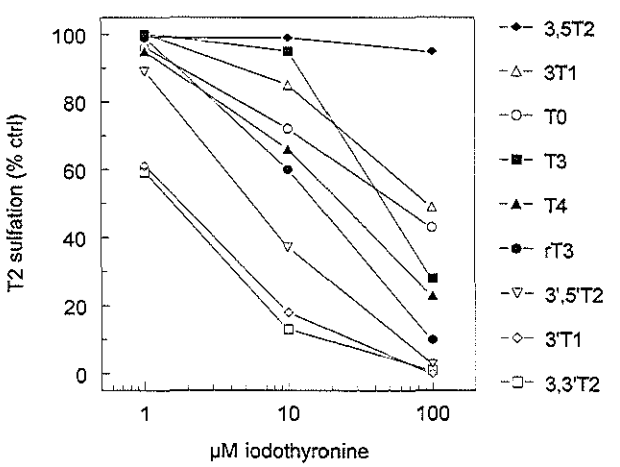

Fig. 5. Effects of 1–100 µM unlabeled iodothyronines on the sulfation of 3,[3'-^{125}I]T2 by male rat liver cytosol. Reaction conditions were 10^5 cpm 3,[3'-^{125}I]T2, 25 µg protein/ml, 50 µM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

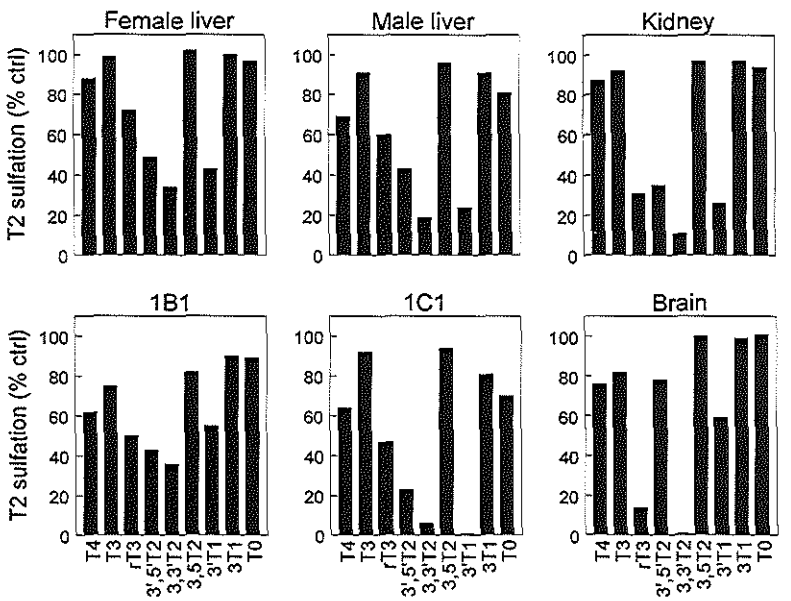

Fig. 6. Effects of 10 µM unlabeled iodothyronines on the sulfation of 1 µM 3,[3'-^{125}I]T2 by male and female rat liver cytosol, male rat kidney and brain cytosol, and by rSULT1B1 and rSULT1C1. Data represent the sulfation of 3,[3'-^{125}I]T2 in the presence of unlabeled iodothyronines as percentage of the control (without addition of unlabeled iodothyronines). Results are the means of 2-3 experiments.

iodines in the inner ring (3,5-T2, T3, T4) showed little or no inhibition. In other words, iodothyronines that showed significant inhibition had 0 or 1 iodine substituent in the inner ring and 1 or 2 iodines in the outer ring.

The inhibition profiles for rat liver and kidney were significantly correlated with those for SULT1B1 and 1C1, with coefficients varying between 0.869 and 0.990. However, in contrast to all other enzyme preparations, 3,3'-T2 sulfation by rat brain cytosol was poorly inhibited by 3'-T1 and 3',5'-T2, and the inhibition profile for rat brain cytosol also showed weaker correlations with those for rSULT1B1 ($r = 0.814$) and 1C1 ($r = 0.633$).

Discussion

In previous studies human SULT1A1, 1A2, 1A3, 1B1, 1C1 and 1E1 have been identified as important enzymes for iodothyronine sulfation in humans (61,62,69-72). Rat SULT1A1, 1B1, 1C1 and 1E1 show 79, 74, 63 and 70%, respectively, amino acid sequence identity with their human

homologs; and about 50% identity among themselves. Sulfation of T3 by rat SULT1B1 and 1C1 has been reported previously (39,73,74). In this study we compared kinetic parameters and substrate specificities for the different rat enzymes with these characteristics for male and female rat liver cytosol and male rat kidney and brain cytosol, in an attempt to determine which enzyme forms are involved in iodothyronine sulfation in the different tissues. We used mammalian V79 cells and bacterial *S. typhimurium* cells as expression systems for the different SULT enzymes. Previous studies showed that the different systems give similar results for the various human SULT enzymes (61).

Iodothyronine sulfotransferase activities in rat liver and kidney and of rat SULT1B1 and 1C1 showed very similar substrate specificities. The higher maximum sulfation rates observed in male than in female rat liver cytosol are in agreement with earlier reports on the sex-dependence of T3 sulfation in rats, which is explained by the male-dominant expression of rSULT1C1 (75-79). rSULT1C1 is predominantly expressed in male liver, kidney and spleen, whereas rSULT1B1 expression in liver, kidney and intestine is equal in male and female rats (80-82). The apparent K_m of 3,3'-T2 in liver cytosol is in between the K_m values for SULT1B1 and 1C1; in male liver closer to that for 1C1 and in female liver closer to that for 1B1, supporting a greater contribution of 1C1 in male versus female rat liver. The apparent K_m of 3,3'-T2 in kidney is similar to the K_m for SULT1C1, suggesting that 1C1 is a more important enzyme than 1B1 in rat kidney. It should however be noted that besides 1B1 and 1C1, 1C2, 1C3 and 1D1 may also contribute to iodothyronine sulfation in the different tissues. Furthermore, rat phenol sulfotransferases have been demonstrated to exist not only as homodimers but also as heterodimers (50). Thus, besides 1B1/1B1 and 1C1/1C1 homodimers, tissues such as liver may contain various other heterodimers. Although 1A1 homodimer does not possess sulfotransferase activity towards iodothyronines, it is not excluded that 1A1/1B1 and 1A1/1C1 heterodimers catalyze iodothyronine sulfation. It is clear that substrate specificities and apparent K_m values determined in tissue represent average values for mixtures of homo- and heterodimeric iodothyronine sulfotransferases. Substrate preference and K_m value of 3,3'-T2 in rat brain are different from 1B1 and 1C1. Therefore, other enzyme form(s) seem to be involved in iodothyronine sulfation in rat brain. A possible candidate is the recently cloned rat brain sulfotransferase-like protein rSULT4A1 (83). Compared with liver and kidney, the inhibition profile for rat brain cytosol showed weaker correlations with those for SULT1B1 and 1C1, also indicating the involvement of different enzymes. However, assessment of inhibition profiles may be biased if inhibitors are extensively sulfated themselves by the enzymes under study or other sulfotransferases, resulting in a decrease in their inhibitory potency. For instance, the weaker inhibition of 3'-T1 in rat brain may be explained by its sulfation by different enzymes present in brain.

Concerning the rSULT1C1 variant, mutational analysis should reveal which amino acid substitution (S2A, T60A or S96P) contributes most to the 10-fold lower affinity of the rSULT1C1 variant enzyme compared to the wild-type rSULT1C1. Previous studies showed that hSULT1A1 efficiently sulfates iodothyronines, whereas the rat SULT1A1 homolog does not catalyze iodothyronine sulfation (74). The estrogen sulfotransferase hSULT1E1 also efficiently catalyzes iodothyronine sulfation (62). However, since estrone and estradiol are inefficient substrates for the rat homolog rSULT1E1 (43,44), it is not surprising that no catalytic activity toward iodothyronines was detected for this enzyme. Still, iodothyronine sulfation by rSULT1E2 is not excluded. In rats as well as in humans (Kester et al., unpublished observations) hydroxysteroid sulfotransferases do not appear to contribute importantly to iodothyronine sulfation.

In conclusion, rSULT1B1 and 1C1 appear to be important enzyme forms for sulfation of iodothyronines in rat liver and kidney, with proportionally greater contributions in kidney than in liver, and in male than in female liver. Other, still unidentified enzymes appear to be responsible for iodothyronine sulfation in rat brain. Further studies are needed to determine the role of these sulfotransferases in the regulation of (fetal) thyroid hormone status.

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Chapter 4

Characterization of iodothyronine sulfatase activities in human and rat liver and placenta

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Abstract

In conditions associated with high serum iodothyronine sulfate concentrations, e.g. during fetal development, desulfation of these conjugates may be important in the regulation of thyroid hormone homeostasis. However, little is known about which sulfatases are involved in this process. Therefore, we investigated the hydrolysis of iodothyronine sulfates by homogenates of V79 cells expressing the human arylsulfatases ARSA, ARSB and steroid sulfatase ARSC, as well as tissue fractions of human and rat liver and placenta. We found that only the microsomal fraction from liver and placenta hydrolyzed iodothyronine sulfates. Among the recombinant enzymes only the endoplasmic reticulum-associated ARSC showed activity towards iodothyronine sulfate; the soluble lysosomal ARSA and ARSB were inactive. Recombinant ARSC as well as human placenta microsomes hydrolyzed iodothyronine sulfates with a substrate preference for 3,3'-diiodothyronine sulfate (3,3'-T2S) ~ T3S >> rT3S ~ T4S, whereas human and rat liver microsomes showed a preference for 3,3'-T2S > T3S >> rT3S ~ T4S. ARSC and the tissue microsomal sulfatases were all characterized by high apparent K_m values (>50 μM) for 3,3'-T2S and T3S. Iodothyronine sulfatase activity determined using 3,3'-T2S as a substrate was much higher in human liver microsomes than in human placenta microsomes, although ARSC is expressed at higher levels in human placenta than in human liver. The ratio of E1S *vs* T2S hydrolysis in human liver microsomes (~0.2) differed largely from that in ARSC homogenate (80) and human placenta microsomes (150). These results suggest that ARSC accounts for the relatively low iodothyronine sulfatase activity of human placenta, and that additional arylsulfatase(s) contribute to the high iodothyronine sulfatase activity in human liver. Further research is needed to identify these iodothyronine sulfatases, and to study the physiological importance of the reversible sulfation of iodothyronines in thyroid hormone metabolism.

Introduction

Sulfation is an important metabolic pathway which facilitates the inactivation and elimination of lipophilic exogenous and endogenous compounds, including thyroid hormones, by increasing their water solubility (1-3). A more important purpose for the sulfation of thyroid hormones is to facilitate their degradation by the type I iodothyronine deiodinase (D1) (4-7). D1 catalyzes the outer ring deiodination (ORD; activation) of T4 to T3 as well as the inner ring deiodination (IRD; inactivation) of T4 to rT3 and of T3 to 3,3'-diiodothyronine (T2) (5). Since IRD of sulfated T4 and T3 by D1 is accelerated 40 to 200-fold, whereas ORD of T4 sulfate (T4S) is completely blocked (4-7), sulfation has an important role in the irreversible inactivation of thyroid hormone by D1. However, when D1 activity is low or clearance of iodothyronine sulfates is impaired otherwise, inactivation of thyroid hormone by sulfation may be reversible due to the expression of arylsulfatases in different tissues (8-10), or the presence of bacterial sulfatases in the intestine (11). Strongly elevated iodothyronine sulfate concentrations have been found in fetal and neonatal serum, and amniotic fluid in humans and sheep (12-16). Thyroid hormone is essential for the normal fetal development of several organs, such as the brain (17-20). Therefore, it has been speculated that these iodothyronine sulfates, in particular T3S, function as a pool of inactive thyroid hormone, from which the active hormone is released in a tissue-specific and development stage-dependent manner (7,9,12,14,21). Iodothyronine sulfatase activities are present in human fetal liver and lung, and become undetectable in lung after birth (22). In rats, which are born immature compared with humans, hepatic T3S sulfatase activity progressively increases after birth until two months of age (23).

Hydrolysis of sulfate conjugates is an enzymatic process, and multiple arylsulfatases have now been identified (24). Arylsulfatase A (ARSA) and arylsulfatase B (ARSB) are soluble enzymes, localized in lysosomes. So far, sulfated glycolipids have been identified as endogenous substrates for ARSA, while ARSB has a known substrate specificity for dermatan sulfate and chondroitin sulfate (25). These sulfatases are widely distributed, although in the pig ARSA activity is 20 to 60 times higher in the thyroid than in other tissues (26). Arylsulfatase C (ARSC), also termed steroid sulfatase, is located in the endoplasmic reticulum, and hydrolyzes steroid sulfates such as dehydroepiandrosterone sulfate (DHEAS), estrone sulfate (E1S) and cholesterol sulfate (25). We have recently demonstrated that iodothyronines are good substrates for estrogen sulfotransferase (27). Therefore, it seems logical to assume that iodothyronine sulfates are also good substrates for the steroid sulfatase ARSC. This isoenzyme is expressed in many tissues, among which placenta and liver (28-31). In the placenta, ARSC plays a major role in estrogen biosynthesis from DHEAS, which is mainly produced in the fetal adrenal gland and converted to

16-α-hydroxy-DHEAS by the fetal liver (32). Recently, a group of novel ARS genes was identified, clustered on Xp22.3 (33-35), near the ARSC gene. ARSDα and β and ARSF have been localized in the endoplasmic reticulum, whereas ARSE is located in the Golgi apparatus (35,36). The endogenous substrates for these arylsulfatases remain to be identified, although neither ARSE nor ARSF hydrolyzes steroid sulfates (33-36). ARSD does not appear to act as a conventional arylsulfatase since no such activity has yet been determined for the recombinant protein (34,35). They also differ from ARSC in that they are thermolabile.

Earlier studies demonstrated T3S sulfatase activities in human and rat liver microsomes, and in rat hepatocytes (9,21). However, not much is known about which sulfatases are responsible for the hydrolysis of sulfated iodothyronines. Therefore, we studied the arylsulfatases ARSA, ARSB and ARSC, and the sulfatase activities in human and rat liver and placenta, using iodothyronine sulfates as substrates, to determine if these arylsulfatases are involved in hydrolysis of thyroid hormone sulfates in tissues.

Materials and Methods

Materials

Cytosolic and microsomal fractions of male Wistar rat and human liver were obtained as previously described (4,37). Normal human placental tissue was obtained at spontaneous, full-term delivery, and rat placenta after cesarean section at gestational age E20. Approval was obtained from institutional committees. Cytosol and microsomes were prepared as described for human liver (4). Human ARSA, ARSB and ARSC cDNA clones were kindly provided by Prof. K. von Figura (University of Göttingen, Göttingen, Germany) and expressed in V79 Chinese hamster lung fibroblast cells as previously described (25).

T4, rT3, 3,3'-T2 and 3-iodothyronine (3-T1) were obtained from Henning (Berlin, Germany); T3, estrone sulfate (E1S), 6-n-propyl-2-thiouracil (PTU) and bis-tris propane (BTP) from Sigma (St. Louis, MO); [3',5'-^{125}I]T4, [3'-^{125}I]T3 and [^{3}H]E1S were obtained from Amersham (Amersham, UK); [3',5'-^{125}I]rT3 and 3,[3'-^{125}I]T2 were prepared by radioiodination of 3,3'-T2 and 3-T1, respectively (5). ^{125}I-labeled and unlabeled T4S, rT3S, T3S and 3,3'-T2S were prepared by reaction of labeled and unlabeled T4, rT3, T3 and 3,3'-T2 with chlorosulfonic acid in dimethylformamide. They were purified by LH-20 chromatography (38).

Sulfatase assay

Iodothyronine sulfatase activity was assayed by incubation of usually 0.1 μM unlabeled and 100,000 cpm ^{125}I-labeled T4S, rT3S, T3S or 3,3'-T2S, and usually 0.1 or 1 mM PTU (to block D1 activity), for 60 min at usually 37 C with the indicated amounts of tissue cytosol or microsomes or V79 cell homogenate in 0.2 ml buffer. Optimal assay conditions for the different sulfatases were determined by testing different buffers (0.1 M sodium acetate, sodium citrate, Tris/HCl, sodium phosphate or BTP/HCl), pH values and temperatures. The reactions were started by addition of enzyme in ice-cold buffer, and stopped by addition of 0.8 ml 0.1 M HCl. The mixtures were analyzed for T4, rT3, T3 or 3,3'-T2 formation by chromatography on Sephadex LH-20 minicolumns as previously described (39). Desulfation in complete reaction mixtures was corrected for background radioactivity detected in the corresponding Sephadex LH-20 fractions of control incubations without enzyme.

Estrogen sulfatase activity was analyzed by incubation of 0.1 μM [^{3}H]E1S for 0 (blank) or 30 min at 37 C with the indicated amounts of tissue microsomes or V79 cell homogenate in 0.1 ml 0.1 M Tris/HCl (pH 7.2). The reactions were stopped by the addition of 0.4 ml 0.1 M Tris/HCl (pH 8.8), and the mixtures were extracted with 2.5 ml chloroform. Sulfate hydrolysis was quantified by counting 0.25 ml of the aqueous phase.

Results

Figure 1 shows the pH profiles of the desulfation of 0.1 μM 3,3'-T2S by rat liver and human liver microsomes and recombinant human ARSC which were obtained using acetate and BTP/HCl buffers. Rat liver microsomal sulfatase showed an optimum at pH 6.0-6.5, human liver microsomal sulfatase at pH 6.0-7.5, and ARSC around pH 7.0. At neutral pH, the different enzymes showed similar sulfatase activities in BTP/HCl and Tris/HCl buffers but much lower activities in phosphate buffer (Fig. 2A). At acidic pH values, incubations of the different enzymes, in particular human liver, in citrate buffer strongly inhibited their 3,3'-T2S sulfatase activities compared with incubations in acetate buffer (Fig. 2B). Similar results were obtained in buffers with or without 2 mM EDTA (not shown).

Figure 3 demonstrates the effects of temperature on the desulfation of 0.1 μM 3,3'-T2S by human and rat liver microsomes. The optimal temperature for human liver microsomes is 50 C and for rat liver microsomes even 70 C or higher.

Figure 4 presents the desulfation of 0.1 μM T4S, T3S, rT3S and 3,3'-T2S by recombinant human ARSC and human and rat placenta and liver microsomes at pH 7.2. ARSC showed similar activities towards 3,3'-T2S and T3S, whereas both rT3S and T4S were poor substrates for this

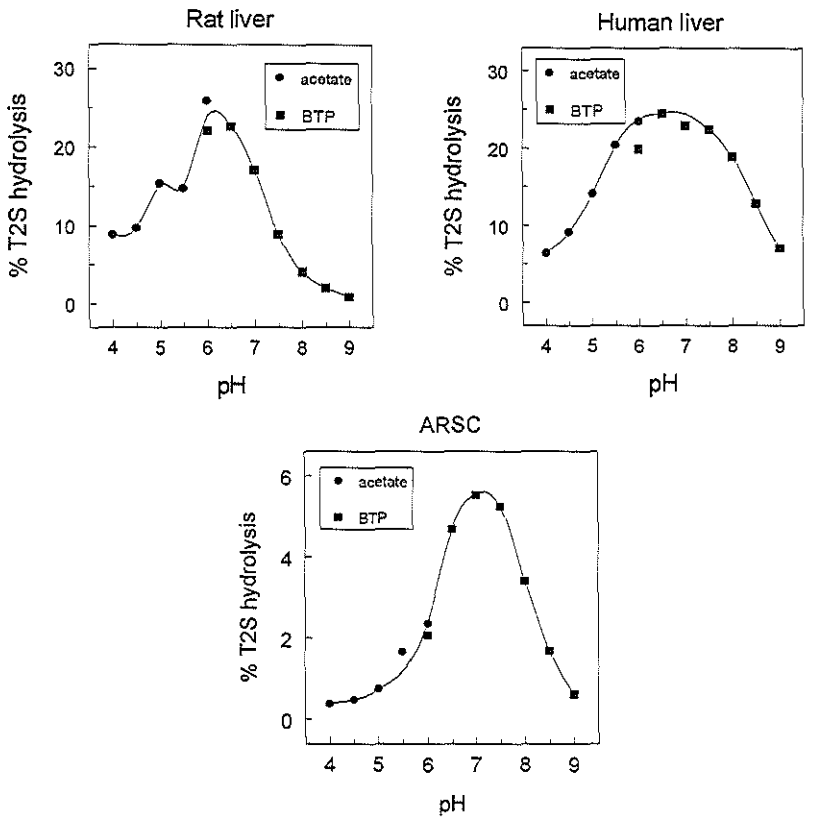

Fig. 1. Effects of pH on 3,3'-T2S desulfation by rat liver or human liver microsomes or ARSC. Reaction conditions were: 0.1 μM ^{125}I-labeled 3,3'-T2S, 0.25 (rat liver, ARSC) or 0.05 (human liver) mg protein/ml, 0.1 mM PTU, and 60 min incubation in 0.1 M sodium acetate or BTP/HCl. Results are the means of triplicate determinations from a representative experiment.

enzyme. The substrate specificity of human placenta microsomes was similar to that of ARSC. Very high desulfation rates were observed in human liver microsomes, with a strong substrate preference for 3,3'-T2S which was hydrolyzed ~4 times faster than T3S (i.e. desulfation rates of ~7.2 and 1.8 pmol/min/mg) and >10 times faster than the relatively poor substrates rT3S and T4S. Rat liver microsomes also showed a substrate preference for 3,3'-T2S which was desulfated 2 times more rapidly than T3S; in rat placenta microsomes low desulfation rates (i.e. <0.1 pmol/min/mg) were observed with all iodothyronine sulfates. We also tested steroid sulfatase activities of ARSC and of human placenta and liver microsomes. Table 1 compares E1S and T2S sulfatase activities of ARSC and the different human tissue microsomes. The low ratio of E1S *vs*

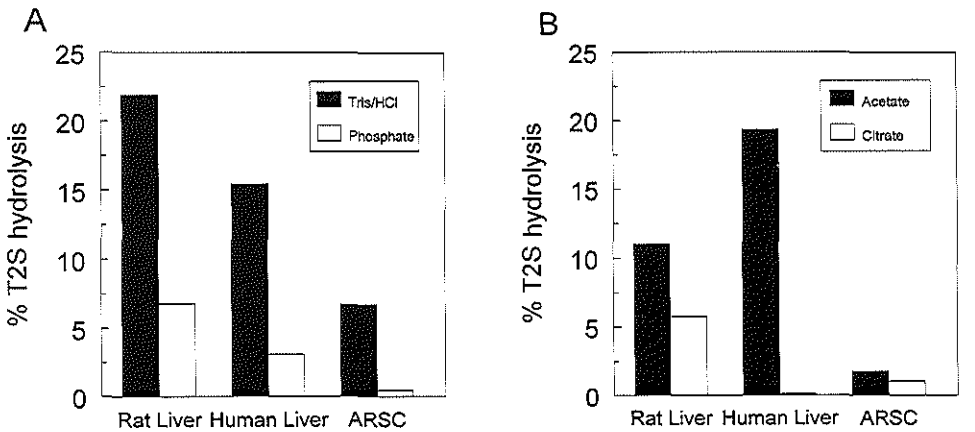

Fig. 2. Effects of buffer on 3,3'-T2S desulfation by rat liver and human liver microsomes, and ARSC at pH 7.2 (A) or 5.5 (B). Reaction conditions were: 0.1 μM ^{125}I-labeled 3,3'-T2S, 0.25 (rat liver, ARSC) or 0.05 (human liver) mg protein/ml, 0.1 mM PTU, and 60 min incubation in 0.1 M sodium phosphate (pH 7.2), 0.1 M Tris/HCl (pH 7.2), 0.1 M sodium acetate (pH 5.5) or 0.1 M sodium citrate (pH 5.5). Results are the means of triplicate determinations from a representative experiment.

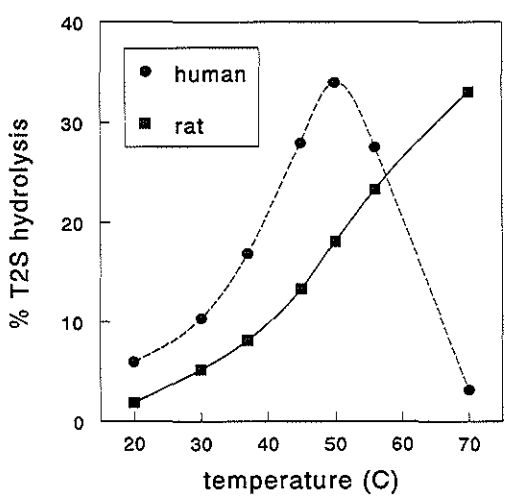

Fig. 3. Effects of temperature on desulfation of 3,3'-T2S by human or rat liver microsomes. Reaction conditions were: 0.1 μM 3,[3'-^{125}I]T2S, 0.25 (rat liver) or 0.025 (human liver) mg protein/ml, 1 mM PTU, and 60 min incubation in 0.1 M Tris/HCl (pH 7.2) at 20-70 C. Results are the means of two closely agreeing experiments.

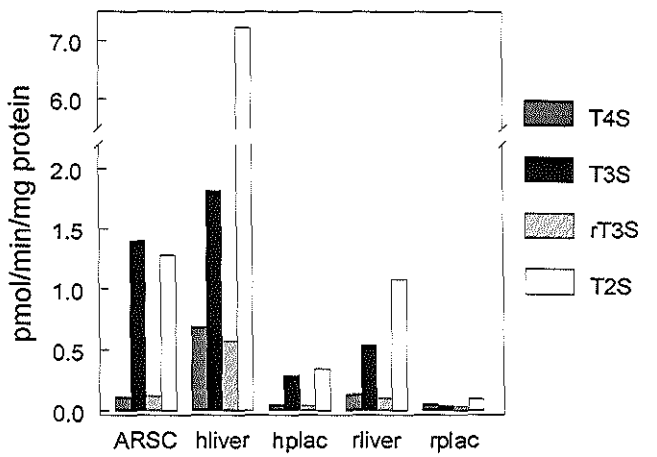

Fig. 4. Desulfation of iodothyronine sulfates by ARSC, human or rat liver microsomes, or human or rat placenta microsomes. Reaction conditions were: 0.1 μM ^{125}I-labeled T4S, T3S, rT3S or 3,3'-T2S, 0.25 (ARSC and rat liver), 0.05 (human liver) or 0.5 (human and rat placenta) mg protein/ml, 0.1 mM PTU, and 60 min incubation in 0.1 M Tris/HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

Table 1. Hydrolysis of E1S and 3,3'-T2S by human ARSC, liver and placenta

Enzyme source	Hydrolysis		
	E1S	3,3'-T2S	E1S/3,3'-T2S
	(pmol/min/mg)		
ARSC-V79 homogenate	100	1.3	81
human liver microsomes	1.6	7.2	0.21
human placenta microsomes	52	0.34	150

Reaction conditions were: 0.1 μM 3,[3'-^{125}I]T2S, 0.1 mM PTU and 0.25 (ARSC), 0.05 (liver) or 0.5 (placenta) mg protein/ml, or 0.1 μM [^{3}H]E1S and 0.005 (ARSC), 0.5 (liver) or 0.01 (placenta) mg protein/ml, and 60 min incubation in 0.1 M Tris/HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

T2S hydrolysis in liver microsomes differs largely from the high preference for E1S *vs* T2S hydrolysis by ARSC and human placenta microsomes. Tested at their optimum pH 5.5, the soluble ARSA and ARSB as well as rat and human liver cytosols, showed very low activity towards all iodothyronine sulfates (results not shown).

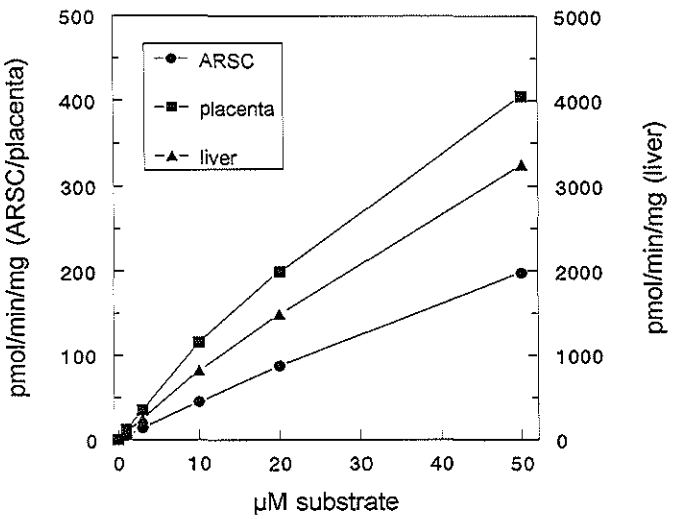

Fig. 5. Effects of substrate concentration on the desulfation of 3,3'-T2S by ARSC, human placenta or human liver microsomes. Reaction conditions were: 1-50 µM 3,[3'-^{125}I]T2S, 0.05 (liver), 0.25 (ARSC) or 0.5 (placenta) mg protein/ml, 1 mM PTU, and 60 min incubation in 0.1 M Tris/HCl (pH 7.2). Results are the means of triplicate determinations from a representative experiment.

Figure 5 shows the desulfation of 3,3'-T2S by ARSC, human liver and human placenta microsomes as a function of the substrate concentration. As no saturation was reached even at the highest concentration of 50 µM, K_m and V_{max} values could not be calculated. Similar results were obtained when T3S was used as substrate. Apparently, all these sulfatases have low affinity for iodothyronine sulfates, with K_m values higher than 25 µM.

Discussion

Normally, serum T4S and T3S levels are low (12,40,41). This is explained by the very rapid deiodination of these conjugates, since sulfation strongly induces the D1-catalyzed IRD of both T4 and T3 (7). However, under certain (patho)physiological conditions, e.g. during fetal development and non-thyroidal illness, possibly due to diminished D1 activity, plasma concentrations of iodothyronine sulfates are increased (12,13,16,41). T3S is considered to be biologically inert, as it has lost its affinity for the T3 receptors (42). It could however serve as a reservoir, from which active thyroid hormone is regenerated by tissue sulfatases or bacterial sulfatases in the intestine (8-11).

Recently, much research has been done to develop inhibitors of steroid sulfatase (ARSC) because of their potential for the treatment of estrogen-dependent breast cancers (43-54). These studies have revealed some important structure-activity relations for compounds binding to the active site of ARSC. Furthermore, the crystal structures of arylsulfatases A and B have recently been elucidated (55,56). Although the overall amino acid sequence homology is only about 20-30% between different arylsulfatases, the protein structures of all sulfatases share some important features. The active site of eukaryotic sulfatases contains a metal ion, probably Mg^{2+} (56,57), and a formylglycine, generated by post-translational modification of a cysteine residue (58-61). Residues interacting with the Mg^{2+} and the formylglycine are conserved among the members of the sulfatase family. Uhlhorn-Dierks et al. (57) proposed a catalytic mechanism for the hydrolysis of sulfates by sulfatases based on their structure and mutational analyses. An intermediate enzyme-sulfate complex is formed by the covalent binding of sulfate to the hydrated formylglycine (i.e. dihydroxy-alanine). When the active site formylglycine is replaced by a serine (i.e. hydroxy-alanine), the intermediate enzyme-sulfate complex is trapped (62), which indicates that the second hydroxyl group of hydrated formylglycine is needed for sulfate release (57,62).

Crystallographic analyses (55,56,63) also revealed structural homology between alkaline phosphatases and arylsulfatases. A functional relationship between the enzymes was shown by O'Brien et al. (64), who demonstrated that alkaline phosphatase exhibits a low level of sulfatase activity. They also showed inhibition of the phosphatase as well as the sulfatase activities of alkaline phosphatase by inorganic phosphate (64). Anderson et al. reported on steroidal and non-steroidal phosphates which inhibited steroid sulfatase activity (43). We demonstrated inhibition of the iodothyronine sulfatase activities by inorganic phosphate and citrate. Perhaps, these anions block the active site of iodothyronine sulfatases. An alternative explanation is that citrate and phosphate inhibit iodothyronine sulfatase activity by complexing the enzyme-bound Mg^{2+}. However, we did not observe inhibition of iodothyronine sulfatase activity in the presence of EDTA. Our results strongly suggest that iodothyronine sulfatase activities determined in phosphate buffers as reported by others (9,23) represent a marked underestimation of true enzyme levels.

We showed a pH optimum for rat liver microsomes at pH 6.0-6.5, for human liver microsomes at pH 6.0-7.5 and for arylsulfatase C at pH 7.0. The broader peak for the human and rat liver microsomes might indicate that different sulfatases, with different pH optima, are involved in the desulfation of 3,3'-T2S. Kung et al. (9) observed T3S sulfatase activities in human and rat liver microsomes. E1S and DHEAS, both substrates for ARSC, inhibited T3S hydrolysis with IC_{50} values of ~10 μM. The fact that high levels of E1S only partially inhibited T3S desulfation,

whereas high DHEAS concentrations produced complete inhibition, support the involvement of multiple sulfatases, among which possibly ARSC (9). However, these analyses were done in phosphate buffer, which may strongly affect the contribution of different sulfatases.

The optimal temperature of iodothyronine sulfatase activities amounts to 50 C in human liver microsomes and to at least 70 C in rat liver microsomes. The high thermostability of these sulfatases is in agreement with the temperature optimum of 60 C for ARSC (65). ARSC and the sulfatase activities in human liver and placenta microsomes have high K_m values for iodothyronine sulfates. The different substrate specificity of the iodothyronine sulfatase activity in human liver *versus* ARSC and placenta, plus the finding that the ratio between E1S and T2S hydrolysis in human liver differs largely from that in ARSC and placenta, suggest that in human liver additional sulfatases to ARSC contribute to hydrolysis of iodothyronine sulfates, in particular T2S. However, ARSD, ARSE and ARSF are no likely candidates, as a) both ARSE and ARSF are thermolabile, whereas iodothyronine sulfatase activity appears thermostable (33,34) and b) ARSD does not possess arylsulfatase activity (34,35).

It is remarkable that, while 3,3'-T2 is the preferred substrate for sulfotransferases, 3,3'-T2S is the preferred substrate for (human liver) sulfatase. Thus, reversible sulfation/desulfation seems a more important metabolic pathway for 3,3'-T2 than for T4, T3 and rT3. This may reflect restrictions in the active sites of the sulfotransferase and sulfatase concerned to accomodate bulky substrates with more than 2 iodine substituents. However, a physiological role for 3,3'-T2 is not excluded. Although its affinity for the nuclear thyroid hormone receptors is low (17), 3,3'-T2 has been shown to stimulate mitochondrial respiration in different tissues (66).

In conclusion, we have identified arylsulfatase C as a high-K_m iodothyronine sulfatase, which is most likely the main enzyme responsible for the hydrolysis of iodothyronine sulfates in human placenta and to some extent in human liver. Further investigations are needed to determine the possible importance of other, still unidentified, microsomal sulfatases in hydrolysis of iodothyronine sulfates in the liver and perhaps other tissues. This information may contribute to the understanding of the role of sulfation-desulfation in the regulation of thyroid hormone bioactivity, in particular during fetal development.

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Chapter 5

Characterization of human iodothyronine sulfotransferases

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Abstract

Sulfation is an important pathway of thyroid hormone metabolism that facilitates the degradation of the hormone by the type I iodothyronine deiodinase, but little is known about which human sulfotransferase isoenzymes are involved. We have investigated the sulfation of the prohormone T4, the active hormone T3 and the metabolites rT3 and 3,3'-diiodothyronine (3,3'-T2) by human liver and kidney cytosol as well as by recombinant human SULT1A1 and SULT1A3, previously known as phenol-preferring and monoamine-preferring phenol sulfotransferase, respectively. In all cases, the substrate preference was 3,3'-T2 >> rT3 > T3 > T4. The apparent Km values of 3,3'-T2 and T3 (at 50 μM 3'-phosphoadenosine-5'-phosphosulfate (PAPS)) were 1.02 and 54.9 μM for liver cytosol, 0.64 and 27.8 μM for kidney cytosol, 0.14 and 29.1 μM for SULT1A1, and 33 and 112 μM for SULT1A3, respectively. The apparent Km value of PAPS (at 0.1 μM 3,3'-T2) was 6.0 μM for liver cytosol, 9.0 μM for kidney cytosol, 0.65 μM for SULT1A1 and 2.7 μM for SULT1A3. The sulfation of 3,3'-T2 was inhibited by the other iodothyronines in a concentration-dependent manner. The inhibition profiles of the 3,3'-T2 sulfotransferase activities of liver and kidney cytosol obtained by addition of 10 μM of the various analogs were better correlated with the inhibition profile of SULT1A1 than with that of SULT1A3. These results indicate similar substrate specificities for iodothyronine sulfation by native human liver and kidney sulfotransferases and recombinant SULT1A1 and SULT1A3. Of the latter, SULT1A1 clearly shows highest affinity for both iodothyronines and PAPS, but it remains to be established if it is the prominent isoenzyme for sulfation of thyroid hormone in human liver and kidney.

Introduction

Sulfation is a detoxication reaction, which increases the water solubility of a variety of endogenous and exogenous lipophilic compounds, thus facilitating their excretion in bile and/or urine (1-3). Sulfation is also an important pathway for the metabolism of thyroid hormone, increasing the hydrophilicity and the biliary excretion of the hormone. However, the major purpose of sulfation of thyroid hormone is to facilitate its degradation by the type I iodothyronine deiodinase (D1) (4,5). This selenoenzyme catalyzes the outer ring deiodination (ORD) as well as the inner ring deiodination (IRD) of different iodothyronines, including the ORD of the prohormone T4 to the active hormone T3, and the IRD of T4 and T3 to the inactive metabolites rT3 and 3,3'-diiodothyronine (3,3'-T2), respectively (6,7). The preferred substrate for D1 is rT3, which is converted by ORD to 3,3'-T2 (6,7).

An intriguing characteristic of D1 is that its deiodination of a number of iodothyronines is accelerated by sulfation of their phenolic hydroxyl group (4,5). Thus, IRD of both T4 sulfate (T4S) and T3 sulfate (T3S) by rat D1 is 40-200 times faster than the deiodination of the nonsulfated substrates. In contrast, ORD of T4 by rat D1 is completely blocked by sulfation (4,5). This is not a general phenomenon since ORD of rT3 by rat D1 is not affected by sulfation, whereas ORD of 3,3'-T2 by rat D1 is accelerated ~50-fold by sulfation of this compound (4,5). Similar findings have been obtained with human and dog D1 (8,9). The facilitated deiodination of sulfated iodothyronines is a unique property of D1. Neither the type II iodothyonine deiodinase (D2), which catalyzes only ORD, *e.g.* T4 to T3 and rT3 to 3,3'-T2, nor the type III iodothyronine deiodinase (D3), which catalyzes only IRD, *e.g.* T4 to rT3 and T3 to 3,3'-T2, is capable of catalyzing the deiodination of sulfated iodothyronines (10,11, T.J. Visser and E. Kaptein, unpublished work).

Serum concentrations of T4S, T3S, rT3S and 3,3'-T2S are low in normal human subjects, but they are high in fetal and cord blood, in patients with nonthyroidal illness, and in patients treated with propylthiouracil or iopanoic acid, both inhibitors of D1 (12-19). The serum T3S/T3 ratio is also increased in hypothyroid patients (13). High serum T4S, T3S, rT3S and 3,3'-T2S levels have also been detected in serum, bile, allantoic fluid and amniotic fluid of fetal sheep (19-22). The high serum iodothyronine sulfate levels during nonthyroidal illness, hypothyroidism and fetal development have been ascribed to a low peripheral D1 activity in these conditions (4,5,11). These results are in accordance with experimental findings in rats, showing marked increases in the serum concentration and biliary excretion of iodothyronine sulfates in animals with impaired hepatic and renal D1 activities due to administration of D1 inhibitors or selenium deficiency (23-27). These changes are not caused by an increased sulfation of iodothyronines but, rather, by a decreased clearance of the sulfated iodothyronines by D1 (24,28). Thus, sulfation is a primary

step leading to the irreversible degradation of T4 and T3 by D1. However, if D1 activity is low, inactivation of thyroid hormone by sulfation is reversible due to expression of sulfatases in different tissues and by intestinal bacteria (11,29-31). It has been speculated that especially in the fetus T3S has an important function as a reservoir from which active T3 may be released in a tissue-specific and time-dependent manner (5,11).

Sulfation of the hydroxyl group of a variety of substrates is catalyzed by a family of homologous sulfotransferases, located in the cytoplasmic fraction of different tissues, such as liver, kidney, intestine and brain (1-3). All of these isoenzymes use 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as sulfate donor (1-3). On the basis of substrate specificity and amino acid sequence homology, two sulfotransferase families have been recognized in human tissues, *i.e.* phenol sulfotransferases (including estrogen sulfotransferases) and hydroxysteroid sulfotransferases (1-3). It is not known which sulfotransferases are involved in the sulfation of iodothyronines in human tissues. Previous studies have suggested a role for the enzymes termed phenol-preferring phenol sulfotransferase (P-PST) and monoamine-preferring phenol sulfotransferase (M-PST), in the sulfation of T3 in human liver and intestine (32-33). Recently, a large number of human and rat sulfotransferases have been cloned and characterized, including human SULT1A1 and SULT1A3, which represent P-PST and M-PST, respectively (34-36), under a new nomenclature system. Here we report the results of a comparison of the kinetic profiles of the sulfation of iodothyronines by human liver and kidney cytosol and by recombinant preparations of human SULT1A1 and SULT1A3.

Materials and Methods

Materials

[3',5'-^{125}I]T4 and [3'-^{125}I]T3 were obtained from Amersham (Aylesbury, UK); T4, T3 and PAPS from Sigma (St. Louis, MO); rT3, 3,5-T2, 3,3'-T2, 3',5'-T2, 3- and 3'-iodothyronine (T1) and thyronine (T0) from Henning (Berlin, Germany); and Sephadex LH-20 from Pharmacia (Woerden, The Netherlands). 3,[3'-^{125}I]T2 and [3',5'-^{125}I]rT3 were prepared by radioiodination of 3-T1 and 3,3'-T2, respectively, as previously described (37).

Normal adult human liver and kidney tissue was obtained at surgery for liver and kidney tumors. Approval was obtained from the Medical Ethical Committee of the Erasmus University Medical School and Hospital. Tissue was homogenized in 0.25 M sucrose, 10 mM HEPES (pH 7.0) and 1 mM dithiothreitol, and cytosol was prepared as previously described (8). SULT1A1 cDNA cloned by Wilborn *et al.* (34) and SULT1A3 cloned by Ganguly *et al.* (36) were kindly provided by Dr. C.N. Falany (University of Alabama, Birmingham, AL) and expressed in

Salmonella typhimurium as previously described (38). Human SULT1A3 cDNA was also cloned from human platelets and expressed in V79 cells (35). Bacterial and V79 cell cytosol was prepared for characterization of recombinant sulfotransferase activities (35,38). Protein was measured with the Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands) using BSA as the standard.

Sulfotransferase assay

Iodothyronine sulfotransferase activities were analyzed by incubation of usually 0.1 μM T4, T3, rT3 or 3,3'-T2 and 100,000 cpm of the ^{125}I-labeled compound for 30 min at 37 C with the indicated amounts of liver or kidney cytosol or recombinant sulfotransferase preparation in the presence or absence (blank) of 50 μM PAPS in 0.2 ml 0.1 M phosphate (pH 7.2) and 2 mM EDTA (39). Similar results were obtained in the absence of EDTA. The reactions were started by addition of enzyme diluted in ice-cold buffer, and stopped by addition of 0.8 ml 0.1 M HCl. The mixtures were analyzed for sulfoconjugate formation by chromatography on Sephadex LH-20 minicolumns as previously described (39). Sulfation in reaction mixtures with PAPS was corrected for background radioactivity detected in the corresponding Sephadex LH-20 fractions of the blanks. Incubations were carried out in triplicate, and the coefficient of variation was less than 10%.

Results

Figure 1 shows the sulfation of 0.1 μM T4, T3, rT3 and 3,3'-T2 by human liver and kidney cytosol, SULT1A1 and SULT1A3 in the presence of 50 μM PAPS. All enzyme preparations display a strong substrate preference for 3,3'-T2, which is sulfated approximately 2 orders of magnitude more rapidly than T3 and rT3, whereas T4 is a poor substrate for these human sulfotransferases.

Figure 2 presents the sulfation of 3,3'-T2 by human liver cytosol in the presence of PAPS as a function of incubation time and cytosolic protein concentration. Under the conditions used, 3,3'-T2 sulfation was linear with incubation time up to 45 min, when about 50% of the substrate was converted (Fig. 2A). The subsequent decrease in sulfation rate probably is due to depletion of substrate rather than depletion of cofactor, since PAPS was added in large excess (50 μM). Initially, 3,3'-T2 sulfation showed a more than proportional increase with the cytosolic protein concentration (Fig. 2B). For instance, an increase in the cytosolic protein concentration from 25 to 50 μg/ml resulted in a 3-fold increase in 3,3'-T2S formation. Further increases in protein concentration resulted in roughly linear increases in 3,3'-T2 sulfation until significant substrate

depletion occurred. Similar results were obtained when 3,3'-T2 sulfation by human kidney cytosol was analyzed as a function of the cytosolic protein concentration (data not shown).

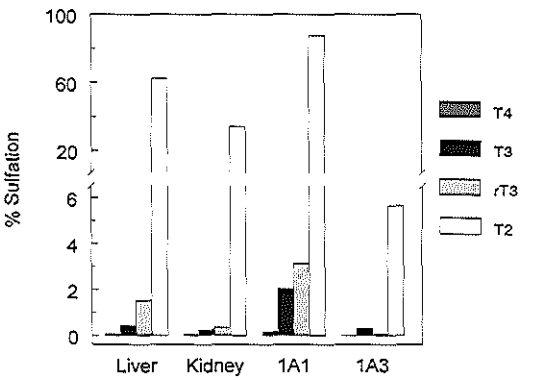

Fig. 1. Sulfation of iodothyronines by human liver and kidney cytosol, SULT1A1 and SULT1A3. Reaction conditions: 0.1 μM ^{125}I-labeled T4, T3, rT3 or 3,3'-T2, 0.1 mg protein/ml, 50 μM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

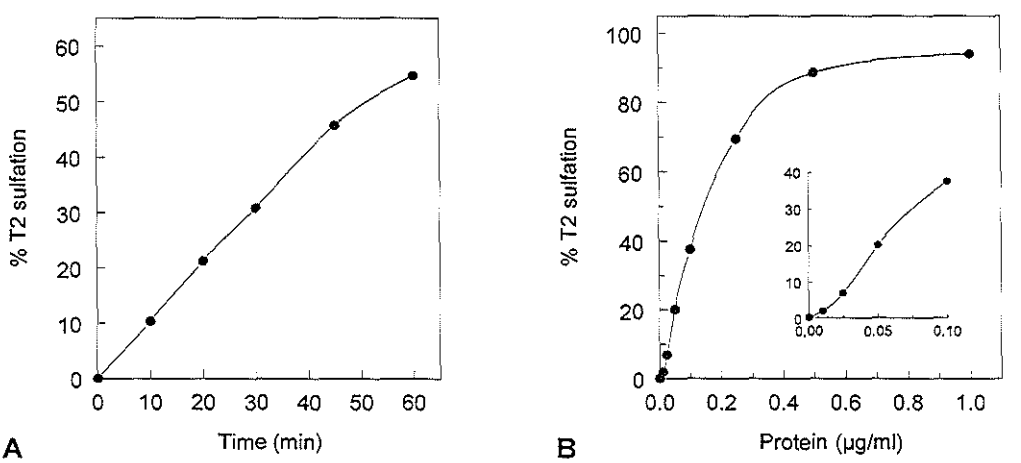

Fig. 2. Effects of incubation time (A) and protein concentration (B) on the sulfation of 3,3'-T2 by human liver cytosol. Reaction conditions: 1 μM 3,[3'-^{125}I]T2, 50 μg protein/ml (A), 50 μM PAPS, and 20 min incubation (B). Results are the means of triplicate determinations from a representative experiment.

Figure 3 shows the sulfation of 3,3'-T2 by human liver (A) and kidney (B) cytosol as a function of the substrate concentration. In both tissues, saturation kinetics were observed in the range of the 3,3'-T2 concentrations tested, with maximum sulfation rates being obtained at 3 μM 3,3'-T2. At

higher 3,3'-T2 concentrations a decrease in sulfation rate was observed, suggesting substrate inhibition. The double-reciprocal plots of sulfation rates *vs* 3,3'-T2 concentration were linear, allowing the calculation of apparent K_m values for 3,3'-T2 and V_{max} values. Table 1 presents the kinetic parameters for 3,3'-T2 sulfation by human liver and kidney cytosol at 50 μM PAPS determined in different experiments. The mean apparent K_m value for 3,3'-T2 amounted to 1.02 μM in liver and 0.64 μM in kidney cytosol. The data presented were obtained using 3 different kidney samples and 2 different cytosol preparations from the same liver, while very similar results were obtained with 59 other liver samples (R.A.H. Gilissen, M.W.H. Coughtrie, E. Kaptein, T.J. Visser, unpublished work). Table 1 also gives the kinetic parameters for the sulfation of T3 determined under similar conditions. Compared with 3,3'-T2, apparent K_m values for T3 were approximately 50-fold higher, *i.e.* 54.9 μM in liver and 27.8 μM kidney, while apparent V_{max} values were roughly 10-fold lower.

Table 1. Kinetic parameters of human iodothyronine sulfotransferases

Enzyme source	K_m (μM)	V_{max} (pmol/min/mg protein)
Variable substrate: 3,3'-T2 (50 μM PAPS)		
Liver cytosol	1.02 ± 0.11	337 ± 88
Kidney cytosol	0.64 ± 0.17	38.5 ± 18.1
SULT1A1 (*Salmonella*)	0.12 ± 0.05	465 ± 184
SULT1A3 (*Salmonella*)	31.2 ± 2.0	782 ± 239
SULT1A3 (V79 cells)	34.7 ± 6.1	2097 ± 474
Variable substrate: T3 (50 μM PAPS)		
Liver cytosol	54.9 ± 2.6	22.7 ± 8.6
Kidney cytosol	27.8 ± 2.6	2.7 ± 0.1
SULT1A1 (*Salmonella*)	29.1 ± 12.3	239 ± 82
SULT1A3 (*Salmonella*)	112 ± 23	158 ± 94
Variable substrate: PAPS (0.1 μM 3,3'-T2)		
Liver cytosol	6.00 ± 0.25	21.4 ± 3.4
Kidney cytosol	8.95 ± 0.39	5.8 ± 5.2
SULT1A1 (*Salmonella*)	0.65 ± 0.12	177 ± 34
SULT1A3 (V79 cells)	2.70 ± 0.19	4.9 ± 0.2

Data are presented as the means ± SD of 2-5 experiments.

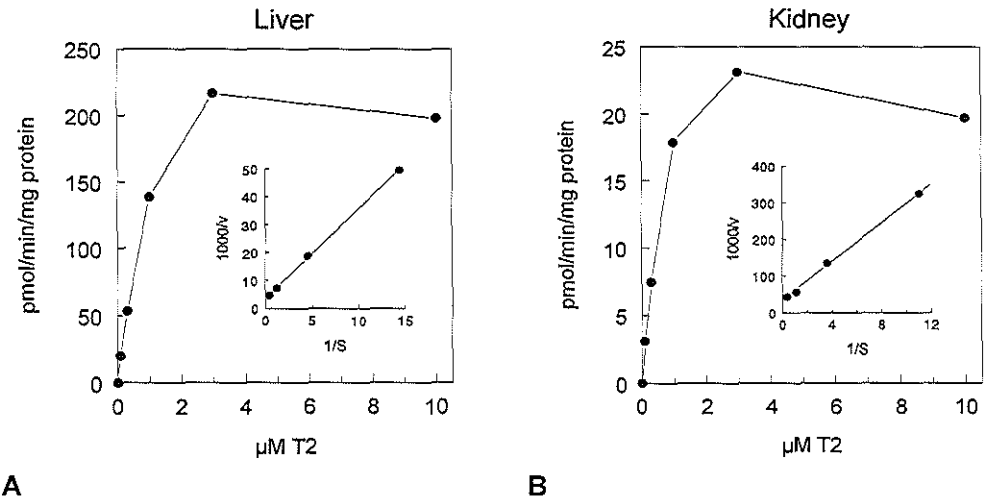

Fig. 3. Effects of substrate concentration on the sulfation of 3,3'-T2 by human liver (A) or kidney cytosol (B). The *insets* show the double-reciprocal plot. Reaction conditions: 0.1-3 μM 3,[3'-^{125}I]T2, 50 (A) or 100 (B) μg protein/ml, 50 μM PAPS, and 60 min incubation.

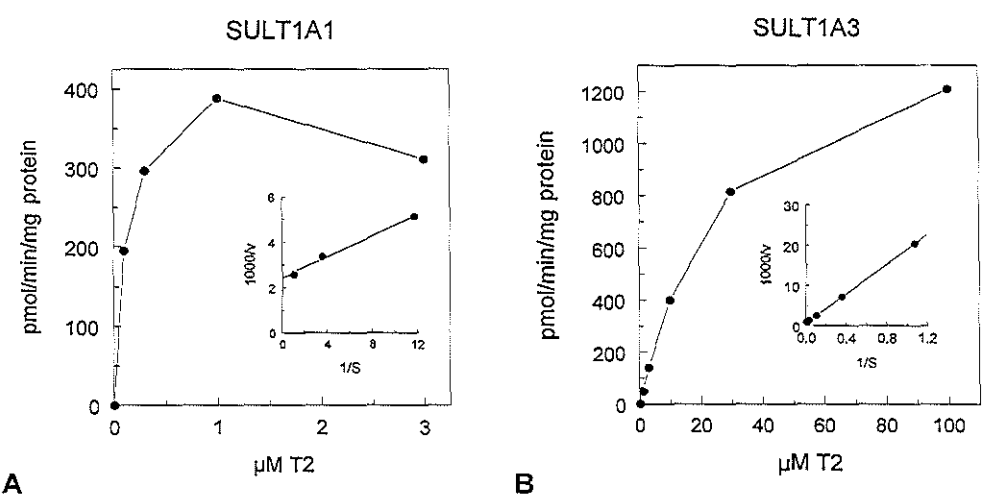

Fig. 4. Effects of substrate concentration on the sulfation of 3,3'-T2 by SULT1A1 (A) and SULT1A3 (B). The *insets* show the double-reciprocal plot. Reaction conditions: 0.1-100 μM 3,[3'-^{125}I]T2, 5 (A) or 100 (B) μg protein/ml, 50 μM PAPS, and 30 min incubation.

Figure 4 depicts the sulfation of 3,3'-T2 by SULT1A1 (A) and SULT1A3 (B) as a function of the substrate concentration. Maximum sulfation rates were obtained at lower 3,3'-T2 concentrations for SULT1A1 than for SULT1A3, with SULT1A1 showing clear substrate inhibition at 3,3'-T2 levels above 1 μM. From the linear double-reciprocal plots apparent K_m and V_{max} values were calculated. Since the enzymes expressed in *Salmonella* and V79 cells were tested as crude cytosol, the maximum sulfation rates determined in these experiments are not representative of the differences in k_{cat} values between the different isoenzymes. The kinetic parameters determined in different experiments are summarized in Table 1, showing mean K_m values of 0.14 and 33 μM for 3,3'-T2 sulfation by SULT1A1 and SULT1A3, respectively. Apparent K_m values were identical for SULT1A3 expressed in either *Salmonella* or V79 cells. Again, T3 sulfation was characterized by much higher apparent K_m values, *i.e.* 29.1 μM for SULT1A1 and 112 μM for SULT1A3, and lower apparent V_{max} values compared with 3,3'-T2 (Table 1).

Figure 5 shows the effects of increasing PAPS concentration on the sulfation of 0.1 μM 3,3'-T2 by human liver (A) and kidney (B) cytosol. Sulfation approached maximum rates at PAPS concentrations of 30 μM or more. The double-reciprocal plots of these data were linear, from which K_m and V_{max} values (at 0.1 μM 3,3'-T2) were calculated. Table 1 summarizes the results from different experiments. The mean apparent K_m value for PAPS amounted to 6 μM in liver and 9 μM in kidney cytosol.

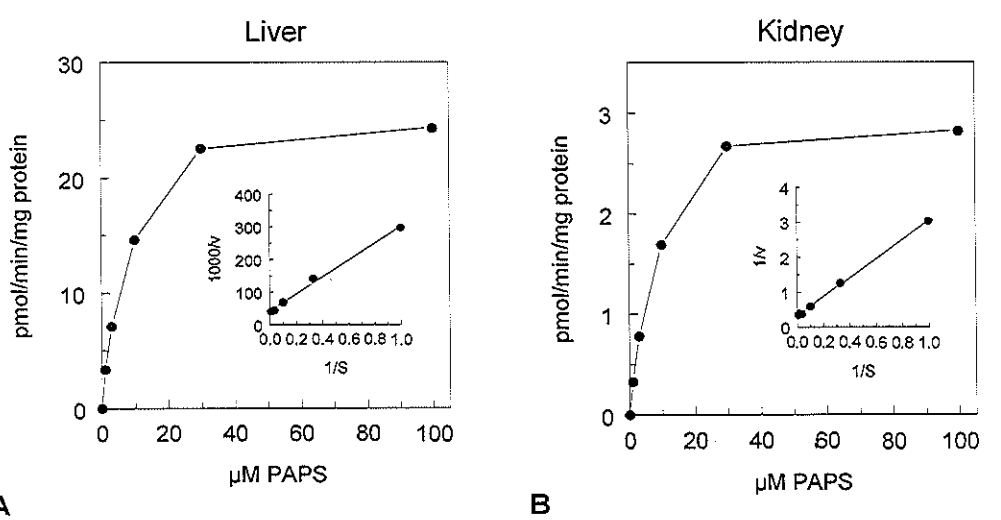

Fig. 5. Effects of cofactor concentration on the sulfation of 3,3'-T2 by human liver (A) or kidney (B) cytosol. The *insets* show the double-reciprocal plot. Reaction conditions: 0.1 μM 3,[3'-^{125}I]T2, 50 (A) or 100 (B) μg protein/ml, 1-100 μM PAPS, and 30 min incubation.

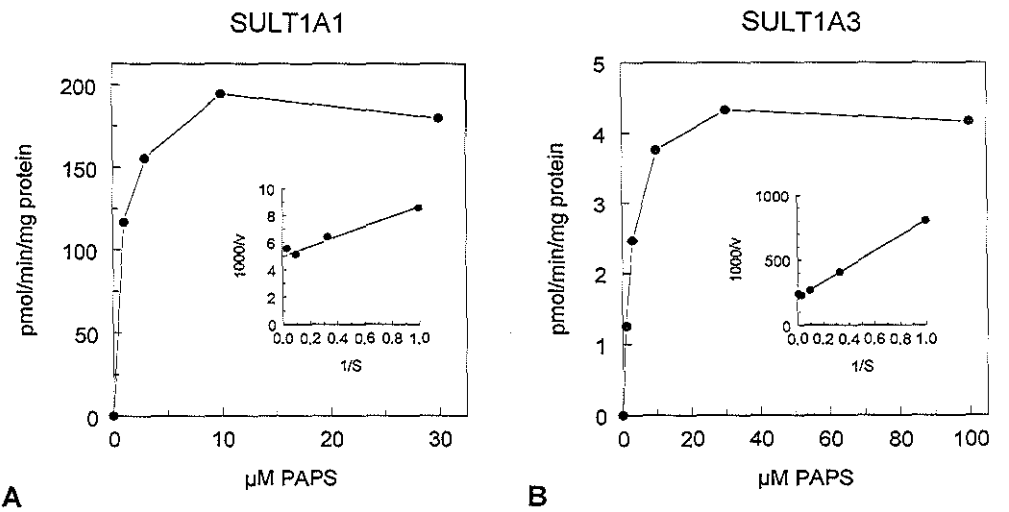

Fig. 6. Effects of cofactor concentration on the sulfation of 3,3'-T2 by SULT1A1 (A) and SULT1A3 (B). The *insets* show the double-reciprocal plot. Reaction conditions: 0.1 µM 3,[3'-^{125}I]T2, 5 (A) or 100 (B) µg protein/ml, 1-100 µM PAPS, and 30 min incubation.

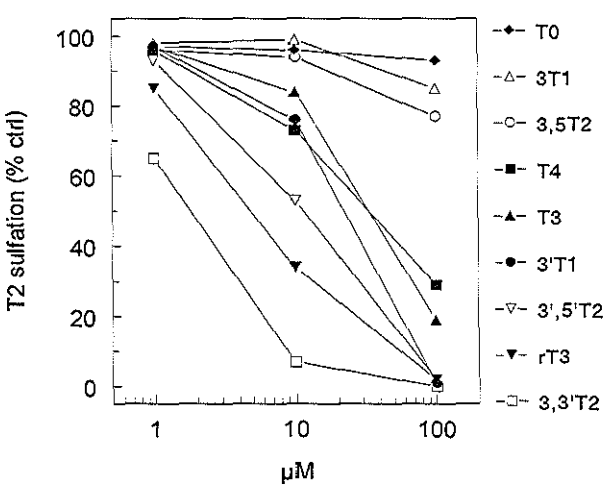

Fig. 7. Effects of 1-100 µM unlabeled iodothyronines on the sulfation of 3,[3'-^{125}I]T2 by human liver cytosol. Reaction conditions: 1 µM 3,[3'-^{125}I]T2, 50 µg protein/ml, 50 µM PAPS, and 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

Figure 6 presents the sulfation of 0.1 μM 3,3'-T2 by SULT1A1 (A) and SULT1A3 (B) as a function of the PAPS concentration. The PAPS concentration required for maximum sulfation rates was lower for SULT1A1 than for SULT1A3. The apparent K_m values for PAPS (at 0.1 μM 3,3'-T2) calculated from the linear Lineweaver-Burk plots, amounted to 0.65 and 2.7 μM, respectively.

Figure 7 demonstrates the effects of increasing concentrations (1-100 μM) of unlabeled iodothyronines on the sulfation of 3,[3'-^{125}I]T2 (1 μM) by human liver cytosol. T0 had no effect, while 3-T1 and 3,5-T2 produced only 10-20% inhibition at the highest concentration tested. All other iodothyronines inhibited the sulfation of labeled 3,3'-T2 dose-dependently, with potencies decreasing in the order 3,3'-T2 > rT3 > 3',5'-T2 > 3'-T1 > T3 ~ T4.

Table 2. Inhibition of the sulfation of 3,3'-T2 by iodothyronine analogs

Iodothyronine	% Inhibition			
	Liver	Kidney	SULT1A1	SULT1A3
T4	17 ± 3	7 ± 5	32 ± 7	0 ± 1
T3	4 ± 2	3 ± 4	24 ± 4	1 ± 2
rT3	43 ± 4	42 ± 10	86 ± 1	1 ± 1
3',5'-T2	47 ± 7	41 ± 14	86 ± 2	6 ± 1
3,3'-T2	87 ± 2	88 ± 2	95 ± 1	19 ± 2
3,5-T2	5 ± 7	-2 ± 2	5 ± 2	-1 ± 1
3'-T1	71 ± 12	61 ± 18	99 ± 1	38 ± 2
3-T1	5 ± 10	1 ± 1	3 ± 2	-1 ± 1
T0	-1 ± 2	0 ± 1	-2 ± 6	-2 ± 1

1 μM 3,[3'-^{125}I]T2 was incubated for 30 min at 37 C with appropriate amounts of enzyme protein in the absence or presence of 10 μM unlabeled iodothyronine. Percentage inhibition is calculated from the 3,[3'^{125}I]T2 sulfation rate in the presence *vs* that in the absence of analog. Data are presented as the means ± SD of 3-6 experiments using 3 liver and 3 kidney samples.

Table 2 compares the effects of unlabeled iodothyronines (10 μM) on the sulfation of 3,[3'-^{125}I]T2 (1 μM) by human liver and kidney cytosol, SULT1A1 and SULT1A3. In general, the magnitude of inhibition of the sulfotransferase activities by the various iodothyronine analogs decreased in the order SULT1A1 > liver ~ kidney > SULT1A3. The inhibition profiles of the tissue sulfotransferase activities were better correlated with the inhibition profile of SULT1A1 than with that of SULT1A3 (liver *vs* SULT1A1: r=0.936, p<0.01; liver *vs* SULT1A3: r=0.793, p<0.01; kidney *vs* SULT1A1: r=0.920, p<0.01; kidney *vs* SULT1A3: r=0.751, p<0.01).

Discussion

Several human phenol sulfotransferases have been cloned and characterized, including SULT1A1, SULT1A2, SULT1A3, SULT1B1 and SULT1C1 (1-3,34-36,40-50). SULT1A1 represents what has been known for some time as the phenol-preferring phenol sulfotransferase (P-PST), while SULT1A3 was previously known as the monoamine-preferring phenol sulfotransferase (M-PST) (1-3). The SULT1A isoenzymes are equally large proteins consisting of 295 amino acids. A significant degree of homology exists between the human SULT1A proteins, with 92% amino acid identity between SULT1A1 and SULT1A3, and an even greater degree of homology between SULT1A1 and SULT1A2, the genes of which are located closely together on chromosome 16 (2,34-36,40-46). Specifically, SULT1A1 and SULT1A2 differ in 11-15 amino acid positions, SULT1A1 and SULT1A3 differ in 20-23 amino acid positions, and SULT1A2 and SULT1A3 differ in 30-31 amino acid positions. Different allelic variants have been identified for SULT1A1 and SULT1A2 but sofar not for SULT1A3 (2,34-36,40-46).

Sulfation of T3 by P-PST and M-PST purified from human liver and intestine has been reported previously (32,33), but it remains to be determined which SULT1A isoenzyme is most important for thyroid hormone sulfation in human liver and other tissues. In addition to the members of the SULT1A family, two other human phenol sulfotransferases, SULT1B1 (49,50) and SULT1C1 (47,48), have been cloned recently. Whereas it is unknown if isoenzymes homologous to SULT1A2 and SULT1A3 exist in rats, the rat homologs of human SULT1A1, SULT1B1 and SULT1C1 have been cloned and characterized regarding their activity towards iodothyronines (51-53). These studies have demonstrated that both rat SULT1B1 and SULT1C1 catalyze the sulfation of different iodothyronines, in particular 3,3'-T2, whereas rat SULT1A1 is completely inactive. Human SULT1B1 has recently also been shown to have sulfotransferase activity towards iodothyronines (50), but sulfation of iodothyronines by human SULT1C1 has not yet been reported.

We demonstrate that both human SULT1A1 and SULT1A3 are capable of catalyzing the sulfation of iodothyronines. This is not surprising since the sulfation of T3 by P-PST and M-PST purified from human liver and intestine has been reported previously (32,33). We have also recently demonstrated effective sulfation of iodothyronines by human SULT1A2 (M.H.A. Kester, M.W.H. Coughtrie, H.R. Glatt, T.J. Visser, unpublished work). It appears that small differences in amino acid sequence can effect large differences in sulfotransferase activity. The high activity of human SULT1A1 in contrast to the complete lack of iodothyronine sulfotransferase activity of rat SULT1A1 is remarkable, considering the high degree of amino acid sequence identity (80%) between these orthologous proteins (2,51). Likewise, the smaller (8%) difference in amino acid

sequence between human SULT1A1 and SULT1A3 (34-36,46) is associated with a more than 200-fold difference in K_m value for 3,3'-T2, a 4-fold difference in K_m value for T3, and 4-fold difference in K_m value for PAPS. It should be noted that the apparent K_m value of 3,3'-T2 for SULT1A1 presented here is about 10-fold lower than that mentioned earlier (53), which may be due to partial inactivation through oxidation (54) of the enzyme preparation used previously.

The main purpose for comparing the substrate specificities and kinetic parameters of native iodothyronine sulfotransferase activities in human liver and kidney with these properties of recombinant sulfotransferases is to try to identify the isoenzymes which contribute most to the sulfation of thyroid hormone in these tissues. The iodothyronine sulfotransferase activities of human liver and kidney cytosol are characterized by similar apparent K_m values for both 3,3'-T2 and PAPS as well as similar substrate specificities, suggesting the involvement of similar isoenzymes. The substrate specificities of the hepatic and renal sulfotransferase activities showed a better correlation with SULT1A1 than with SULT1A3, suggesting that SULT1A1 is a prominent iodothyronine sulfotransferase in human liver and kidney. However, the different iodothyronines showed a lower apparent affinity for the native sulfotransferases than for recombinant SULT1A1, which may be due to the presence of iodothyronine-binding proteins in the tissues. Sulfation of thyroid hormone in human liver and kidney (and possibly other tissues) involves contributions of at least SULT1A1, SULT1A2, SULT1A3 and SULT1B1, and perhaps also SULT1C1. The complexity is further increased by the polymorphic variation in these isoenzymes (2,55) and their tissue-specific expression (56). In addition, it has been demonstrated that functional rat phenol sulfotransferases may either consist of two identical (homodimer) or two different subunits (heterodimer) (57). Our findings of a more than linear increase in iodothyronine sulfotransferase activity with an increase in hepatic or renal cytosolic protein concentration may reflect this requirement for protein dimerization.

The native and recombinant sulfotransferases tested in this study show a marked preference for 3,3'-T2 as the substrate. Both SULT1A1 and SULT1A3 are much less efficient in catalyzing the sulfation of T3, which does not imply that these isoenzymes are not important for T3 sulfation in vivo. This is supported by the significant sulfation of T3 in both human liver and kidney cytosol. Sulfation of T4 is almost undetectable, not only with recombinant SULT1A1 and SULT1A3 but also in human liver and kidney. Nevertheless, high serum T4S levels have been detected in human newborns (14,15), suggesting sulfation of T4 by other isoenzymes.

In conclusion, we have identified SULT1A1 and SULT1A3 as low K_m and high K_m human iodothyronine sulfotransferases, respectively, and obtained evidence that the sulfation of iodothyronines in human liver and kidney is catalyzed by similar enzymes. Further investigations are required to determine the possible importance of other isoenzymes, such as SULT1A2,

SULT1B1 and SULT1C1, and of polymorphic variations in the different sulfotransferases for the sulfation of thyroid hormone in human tissues. This information is essential for the investigation of the regulation of this important pathway of thyroid hormone metabolism under (patho)physiological conditions, in particular during fetal development.

Acknowledgments

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Chapter 6

Sulfation of thyroid hormone by estrogen sulfotransferase

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J Clin Endocrinol Metab. 84: 2577-2580, 1999

Abstract

Sulfation is one of the pathways by which thyroid hormone is inactivated. Iodothyronine sulfate concentrations are very high in human fetal blood and amniotic fluid, suggesting important production of these conjugates *in utero*. Human estrogen sulfotransferase (SULT1E1) is expressed among other tissues in the uterus. Here we demonstrate for the first time that SULT1E1 catalyzes the facile sulfation of the prohormone T_4, the active hormone T_3 and the metabolites rT_3 and 3,3'-diiodothyronine (3,3'-T_2) with preference for $rT_3 \sim 3,3'\text{-}T_2 > T_3 \sim T_4$. Thus, a single enzyme is capable of sulfating two such different hormones as the female sex hormone and thyroid hormone. The potential role of SULT1E1 in fetal thyroid hormone metabolism needs to be considered.

Introduction

Thyroid hormone is essential for the development of different tissues, in particular the brain, and requires the binding of the active hormone T_3 to nuclear receptors (1). Sulfation is one of the pathways by which T_3 and other iodothyronines, including the prohormone T_4, are metabolized (2). This is an inactivating pathway since T_3 sulfate (T_3S) has lost its affinity for the T_3 receptors (3). Moreover, sulfation of T_3 and its prohormone T_4 strongly facilitates their degradation through inner ring deiodination by the type I iodothyronine deiodinase in liver (2). Iodothyronine sulfate concentrations are very high in the human fetal circulation and in the amniotic fluid (4), suggesting important production of these conjugates *in utero*. Human estrogen sulfotransferase (SULT1E1) is known to be expressed, among others, in the endometrium (5,6). In this study we tested the possible sulfation of T_4, T_3 and the metabolites rT_3 and 3,3'-diiodothyronine (3,3'-T_2) by recombinant human SULT1E1 in comparison with the sulfation of estrone (E_1) and 17β-estradiol (E_2).

Materials and Methods

Materials

[3',5'-125I]T_4, [3'-125I]T_3, [^{3}H]E_1 and [^{3}H]E_2 were obtained from Amersham (Amersham, UK); T_3, E_2 and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) from Sigma (St. Louis, MO); T_4, rT_3 and 3,3'-T_2 from Henning Berlin GmbH (Berlin, Germany); and E_1 from Ikapharm (Ramat, Israel). 3,[3'-^{125}I]T_2 and [3',5'-^{125}I]rT_3 were prepared as previously described (7). Human SULT1E1 was expressed in *S. typhimurium* as previously described (8) and used without further purification. Expression in *E. coli* and purification of human SULT1A1, SULT1A3 and SULT1E1 have also been described previously (9,10). Cloning, expression and purification of human SULT1B1 (11) will be described in detail elsewhere. Briefly, the clone was isolated from human liver cDNA by PCR, cloned into the vector pET11a and expressed in *E. coli*. Protein was purified as described (9,10).

Sulfotransferase assays

Iodothyronine sulfotransferase activities were analyzed by incubation of usually 0.1 μM T_4, T_3, rT_3 or 3,3'-T_2 and 10^5 cpm of the ^{125}I-labeled compound for 30 min at 37 C with the indicated amounts of recombinant sulfotransferase in the absence (blank) or presence of 50 μM PAPS in 0.2 ml 0.1 M phosphate (pH 7.2) and 2 mM EDTA. The reactions were stopped by addition of 0.8 ml 0.1 M HCl, and the mixtures were analyzed for sulfate formation as previously described (7). Estrogen sulfotransferase activity was analyzed by incubation of 1-3 nM ^{3}H-labeled E_1 or E_2 for

30 min at 37 C with the indicated amount of recombinant SULT1E1 in the absence (blank) or presence of 50 µM PAPS in 0.2 ml phosphate-EDTA buffer. The reactions were stopped by addition of 2 ml ice-cold water, and the mixtures were extracted with 2 ml dichloromethane. Sulfate formation was quantified by counting 1 ml of the aqueous phase. Enzymatic sulfation was corrected for background radioactivity estimated in the blanks. Kinetic parameters were determined by Lineweaver-Burk analysis of the sulfation of varying substrate concentrations. Apparent K_i values were calculated from the change in slope of the Lineweaver-Burk plot in the presence of a fixed inhibitor concentration.

Results and Discussion

Figure 1 shows the sulfation of E_1, E_2, T_4, T_3, rT_3 and 3,3'-T_2 by recombinant human SULT1E1 as a function of the enzyme concentration. The results show that not only the estrogens but also the different iodothyronines are sulfated by human SULT1E1. Under the conditions used, sulfation of E_1 and E_2 requires the lowest enzyme concentrations. Substantially more enzyme is needed for sulfation of 3,3'-T_2 and rT_3, while sulfation of T_3 and T_4 requires the highest enzyme concentrations.

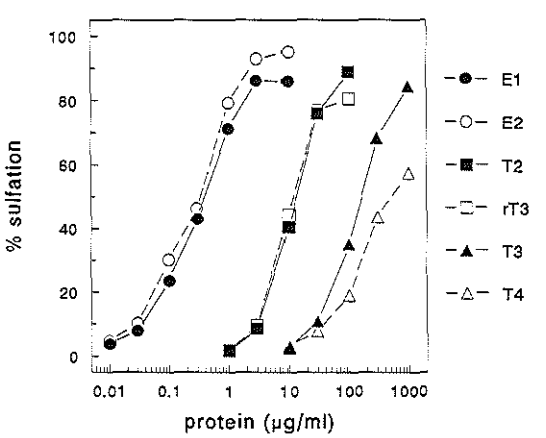

Fig. 1. Sulfation of estrogens and iodothyronines by increasing concentrations of human SULT1E1. Reaction conditions: 3 nM E_1 or E_2, 0.1 µM T_4, T_3, rT_3 or 3,3'-T_2, 50 µM PAPS, and 30 min incubation.

Significant sulfation of iodothyronines, in particular 3,3'-T_2, has been demonstrated previously in human liver and kidney as well as with recombinant human SULT1A1, SULT1A3 and SULT1B1 (11,12). Figure 2 compares the sulfation of the different iodothyronines by purified recombinant

human SULT1A1 (13), SULT1A3 (14), SULT1B1 (11) and SULT1E1 (15). In agreement with previous studies, 3,3'-T_2 is by far the preferred substrate for SULT1A1, SULT1A3 and SULT1B1, its sulfation rates being orders of magnitude higher than those for T_3 and rT_3, whereas sulfation of T_4 is negligible. Although 3,3'-T_2 is a better substrate for SULT1A1 than for SULT1E1 and T_3 is sulfated at similar rates by the different isoenzymes, SULT1E1 is much more effective in catalyzing the sulfation of T_4 and, in particular, rT_3 than any other isoenzyme tested.

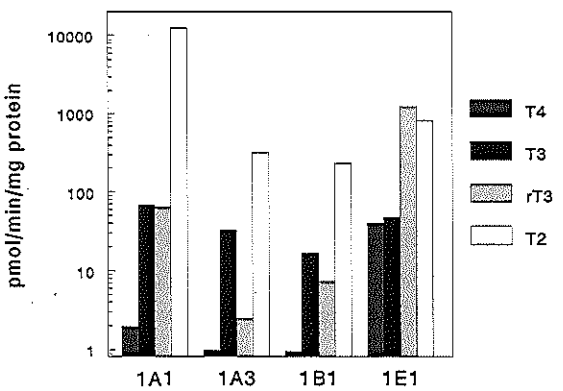

Fig. 2. Sulfation of iodothyronines by purified human sulfotransferases. Reaction conditions: 0.1 μM iodothyronines, appropriate concentrations of enzymes, 50 μM PAPS, and 30 min incubation.

Figure 3A shows the Lineweaver-Burk analysis of the sulfation of the iodothyronines by human SULT1E1, and the values for the kinetic parameters are presented in Table 1. The apparent K_m values for the different iodothyronines are in the micromolar range. They are 5-10 times lower while V_{max} values are 2-8 times higher for rT_3 and 3,3'-T_2 than for T_3 and T_4. Reflecting catalytic efficiency, the V_{max}/K_m ratio decreases in the order $rT_3 \sim$ 3,3'-$T_2 > T_3 \sim T_4$. Lineweaver-Burk analysis of the sulfation of E_2 by SULT1E1 yielded an apparent K_m value of 4 nM (Table 1) in close agreement with reported data (15,16). Similar K_m and V_{max} values were obtained using E_1 as substrate (not shown). Although V_{max} values are lower for E_1 and E_2 than for rT_3 and 3,3'-T_2, their ~10^3-fold lower apparent K_m values indicate that the estrogens have much higher affinity for SULT1E1 than the iodothyronines.

The different iodothyronines dose-dependently inhibited the sulfation of estrogens by human SULT1E1. The nature of this inhibition was studied by Lineweaver-Burk analysis (Fig. 3B). The results demonstrate that the iodothyronines are mixed-type inhibitors of E_2 sulfation. The apparent K_i values for the iodothyronines are in agreement with their apparent K_m values (Table 1).

However, the apparent K_m value for T_4 is higher than its apparent K_i value, which may be due to significant protein binding of T_4 at the higher protein concentrations required for its sulfation than for E_2 sulfation. Conversely, E_1 and E_2 were found to be potent inhibitors of the sulfation of iodothyronines, using 3,3'-T_2 as the substrate (not shown). That iodothyronines are not pure competitive inhibitors of the sulfation of estrogens by SULT1E1 may be explained by recent findings of two substrate-binding sites on human SULT1E1, the active site as well as an allosteric binding site (16).

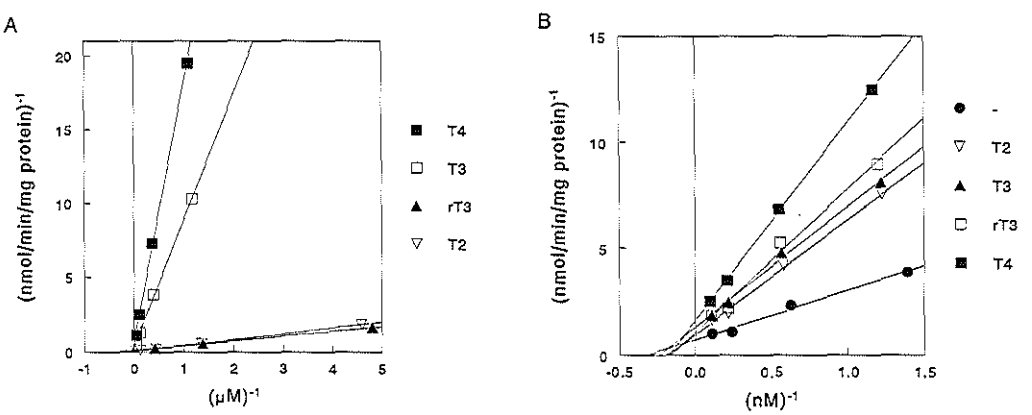

Fig. 3. Kinetics of human SULT1E1. A. Lineweaver-Burk analysis of the sulfation of iodothyronines. Reaction conditions: 0.3-30 μM iodothyronine, 10-100 μg protein/ml, 50 μM PAPS, and 30 min incubation. B. Lineweaver-Burk analysis of the inhibition of the sulfation of E_2 by iodothyronines. Reaction conditions: 1-10 nM E_2, 0.05 μg protein/ml, 50 μM PAPS, and 30 min incubation in the absence or presence of 5-10 μM T_4, 20 μM T_3, 2 μM rT_3, or 5 μM 3,3'-T_2.

Table 1. Kinetic parameters of iodothyronine and estrogen sulfation by human SULT1E1

Substrate/ competitor	K_i (μM)	K_m (μM)	V_{max} (nmol/min/mg)
E_2	-	0.003 - 0.006	1.1 - 2.8
3,3'-T_2	3.0 - 4.3	3.5 - 6.0	8.9 - 15.3
rT_3	0.6 - 0.9	1.7 - 2.6	4.5 - 8.0
T_3	12.3 - 18.8	15.3 - 36.1	2.2 - 4.4
T_4	2.3 - 2.4	22.6 - 24.6	1.4 - 1.4

Data are presented as the range of values from 2-3 experiments.

These studies indicate that thyroid hormone is sulfated importantly by human SULT1E1. Although the estrogens E_1 and E_2 are clearly the preferred substrates for this isoenzyme, T_4 and especially rT_3 are sulfated much better by human SULT1E1 than by any other known sulfotransferase. Whereas human SULT1A1, SULT1A3 and SULT1B1 show an obvious preference for 3,3'-T_2 as the substrate, rT_3 is sulfated by human SULT1E1 as fast as 3,3'-T_2. The K_m values of the estrogens and iodothyronines for SULT1E1 appear unrelated to their concentrations *e.g.* in amniotic fluid (17). The preference of SULT1E1 for estrogens is reflected in their higher sulfated/free ratios in amniotic fluid compared with iodothyronines (4,17).

The purpose of the rapid sulfation of 3,3'-T_2 and rT_3 by human SULT1E1 is unknown. Both metabolites have little affinity for the nuclear T_3 receptors (1). However, 3,3'-T_2 has been shown to stimulate mitochondrial respiration in different tissues (18) and rT_3 may regulate actin polymerization in brain cells (19), actions which are not mediated by the nuclear T_3 receptors. The possibility that rT_3, 3,3'-T_2 or their sulfates serve a physiological function in the fetus is, therefore, not excluded. It is intriguing in this respect that rT_3 and 3,3'-T_2 are the products of T_4 and T_3 deiodination, respectively, by the type III iodothyronine deiodinase which is abundantly expressed in placenta (20) as well as the pregnant uterus (21).

It is astonishing that a single enzyme is capable of conjugating two such completely different hormones as the female sex hormone and thyroid hormone. E_2 is inactivated by sulfation, which is a reversible process as free E_2 is liberated by hydrolysis of the sulfate by steroid sulfatase expressed in different tissues (5,6). Similarly, in the human fetal circulation, T_4S and in particular T_3S may represent a reservoir of inactive thyroid hormone, from which active hormone may be liberated by action of sulfatases expressed in a tissue-specific and developmental stage-dependent manner (2). Our results suggest that the iodothyronine sulfates in the human fetal circulation and amniotic fluid may be derived at least in part from sulfation of thyroid hormone by SULT1E1 in the uterus. This may represent another route for the supply of maternal thyroid hormone to the fetus in addition to placental transfer (22). There is one report suggesting that SULT1E1 is also expressed in human placenta (23). SULT1E1 expression in human endometrium is up-regulated by progesterone (5,6). Preliminary findings suggest low levels of SULT1E1 expression in the uterus during the first 13 weeks of pregnancy, but further studies are needed to explore SULT1E1 expression in human endometrium throughout gestation.

Acknowledgments

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Chapter 7

Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs

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Abstract

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants which exert a variety of toxic effects in animals, including disturbances of sexual development and reproductive function. The estrogenic effects of PCBs may be mediated in part by hydroxylated PCB metabolites (PCB-OHs), but the mechanisms by which they are brought about are not understood. PCBs as well as PCB-OHs show low affinities for both α and β estrogen receptor isoforms. In the present study we demonstrate that various environmentally relevant PCB-OHs are extremely potent inhibitors of human estrogen sulfotransferase, strongly suggesting that they indirectly induce estrogenic activity by increasing estradiol bioavailability in target tissues.

Introduction

The endocrine-disrupting effects of PCBs have received much attention recently, in particular their estrogenic activity which is thought to play an important role in the impaired sexual differentiation and reproductive dysfunction observed in exposed birds, fish, reptiles and mammals (1-5). Also in humans, an increase has been observed over the last 50 years in the incidence of testicular cancer and of abnormal male reproductive tract development in some developed countries (4). Decreasing trends in semen quality and sperm counts have also been reported, but this may not be universal (4). Since similar abnormalities in sexual differentiation and reproductive function have been encountered in male offspring of women treated during pregnancy with the potent estrogen diethylstilbestrol (DES) to prevent miscarriage (6), it has been hypothesized that increased exposure to estrogenic and other endocrine-active chemicals, in particular during fetal and neonatal life, may contribute to the above-mentioned defects. This hypothesis is supported by laboratory animal studies showing disruption of endocrine pathways in the adult animal after *in utero* or early postnatal exposure to a variety of environmental contaminants including PCBs, polychlorinated dibenzodioxins and dibenzofurans, pesticides such as 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT), plastic additives such as bisphenol A, and detergent additives such as alkylphenols (3,4).

Specific PCB congeners exhibit estrogenic activities in experimental animals, whereas other congeners are associated with anti-estrogenic activities (1,2). There is evidence that the estrogenic (and anti-estrogenic) activities of PCBs are mediated at least in part through hydroxylated metabolites (1,2,7), but the mechanism by which PCB-OHs exert their effects has not been established. It has been shown previously for a large number of PCB-OHs that their affinity for both α and β estrogen receptor subtypes is low (8,9), suggesting that they have little activity as estrogen receptor agonists. However, it is possible that PCBs or PCB-OHs indirectly exert estrogenic activity by inhibiting estradiol (E2) metabolism, thus enhancing cellular E2 bioavailability. Sulfation by estrogen sulfotransferase (EST) is an important pathway for E2 inactivation (10). In this study, we investigated the potential inhibition of human EST (hEST) by hydroxylated PCBs.

Materials and Methods

Materials

[^{3}H]E2 (3.22 MBq/nmol) was obtained from Amersham (Amersham, UK); [^{35}S]PAPS (52.9 MBq/μmol) from NEN (Boston, MA); unlabeled E2 and PAPS from Sigma (St. Louis, MO). The

sources of the various PCB-OHs have been described previously (8,11). Recombinant hEST (12) was expressed in *S. typhimurium* as previously described (13). Cytosolic preparations from these bacteria were used without further purification. EST accounted for 5-7% of the cytosolic proteins. Similar results were obtained with hEST expressed in *E. coli* and purified as previously described (14).

Sulfotransferase assays.

Estrogen sulfotransferase activity was analyzed by incubation of 1 nM [^{3}H]E2 for 30 min at 37 C with recombinant hEST (0.1 μg protein/ml) in the absence (blank) or presence of 50 μM PAPS in 0.2 ml 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA and 1 mM dithiothreitol. The reactions were stopped by addition of 2 ml ice-cold water, and the mixtures were extracted with 2 ml dichloromethane. Sulfate formation was quantified by counting 1 ml of the aqueous phase. Enzymatic sulfation was corrected for background radioactivity estimated in the blanks. Kinetic parameters were determined by Lineweaver-Burk analysis (15) of the sulfation of varying substrate concentrations. Apparent K_i values were calculated from the change in slope of the Lineweaver-Burk plot in the presence of inhibitor (15).

Results and Discussion

The effects of increasing concentrations (0.01-1000 nM) of various PCB-OHs were tested on the sulfation of 1 nM E2 by recombinant hEST. The compounds are numbered as explained in Table 1. The nonhydroxylated compound ***1*** (PCB77) did not affect EST activity even at the highest concentration tested (1000 nM). However, hydroxylation of one of the phenyl rings induced strong inhibitory activity that was dependent on the positions of the substituents in this ring. Figure 1A shows the results with PCB-OHs having the same 3',4'-dichloro-substituted nonphenolic ring. Although the *ortho*-hydroxylated compounds ***28*** and ***30*** were relatively weak inhibitors, increasing potencies were observed with the *meta*-hydroxylated compounds ***23*** and ***26***, and even higher inhibitory activities were observed with *para*-hydroxylated compounds, in particular ***13*** and ***18***. Concentrations as low as 0.1 nM of the latter PCB-OHs significantly inhibited EST activity. Also from the potencies of other PCB-OHs it is concluded that an OH group in the *para* position with two adjacent Cl substituents is required for maximum EST inhibitory potency. (Table 1).

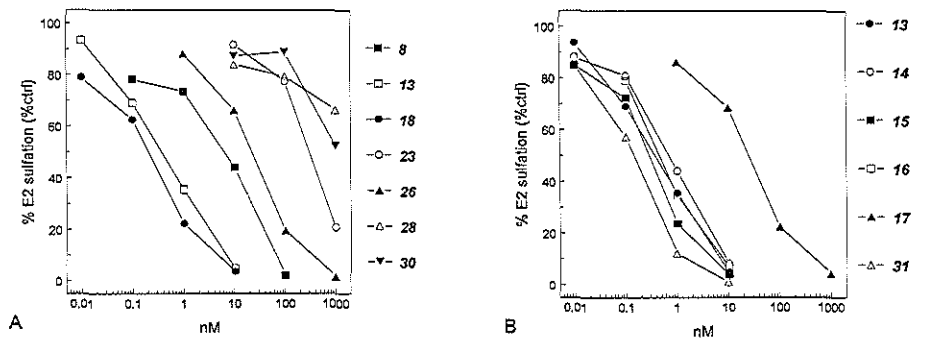

Fig. 1. Inhibition of the sulfation of 1 nM E2 by recombinant hEST by increasing concentrations of PCB-OHs with a 3',4'-dichloro-substituted nonphenolic ring (A) or with a 4-hydroxy-3,5-dichloro-substituted phenolic ring. Results are the means of 2-4 experiments.

Figure 1B compares the effects of PCB-OHs with an identical 4-hydroxy-3,5-dichloro-substituted phenolic ring. Potent inhibition was observed irrespective of whether the nonphenolic ring was substituted with two (3',4' or 3',5') or three (2',3',4' or 3',4',5') Cl atoms, but a marked reduction in inhibitory potency was observed with four (2',3',4',5') Cl substituents. Further analysis of other PCB-OHs indicated that in general the substitution of both *ortho* (2' and 6') positions or of two diametrically opposite (2' and 5') positions negatively affects EST inhibitory potency (Table 1). This suggests that binding of PCB-OHs to hEST is favored by a coplanar structure of the inhibitor and/or that there are steric constraints for accommodation of the substituted nonphenolic ring. However, other di-*ortho* (2,6 and 2,2') Cl substitutions did not decrease EST inhibitory potency, suggesting that the dimensions of the substituted nonphenolic ring are critical. From the concentration-inhibition relationships, IC_{50} values (concentrations producing 50% inhibition) were determined which are presented in Table 1. IC_{50} values for several PCB-OHs (***13***, ***14***, ***15***, ***16***, ***18*** and ***19***) are in the subnanomolar range. All these compounds are characterized by a 4-hydroxy-3,5-dichloro substitution pattern. Compound ***31***, having such a pattern in both rings, is the most potent inhibitor identified in this study, with an IC_{50} value of 0.1 nM (Fig. 1B, Table 1).

To further appreciate the contributions of each phenolic and nonphenolic ring to the inhibitory activity of PCB-OHs towards hEST, the possible effects of a series of single-ring halogenated phenols were tested at a concentration of 1 μM. Figure 2 shows that phenol itself had little effect on EST activity, but halogenation resulted in the generation of marked inhibitory activity. In general, the potency of the halophenols increased with the number and size (I > Br > Cl > F) of the halogen substituents, suggesting that hydroxylated metabolites of polybromobiphenyls (16) may be even more potent inhibitors of hEST than the corresponding PCB-OHs.

Table 1. Potency of inhibition of hEST activity by PCB-OHs

Compound		IC_{50} (nM)
1	3,4,3',4'-tetraCB	>1000
2	**4-OH-2**',4',6'-triCB	610 - 670
3	**4-OH-2**',3',4',5'-tetraCB	640 - 650
4	**4-OH-2**,2',4',6'-tetraCB	230 - 260
5	**4-OH-2**,2',3',4',5'-pentaCB	260 - 370
6	**4-OH-2**,2',3',4',6'-pentaCB	150 - 295
7	**4-OH-2**,2',3',5',6'-pentaCB	280 - 430
8	**4-OH-3**,3',4'-triCB	4.3 - 7.8
9	**4-OH-3**,2',4',6'-tetraCB	220 - 240
10	**4-OH-3**,2',3',4',5'-pentaCB	100 - 120
11	**4-OH-3**,2',3',4',6'-pentaCB	170 - 200
12	**4-OH-3**,2',3',5',6'-pentaCB	260 - 370
13	**4-OH-3,5**,3',4'-tetraCB	0.21 - 0.61
14	**4-OH-3,5**,3',5'-tetraCB	0.47 - 1.00
15	**4-OH-3,5**,2',3',4'-pentaCB	0.28 - 0.30
16	**4-OH-3,5**,3',4',5'-pentaCB	0.38 - 0.50
17	**4-OH-3,5**,2',3',4',5'-hexaCB	20 - 30
18	**4-OH-2,3,5**,3',4'-pentaCB	0.15 - 0.25
19	**4-OH-2,3,5**,2',3',4'-hexaC	0.27 - 0.75
20	**4-OH-2,3,5**,2',4',5'-hexaCB	5.8 - 14
21	**4-OH-2,3,5**,2',3',4',5'-hexaCB	25 - 26
22	**4-OH-2,3,5,6**,2',4',5'-heptaCB	6.8 - 30
23	**3-OH-4,5**,3',4'-tetraCB	210 - 410
24	**3-OH-4,5**,2',3',4'-pentaCB	400 - 580
25	**3-OH-4,5**,3',4',5'-pentaCB	250 - 380
26	**3-OH-2,4,5**,3',4'-pentaCB	21 - 24
27	**3-OH-2,4,5**,2',3',4',5'-heptaCB	9.0 - 13
28	**2-OH-3,4**,3',4'-tetraCB	>1000
29	**2-OH-3,4**,2',3',4'-pentaCB	>1000
30	**2-OH-4,5**,3',4'-tetraCB	720 - >1000
31	**4,4'-$(OH)_2$-3,5,3',5'**-tetraCB	0.10 - 0.19
32	**3,3'-$(OH)_2$-4,4'**-diCB	35 - 52

The substitution pattern in the phenolic ring is indicated in bold.
Data are presented as the range of values determined in 2-4 experiments.

The inhibitory potency of 2,6-dichlorophenol is much lower than observed for PCB-OHs with identically substituted (4-hydroxy-3,5-dichloro) phenolic rings, indicating that the nonphenolic ring contributes importantly to the inhibitory effects of PCB-OHs on hEST. It should be noted that pentachlorophenol and other chlorophenols are also environmental pollutants resulting from their extensive use as preservatives in the wood and paper industry (17). Pentachlorophenol, which is also a major metabolite of the fungicide hexachlorobenzene, has been widely identified in human blood and urine (18,19). Although pentachlorophenol and other halogenated phenols exhibit lower EST inhibitory activity than several PCB-OHs, occupational exposure to these chemicals may be sufficiently high to contribute to endocrine-disrupting effects in exposed subjects.

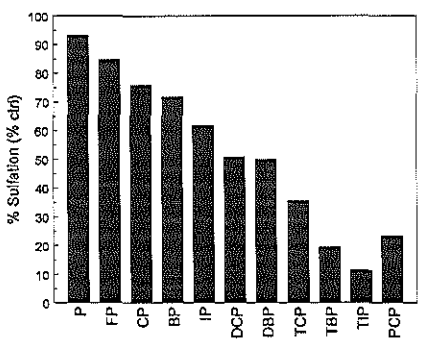

Fig. 2. Effects of different phenols on the sulfation of E2 by recombinant hEST. Sulfation of 1 nM E2 in the presence of 1 μM phenol is expressed as a percentage of that in the absence of inhibitor. P, phenol; FP, 2-fluorophenol; CP, 2-chlorophenol; BP, 2-bromophenol; IP, 2-iodophenol; DCP, 2,6-dichlorophenol; DBP, 2,6-tribromophenol; TCP, 2,4,6-trichlorophenol; TBP, 2,4,6-tribromophenol; TIP, 2,4,6-triiodophenol; PCP, pentachlorophenol. Results are the means of 2 experiments.

The phenolic hydroxyl group in PCB-OHs is essential for potent inhibition of EST activity. Since EST catalyzes the sulfation of the phenolic 3-hydroxyl group of E2 (10), this suggests that PCB-OHs may also be substrates for this enzyme. To gain more insight in the mechanism of EST inhibition by PCB-OHs, the kinetics of this inhibition were studied by Lineweaver-Burk analysis (15) for compounds ***8***, ***16***, ***18***, ***26*** and ***31***) (Fig. 3). The double-reciprocal plots of the rate of E2 sulfation *versus* the E2 concentration in the absence or presence of a single concentration of different PCB-OHs (Fig. 3A) or different concentrations of a single PCB-OH (Fig. 3B) converged at approximately the same point on the *x*-axis. This indicates that these PCB-OHs are noncompetitive inhibitors of E2 sulfation and not competitive inhibitors which would be expected if

they are also substrates for EST. The K_i values derived from these Lineweaver-Burk plots are in good agreement with the corresponding IC_{50} values for the different inhibitors. The noncompetitive type of inhibition can be explained by the presence of two substrate-binding sites on hEST, the active site as well as an allosteric site (20). Our results suggest that the potent inhibition of hEST by PCB-OHs is primarily due to binding of these inhibitors to the second, allosteric site.

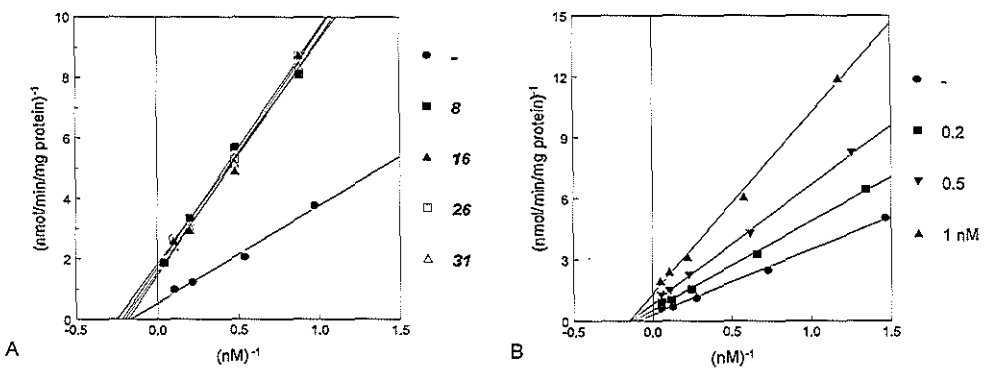

Fig. 3. Lineweaver-Burk plots of the sulfation of E2 by recombinant hEST in the absence or presence of (**A**) 6 nM ***8***, 0.5 nM ***16***, 22 nM ***26*** or 0.1 nM ***31***, or (**B**) 0.2, 0.5 or 1 nM ***18***. Results are representative for 2-4 experiments.

Binding of hydroxylated PCB metabolites to the estrogen receptor is an obvious mechanism by which these compounds could exert their estrogenic activity. However, previous studies have demonstrated that the affinity of PCB-OHs for both α and β estrogen receptor subtypes is in general very low. Among the large number of PCB-OHs tested, compounds ***2*** and ***3*** showed by far the highest affinities for both estrogen receptors which were still >20-fold lower than the affinity of E2 itself (8,9). The results of our study provide a more attractive explanation for the estrogenic activity of PCB-OHs. Several congeners were found to be extremely potent inhibitors of hEST. The IC_{50} and K_i values of different PCB-OHs are up to 50-fold lower than the K_m value of E2 for hEST (4 nM) (21), indicating that these inhibitors have much higher affinity for the enzyme than its natural substrate. To our knowledge, inhibition of hEST is the most potent biological effect described to date regarding the endocrine-disrupting activity of PCBs or their metabolites. It is noteworthy that among the most potent EST inhibitors, ***18*** has been identified as one of the most abundant PCB-OHs in blood and tissues of animals and humans exposed to PCBs (22,23). By inhibiting the formation of inactive E2 sulfate, PCB-OHs can increase E2 bioavailability in target tissues, thereby exerting an indirect estrogenic effect. This may not necessarily be associated with significant changes in circulating levels of E2 and other estrogens but may take place locally in estrogen-sensitive tissues expressing EST, including testis (24), mammary gland (25) and endometrium (26).

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Chapter 8

Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters

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Abstract

Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), polybrominated diphenylethers (PBDEs) and bisphenol A (BPA) derivatives are persistant environmental pollutants, which are capable of interfering with reproductive and endocrine function of birds, fish, reptiles and mammals. Part of the endocrine-disrupting effects may be mediated by their hydroxylated metabolites (PHAH-OHs), the mechanisms of which remain to be identified. PHAH-OHs show low affinity for the estrogen receptors. Alternatively, they may exert their estrogenic effects by inhibiting estradiol (E2) metabolism. Since sulfation of E2 by estrogen sulfotransferase (SULT1E1) is an important pathway for E2 inactivation, inhibition of SULT1E1 may lead to an increased bioavailability of estrogens in tissues expressing this enzyme. Therefore, we studied the possible inhibition of human SULT1E1 by hydroxylated PHAH metabolites, and sulfation of the different compounds by SULT1E1. We found marked inhibition of SULT1E1 by various PHAH-OHs, in particular by compounds with two vicinal halogen substituents around the hydroxyl group which were effective at (sub)nanomolar concentrations. Depending on the structure, the inhibition is primarily competitive or noncompetitive. Most PHAH-OHs are also sulfated by SULT1E1. We also investigated the inhibitory effects of the various PHAH-OHs on E2 sulfation by human liver cytosol, and found that the effects were strongly correlated with their inhibitions of recombinant SULT1E1 ($r = 0.922$). Based on these results, we hypothesize that hydroxylated PHAHs exert their estrogenic effects at least in part by inhibiting SULT1E1-catalyzed E2 sulfation.

Introduction

Recently, many studies have been done on the interaction of environmental chemicals with the endocrine system, which results in reproductive and developmental anomalies in various organisms (1-4). Endocrine-disrupting effects have been observed in adult animals which were exposed *in utero* to polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) (5-7). Hilakivi-Clarke *et al.* demonstrated in rats that exposure to natural estrogens *in utero* advanced puberty onset and increased breast cancer risk in the offspring (8); this may also apply for environmental estrogens. In humans, abnormalities in the development of the reproductive tract, reduced sperm counts (3,9), and increased incidence of germ cell cancer (10) have been related to exposure to endocrine disrupters present in the environment. Furthermore, several studies have reported increased levels of PCBs, 2,2-bis-(4-chlorophenyl)-1,1,1-trichloroethane (DDT) and its metabolite 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene (DDE) in breast cancer patients (11,12). However, recent epidemiological research does not support the hypothesis that women exposed to organochlorines such as PCBs, PCDDs, DDT and DDE have an increased breast cancer risk (13-15).

Hydroxylated metabolites of the PHAHs (PHAH-OHs) may contribute to the afore-mentioned effects. Hydroxylated metabolites of PCBs (PCB-OHs), PCDDs (PCDD-OHs) and PCDFs (PCDF-OHs) and other organohalogens have been identified in blood, bile and urine of animals treated with the parent compound, but also in wildlife samples as well as in environmentally exposed human subjects (16-20). For PCBs, it has been determined that hydroxylated metabolites are partially responsible for the endocrine-disrupting effects. For instance, Crews *et al.* showed that different hydroxylated PCBs altered the sexual differentiation of the turtle (21).

The mechanisms by which PHAH-OHs exert their estrogenic effects are still not understood. Binding affinities for the estrogen receptors α and β are relatively low (22,23). It is possible, however, that PHAH-OHs exert part of their estrogenic effects by increasing the bioavailability of E2 through inhibition of E2 inactivation in target tissues. Sulfation by the specific estrogen sulfotransferase SULT1E1 is an important pathway for the inactivation of E2 (24). The human enzyme has a low K_m value of 4 nM for E2, and is expressed in target tissues such as the endometrium, mammary gland and testis, as well as the liver (25-29). Recently, we demonstrated potent inhibition of SULT1E1 by hydroxylated PCB metabolites (30), in particular compounds with two vicinal chlorine substituents around the hydroxyl group. Here, we have investigated the potency and type of inhibition of human SULT1E1 by other important

PHAH-OHs, such as PCDD-OHs, PCDF-OHs, and hydroxylated polybromodiphenylethers (PBDE-OHs), and halogenated bisphenol A (BPA) derivatives, as well as their sulfation by SULT1E1. To determine the relevance of our findings using recombinant enzyme, we also studied the inhibition of E2 sulfation by native SULT1E1 in human liver cytosol (29). Furthermore, we analyzed the sulfation of the various PHAH-OHs by SULT1E1 and SULT1A1, another phenol sulfotransferase abundantly expressed in human liver (31,32).

Materials and Methods

Materials

E2 and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) were obtained from Sigma (St. Louis, MO), [^{3}H]E2 (3.22 MBq/nmol) was obtained from Amersham (Amersham, UK), and [^{35}S]PAPS (52.9 MBq/μmol) was purchased from NEN (Boston, MA). The sources of the various hydroxylated organohalogens have been described previously (23,33,34). Human SULT1E1 (35) and SULT1A1 (31) were expressed in *S. typhimurium* as previously described (36,37). Cytosolic fractions were prepared and used without further purification (36,37). SULT1E1 accounted for 5-7% of the cytosolic proteins. Similar results were obtained using recombinant SULT1E1 expressed in *E. coli* and purified as previously described (38). Normal human liver was obtained at surgical resection of liver tumors, and cytosol was prepared as previously described (39). Approval was obtained from the Medical Ethical Committee of the Erasmus University Medical Center.

Estrogen sulfotransferase assay

Estrogen sulfotransferase activity was analyzed by measuring the formation of water-soluble [^{3}H]E2 sulfate after incubation of 1 nM [^{3}H]E2 for 30 min at 37 C with recombinant SULT1E1 (0.1 μg total cytosolic protein/ml) in the presence or absence (blanks) of 50 μM of the cofactor PAPS in 0.2 ml 0.1 M sodium phosphate (pH 7.2), 2 mM EDTA and 1 mM dithiothreitol. The reactions were stopped by addition of 2 ml ice-cold water, and unreacted [^{3}H]E2 was removed by extraction with 2 ml dichloromethane. Sulfate formation was quantified by liquid scintillation counting of 1 ml of the aqueous phase. Enzymatic sulfation was corrected for background radioactivity estimated in the blanks. Inhibition of E2 sulfation by PHAH-OHs was assessed by addition of 0.01 nM-10 μM of these compounds to the reaction mixtures. Kinetic parameters for E2 sulfation were determined by Lineweaver-Burk analysis (40) of the sulfation of varying substrate concentrations. Apparent K_i values for inhibitors were calculated from the change in slope of the Lineweaver-Burk plot in the presence of a fixed inhibitor concentration (40).

Sulfation of PHAH-OHs

The above assay of estrogen sulfotransferase activity is based on the sulfation of limited concentrations of radioactive E2 by excess unlabeled PAPS. Since radioactive PHAH-OHs are not available, their sulfation was analyzed in comparison with E2 by determining the transfer of $^{35}SO_3^-$ from [^{35}S]PAPS to an excess of unlabeled substrate. Sulfation of PHAH-OHs by SULT1E1 was compared with their sulfation by the human phenol sulfotransferase SULT1A1. Assay mixtures contained 1 µM PHAH-OH or E2, 0.3 µM [^{35}S]PAPS and 15 µg (recombinant SULT1E1) or 100 µg (recombinant SULT1A1) of total cytosolic protein/ml in a total volume of 150 µl 10 mM potassium phosphate (pH 7.4), and were incubated for 30 min at 37 C. The formation of sulfated products was analyzed using the $BaSO_4$ precipitation method of Foldes and Meek (41) as well as by HPLC. In the former method, unreacted [^{35}S]PAPS and protein were precipitated by successive addition of 200 µl 0.1 M barium acetate, 200 µl 0.1 M barium hydroxide, and 200 µl 0.1 M zinc sulfate. The presumably soluble sulfated products were quantified by liquid scintillation counting of 500 µl of the supernatant. Sulfation was corrected for background radioactivity determined in blanks without substrate. For HPLC analysis, the reactions were stopped by the addition of 150 µl ice-cold methanol. After centrifugation, 100 µl of the supernatant were injected onto a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters Chromatography BV, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 50 mM triethyl ammonium acetate (pH 6.8) at a flow of 1.0 ml/min. The proportion of acetonitrile was increased linearly from 35% to 65% in 15 min and further to 90% in an additional 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Results and Discussion

Structure-activity relationship of SULT1E1 inhibition by PHAH-OHs

We previously showed that various PCB-OHs potently inhibit E2 sulfation by human SULT1E1 (30). This finding suggested that endocrine-disrupting chemicals may act by increasing the bioavailability of hormones through inhibition of hormone-conjugating enzymes in target tissues (30,42). In this study we tested the effects of 0.01 nM-10 µM of different classes of PHAH-OHs on the sulfation of 1 nM E2 by SULT1E1. Figure 1 presents the core structures of the tested compounds, and their exact structural formulas are listed in Table 1. Figure 2 shows the concentration-dependent inhibition of SULT1E1 activity by subsets of the various types of PHAH-OHs, *i.e.* PCDD-OH (Fig. 2A), PCDF-OH (Fig. 2B), PBDE-OH (Fig. 2C), and BPA (Fig. 2D) derivatives. From these concentration-inhibition curves the concentrations causing

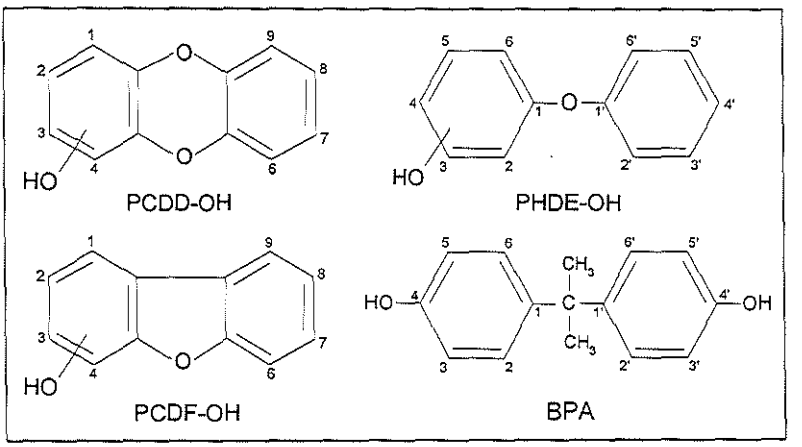

Fig. 1. Core structures of hydroxylated polychlorinated dibenzo-*p*-dioxins (PCDD-OH), polychlorinated dibenzofurans (PCDF-OH), polyhalogenated diphenylethers (PHDE-OH) and bisphenol A (BPA). The position of the hydroxyl group varies between the different PHAH-OHs. Halogen substituents are not indicated.

50% inhibition (IC_{50} values) were determined. Table 1 presents the IC_{50} values and relative potencies compared to the cognate substrate E2 for all compounds tested.

Figure 2A shows the results with PCDD-OHs having the hydroxyl group in position 2, and the same 7,8-dichloro substitution pattern in the nonphenolic ring. Increasing potencies were observed as the number of chlorine substituents surrounding the hydroxyl group increased from 0 to 2, with mean IC_{50} values of 300 nM for 2-OH-7,8-DiCDD, 30 nM for 2-OH-3,7,8-TrCDD and 4 nM for 2-OH-1,3,7,8-TeCDD (Table 1). Figure 2B presents the concentration-inhibition relationships for PCDF-OHs with the hydroxyl group in the 2 or 3 position, *para* or *meta* to the furan oxygen, respectively. Again, the potency increased with increasing number of chlorine substituents adjacent to the hydroxyl group. Furthermore, 3-OH-PCDFs appeared to be slightly more potent inhibitors than 2-OH-PCDFs possessing comparable chlorine substitution patterns. Of all the PCDF-OHs tested, 3-OH-2,4,7,8,9-PeCDF was the most potent inhibitor of E2 sulfation. With a mean IC_{50} value of as low as 0.18 nM this compound has a >30-fold higher affinity for SULT1E1 than the natural substrate E2 (Table 1). As demonstrated in Fig. 2C, all PBDE-OHs tested were relatively weak inhibitors of E2 sulfation by SULT1E1, with IC_{50} values >200 nM (Table 1). Also, the one hydroxylated, polychlorodiphenylether tested, 2-OH-4,2',4'-TrCDE, only inhibited SULT1E1 activity at micromolar concentrations (Table 1). BPA did not affect E2 sulfation at concentrations <1 µM, whereas its derivatives having halogens at all

positions vicinal to the two hydroxyl groups, 3,5,3',5'-TeCBPA and 3,5,3',5'-TeBBPA, were relatively potent inhibitors, with mean IC_{50} values of 40 and 20 nM, respectively (Fig. 2D, Table 1).

Table 1. Potency of inhibition of human SULT1E1 activity by PHAH-OHs

Compound	Code	IC_{50} (nM)	Relative potency
E2		3.8 - 7.1	1
2-hydroxy-7,8-dichlorodibenzo-p-dioxin	2-OH-7,8-DiCDD	200 - 390	0.02
2-hydroxy-3,7,8-trichlorodibenzo-p-dioxin	2-OH-3,7,8-TrCDD	28 - 40	0.17
2-hydroxy-1,3,7,8-tetrachlorodibenzo-p-dioxin	2-OH-1,3,7,8-TeCDD	2.4 - 6.1	1.4
4-hydroxy-1,3,6,7-tetrachlorodibenzofuran	4-OH-1,3,6,7-TeCDF	6.6 - 6.7	0.84
3-hydroxy-2,6,7,8-tetrachlorodibenzofuran	3-OH-2,6,7,8-TeCDF	5.6 - 9.1	0.76
3-hydroxy-2,4,7,8-tetrachlorodibenzofuran	3-OH-2,4,7,8-TeCDF	0.68 - 2.2	4.0
3-hydroxy-2,4,7,8,9-pentachlorodibenzofuran	3-OH-2,4,7,8,9-PeCDF	0.16 - 0.20	31
2-hydroxy-7,8-dichlorodibenzofuran	2-OH-7,8-DiCDF	230 - 560	0.02
2-hydroxy-6,7,8-trichlorodibenzofuran	2-OH-6,7,8-TrCDF	350 - 800	0.01
2-hydroxy-1,3,7,8-tetrachlorodibenzofuran	2-OH-1,3,7,8-TeCDF	5.6 - 6.2	0.97
1-hydroxy-2,4,7,8-tetrachlorodibenzofuran	1-OH-2,4,7,8-TeCDF	240 - 280	0.02
4-hydroxy-2',3,4',5,6'-pentabromodiphenylether	4-OH-3,5,2',4',6'-PeBDE	200 - 240	0.03
4-hydroxy-2',3,4',6'-tetrabromodiphenylether	4-OH-3,2',4',6'-TeBDE	>1000	<0.01
4-hydroxy-2',4',6'-tribromodiphenylether	4-OH-2',4',6'-TrBDE	780 - >1000	<0.01
2-hydroxy-2',4,4'-trichlorodiphenylether	2-OH-4,2',4'-TrCDE	850 - >1000	<0.01
4,4'-isopropylidenediphenol (bisphenol A)	BPA	>10000	<0.001
3,3',5,5'-tetrachlorobisphenol A	3,5,3',5'-TeCBPA	29 - 53	0.15
3,3',5,5'-tetrabromobisphenol A	3,5,3',5'-TeBBPA	12 - 33	0.30

IC_{50} values are presented as the range of values from 2-4 experiments.
Relative potencies are calculated as ratio of the IC_{50} value of E2 over that of inhibitor.

These results demonstrate that, in all classes of PHAH-OHs tested, those with divicinal halogen substituents around the OH group are the most potent inhibitors of E2 sulfation by SULT1E1, which is in agreement with the structure-activity relationship found for SULT1E1 inhibition by single-ring halogenated phenols and hydroxylated PCBs (30). A possible explanation for the increase in potency by vicinal halogen substitutions is the increased dissociation of the OH group. The potency of inhibitors with this substitution pattern decreases in the order PCDF-OH > PCDD-OH > BPA > PBDE-OH derivatives. The planar structures of PCDD-OHs and PCDF-OHs *versus* the non-planar structures of BPAs and PBDE-OHs may play a role in this, since the patterns for inhibition of SULT1E1 by PCB-OHs suggested preferred binding of coplanar compounds to the enzyme (30). This finding fits with the primarily

planar structure of the natural ligand E2. Recently, the crystal structure of the mouse estrogen sulfotransferase has been elucidated (43). Mouse and human SULT1E1 show 77% amino acid sequence identity; both orthologous enzymes have K_m values in the nanomolar range (25,44). The modeling of hydroxylated PHAHs in E2 binding sites of mouse SULT1E1, or human SULT1E1, when its crystal structure also becomes available, should further our understanding of the structural requirements for inhibition of SULT1E1.

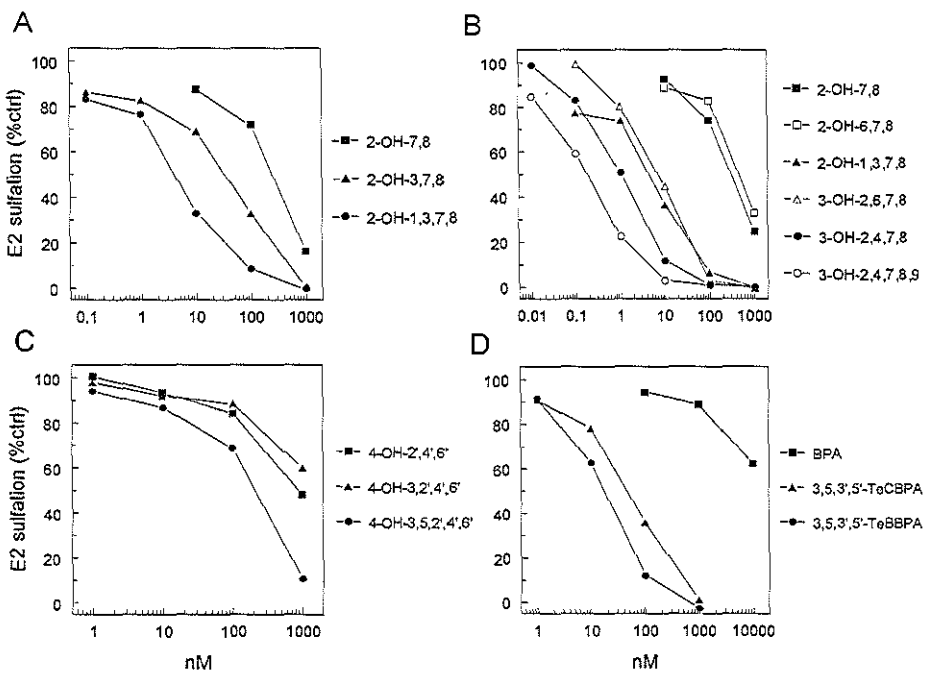

Fig. 2. Inhibition of E2 sulfation by recombinant human SULT1E1 by (A) 0.1-1000 nM PCDD-OHs, (B) 0.01-1000 nM PCDF-OHs, (C) 1-1000 nM PBDE-OHs, or (D) 1-10000 nM BPA or halogenated BPA. Reaction conditions: 1 nM [^{3}H]E2, 0.1 µg of total cytosolic protein/ml, 50 µM PAPS, and 30 min incubation. Results are the means of 2-4 experiments; the coefficient of variation was less than 20 %.

Regarding the varying potencies of the different groups of (hydroxylated) PHAHs, it should be noted that levels of exposure are also different for the various PHAHs. The plasticizer BPA is commonly used in consumer products. Microgram amounts were found in the liquid from vegetable cans with plastic linings; TBBPA and TCBPA are high production volume chemicals which are widely used in consumer electronics and many other products as flame retardants (45). A mean total PBDE level of 5.4 pmol/g lipid weight has been measured in human serum (46). Koistinen *et al.* found that PCDE levels in human adipose tissue range between 2 and 8

ng/g lipid weight; concentrations of PCDD and PCDF congeners in the same human samples varied from <2 to 7700 pg/g lipid weight (47).

Kinetics of SULT1E1 inhibition by PHAH-OHs

The type of inhibition of E2 sulfation by the different PHAH-OHs was studied by Lineweaver-Burk analysis (40) (Fig. 3). Figure 3A shows the double-reciprocal plots of the sulfation rate *versus* the E2 concentration in the presence or absence of a fixed concentration of different PCDD-OHs and PCDF-OHs. Depending on the structure, different types of inhibition were observed. Addition of 2-OH-7,8-DiCDD or 2-OH-7,8-DiCDF changed the slope but had little effect on the y-axis intercept of these plots, indicative of competitive inhibition. However, 2-OH-1,3,7,8-TeCDD and 3-OH-2,4,7,8,9-PeCDF affected both the slope and the y-axis intercept, and the plots converged at about the same point on the x-axis, indicating primarily noncompetitive inhibition by these potent inhibitors. Figure 3B shows the Lineweaver-Burk analysis of the effects of BPA, 3,5,3',5'-TeCBPA and 4-OH-3,5,2',4',6'-PeBDE, indicating that they inhibit E2 sulfation primarily in a noncompetitive manner. The K_i values derived from these plots are in good agreement with the corresponding IC_{50} values for the different inhibitors, amounting to 60 nM for 2-OH-7,8-DiCDD, 2 nM for 2-OH-1,3,7,8-TeCDD, 270 nM for 2-OH-7,8-DiCDF and 0.15 nM for 3-OH-2,4,7,8,9-PeCDF, and to 150 nM for 4-OH-3,5,2',4',6'-PeBDPE, 14 µM for BPA and 35 nM for 3,5,3',5'-TeCBPA.

SULT1E1 is known to have two substrate-binding sites, the active site as well as an allosteric site (24,48). Binding of E2 to the latter site is thought to be largely responsible for the phenomenon of substrate inhibition that is observed at increasing E2 concentrations. The primarily competitive or noncompetitive nature by which the different PHAH-OHs inhibit the sulfation of E2 by SULT1E1 may thus be explained by their preferential affinity for the active or allosteric site, respectively.

Sulfation of PHAH-OHs by SULT1E1 and SULT1A1

Binding of PHAH-OHs to the active site of SULT1E1 is likely to result in their sulfation, which was tested directly by incubating 1 µM of the various compounds with 0.3 µM [^{35}S]PAPS and SULT1E1. These experiments also included different PCB-OHs that have previously been shown to inhibit E2 sulfation by SULT1E1 (30). Product formation was analyzed by the method of Foldes and Meek (41) which involves the precipitation of remaining [^{35}S]PAPS with $BaSO_4$, presumably leaving the radioactive sulfated products in solution. The results of this established procedure were compared with a method developed in our laboratory based on the separation of labeled PAPS and sulfated products by HPLC. Figure 4A shows the HPLC analysis of the

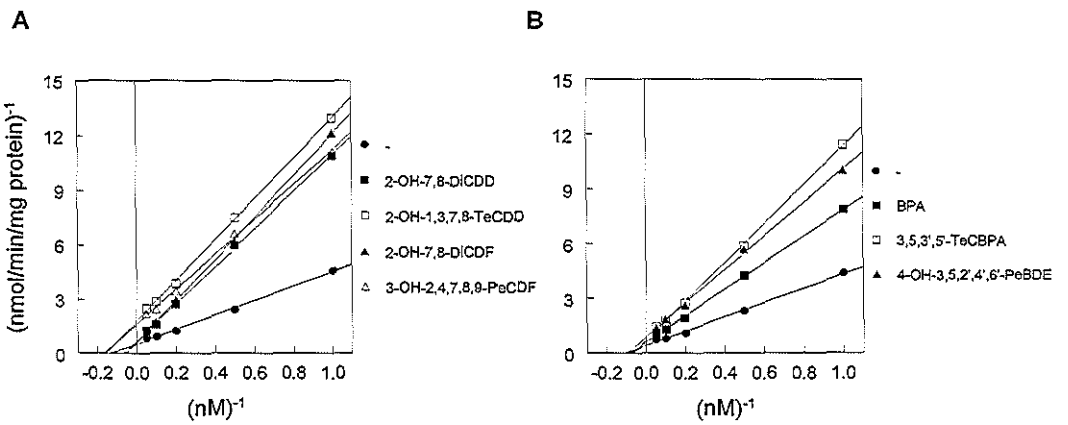

Fig. 3. Lineweaver-Burk analysis of the inhibition of the sulfation of 1-20 nM E2 by 0.1 µg/ml recombinant human SULT1E1 by (A) 250 nM 2-OH-7,8-DiCDD, 4 nM 2-OH-1,3,7,8-TeCDD, 500 nM 2-OH-7,8-DiCDF or 0.2 nM 3-OH-2,4,7,8,9-PeCDF, or (B) 12 µM BPA, 60 nM 3,5,3',5'-TeCBPA or 200 nM 4-OH-3,5,2',4',6'-PeBDE. Results are the means of 2-4 experiments.

sulfation of 2-OH-7,8-DiCDF as a representative example, demonstrating the clear separation between remaining [^{35}S]PAPS and ^{35}S-labeled sulfated product. Figure 4B shows that there was reasonable agreement between the results of the two methods regarding the sulfation of most compounds, although in several instances the $BaSO_4$ precipitation method significantly underestimated the formation of sulfated products in comparison with HPLC analysis. This was especially the case with sulfated compounds that were strongly retarded on the C18 column, suggesting that relatively nonpolar sulfates are partially lost in the $BaSO_4$ precipitation method.

Table 2 shows the results of the HPLC measurements of the sulfation of the various PHAH-OHs by SULT1E1 in comparison with the sulfation of these compounds by the human phenol sulfotransferase SULT1A1. Estrogenic chemicals such as alkylphenols, diethylstilbestrol and BPA have recently been shown to be substrates for human SULT1A1 (49,50). We found that most PHAH-OHs tested in this study were sulfated by SULT1E1 as well as by SULT1A1 (Table 2). It should be noted, however, that these incubations had to be performed with a limited PAPS concentration and excess substrate, which is very different from the conditions used to test the effects of PHAH-OHs on the sulfation of E2 by SULT1E1. Sulfation of the different PHAH-OHs was tested at a single substrate concentration of 1 µM, although the IC_{50} and K_i values for their inhibition of E2 sulfation ranged from <1 nM to >10 µM. Therefore, the findings presented in Table 2 are not representative for the rate of sulfation of the different PHAH-OHs by SULT1E1 under the conditions where they were tested as inhibitors of E2

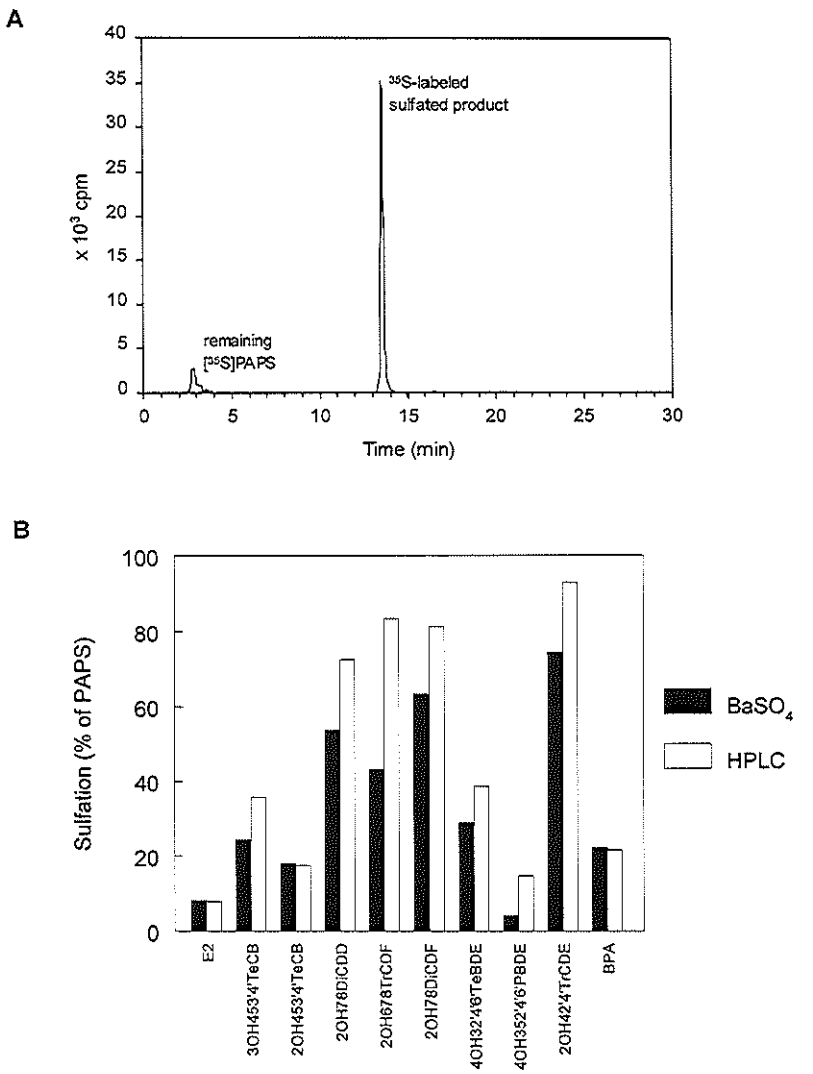

Fig. 4. (A) HPLC analysis of the sulfation of 2-OH-2,7,8-TeCDF by SULT1E1 using [^{35}S]PAPS. (B) Sulfation of PHAH-OHs by SULT1E1 measured by the $BaSO_4$ precipitation method and by HPLC analysis. Reaction conditions: 1 µM substrate, 15 (SULT1E1) or 100 (SULT1A1) µg of total cytosolic protein/ml, 0.3 µM [^{35}S]PAPS, 30 min incubation. Results are the means of triplicate determinations from a representative experiment.

Table 2. Sulfation of PHAH-OHs by human SULT1E1 and SULT1A1

Compound	Sulfation (% of PAPS added)	
	SULT1E1	SULT1A1
4-OH-3,3',4'-TrCB	9.9 ± 1.0	19.3 ± 1.8
4-OH-2,3,5,3',4'-PeCB	0.6 ± 0.8	0.7 ± 1.0
4-OH-3,2',3',4',5'-PeCB	17.6 ± 2.0	6.6 ± 3.1
3-OH-4,5,3',4'-TeCB	33.4 ± 3.3	10.5 ± 3.2
2-OH-4,5,3',4'-TeCB	19.4 ± 2.8	19.0 ± 4.7
4,4'-$(OH)_2$-3,5,3',5'-TeCB	1.0 ± 1.1	18.3 ± 2.8
2-OH-7,8-DiCDD	70.6 ± 2.7	74.1 ± 10.1
2-OH-3,7,8-TrCDD	22.7 ± 9.5	11.4 ± 1.4
2-OH-1,3,7,8-TeCDD	28.2 ± 3.5	31.7 ± 6.9
4-OH-1,3,6,7-TeCDF	6.9 ± 0.3	10.7 ± 1.4
3-OH-2,6,7,8-TeCDF	7.9 ± 1.1	7.3 ± 4.7
3-OH-2,4,7,8-TeCDF	2.5 ± 0.2	3.1 ± 1.1
3-OH-2,4,7,8,9-PeCDF	1.0 ± 0.0	1.0 ± 0.3
2-OH-7,8-DiCDF	82.4 ± 1.7	31.6 ± 4.4
2-OH-6,7,8-TrCDF	81.1 ± 3.1	55.2 ± 8.7
2-OH-1,3,7,8-TeCDF	7.0 ± 1.4	3.3 ± 2.3
1-OH-2,4,7,8-TeCDF	0.0 ± 0.0	24.8 ± 3.4
4-OH-2',4',6'-TrBDE	2.5 ± 1.4	10.7 ± 0.9
4-OH-3,2',4',6'-TeBDE	43.1 ± 6.1	30.4 ± 6.2
4-OH-3,5,2',4',6'-PeBDE	13.3 ± 1.9	0.7 ± 0.7
2-OH-4,2',4'-TrCDE	89.2 ± 5.1	80.5 ± 7.6
BPA	23.6 ± 2.9	62.0 ± 3.2
3,5,3',5'-TeCBPA	7.7 ± 2.5	26.2 ± 1.1
3,5,3',5'-TeBBPA	5.8 ± 0.8	15.9 ± 0.7

Data are presented as the means ± SD from 2-3 experiments.

sulfation. Also, in view of the large variation in saturation of the low-K_m SULT1E1 and the high-K_m SULT1A1 at 1 µM of the various substrates, the data reported in Table 2 are not representative for the substrate preferences of these isoenzymes at lower, more relevant PHAH-OH concentrations. The *in vivo* significance of sulfation of hydroxylated PHAHs in PHAH metabolism remains to be established.

Effects of PHAH-OHs on E2 sulfation by human liver cytosol

We also studied the inhibition of E2 sulfation by various PHAH-OHs using human liver cytosol as a source of native human SULT1E1. Figure 5 compares the effects of different PHAH-OHs (0.1 or 1 µM) on E2 sulfation by SULT1E1 and human liver cytosol, showing a

strong correlation between the inhibitions of the recombinant and native enzymes (r = 0.922). In general, human liver enzyme was less potently inhibited than recombinant human SULT1E1. Although SULT1E1 is the better enzyme for E2 sulfation, with a K_m value of ~4 nM, SULT1A1 also catalyzes E2 sulfation, with a K_m value of 2-5 μM (51). The potencies of inhibition of SULT1E1 differ by orders of magnitude from those by which the different PHAH-OHs inhibit human SULT1A1 activity, which is characterized by apparent K_i values in the micromolar range (33). The non-linear relationship between the rates of E2 sulfation by recombinant SULT1E1 and human liver cytosol in the presence of various PHAH-OHs may be explained by the presence also of SULT1A1 in human liver. However, at nanomolar concentrations, E2 is predominantly sulfated in human liver by SULT1E1 (29), suggesting that the non-linear relationship is not due to significant sulfation of E2 in human liver by SULT1A1. A more likely explanation is suggested by our findings that the PHAH-OHs are sulfated by hSULT1E1 as well as by hSULT1A1. Therefore, compared to recombinant SULT1E1, larger amounts of the PHAH-OHs will be metabolized in human liver cytosol, decreasing their inhibitory effects on native SULT1E1. An additional explanation may be that, compared to recombinant SULT1E1, human liver cytosol contains more proteins to which PHAH-OHs bind, diminishing their availability for native SULT1E1.

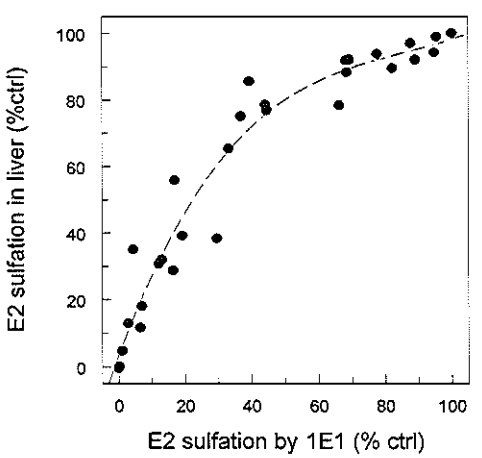

Fig. 5. Sulfation of E2 by human liver cytosol *vs* hSULT1E1 in the presence of different PHAH-OHs. Reaction conditions: 1 nM [^{3}H]E2, 0.1 or 1 μM PHAH-OH, 0.1 (SULT1E1) or 10 (human liver) μg total cytosolic protein/ml, 50 μM PAPS, and 30 min incubation. The PHAH-OHs tested were the PCDD-OHs and PCDF-OHs listed in Table 1 as well as 12 different PCB-OHs (compounds 5, 8, 10, 14, 16, 17, 23, 24, 26, 28, 31 and 32 from Ref. 30). Results are expressed as percentage of control E2 sulfation in the absence of inhibitor, and presented as the means of 2-4 experiments (SULT1E1) or as the means of triplicate determinations from a representative experiment (human liver cytosol).

Concluding remarks

In this study, we have demonstrated that E2 sulfation catalyzed by recombinant human SULT1E1 and by human liver sulfotransferase are both potently inhibited by different classes of PHAH-OHs, with IC_{50} values for recombinant SULT1E1 inhibition in the low- or even sub-nanomolar range. The potent inhibitors 2-OH-3,7,8-TrCDD, 2-OH-1,3,7,8-TeCDD, 3-OH-2,4,7,8-TeCDF and 3-OH-2,4,7,8,9-PeCDF, with IC_{50} values of 34, 4.1, 1.4 and 0.18 nM, respectively, have been identified in mammalian species (20). The most potent inhibitors have an even higher affinity for human SULT1E1 than its cognate substrate E2 which has a K_m value of 4 nM for the enzyme (25). Therefore, we hypothesize that part of the estrogenic activity of PHAHs is explained by an increase in E2 bioavailability through inhibition of human SULT1E1 by hydroxylated PHAH metabolites. Such a mechanism for the pseudo-estrogenic activity of PHAH-OHs is particularly relevant for estrogen-responsive tissues which express SULT1E1, such as the endometrium, mammary gland and testis (26-28). The effects of PHAH-OHs on the regulation of local estrogen levels in these tissues will depend on a variety of factors, such as the supply or local generation of the various PHAH metabolites, their potency in inhibiting SULT1E1, their rate of inactivation by SULT1E1 and other isoenzymes such as SULT1A1, their urinary or biliary excretion rates, and also on the reversal of the sulfation of E2 and the inhibitors by local estrogen sulfatase expression (52).

PHAHs are known also to affect the thyroid hormone system. In laboratory animals, plasma T4 is markedly decreased as a result of competitive binding of the hydroxylated metabolites to the plasma carrier transthyretin and induction of hepatic UDP-glucuronyltransferases by the PHAHs themselves (53). PHAH-OHs have also been reported to inhibit the *in vitro* deiodination of thyroid hormone by the type I iodothyronine deiodinase as well as the sulfation of the hormone by human SULT1A1 (33). During human fetal development, sulfation is an important pathway of thyroid hormone inactivation (54). We have recently demonstrated that human SULT1E1 also efficiently catalyzes the sulfation of iodothyronines, among which the prohormone T4 and the active hormone T3 (25). Inhibition of SULT1E1 may thus also have thyroid hormone-disrupting effects during fetal development. Further studies should determine to what extent estrogen and possibly thyroid hormone levels are disrupted by hydroxylated PHAHs through inhibition of estrogen sulfotransferase.

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Chapter 9

General discussion

Ontogeny of iodothyronine sulfotransferases and deiodinases; importance of thyroid hormone sulfation during fetal development

Thyroid hormone metabolism is one of the mechanisms regulating fetal thyroid status. To test the hypothesis that sulfation is an important reversible pathway of thyroid hormone inactivation during fetal development, we analyzed the expression of the various thyroid hormone metabolizing enzymes in different fetal rat tissues (Chapter 2). In general, D3 activities were high in placenta and fetal brain, and highest D1 activities were found in the liver at late fetal stages, the D1 expression level just before birth being comparable to that of the adult liver. Also for sulfotransferase activities tissue-specific and development stage-dependent patterns were found, but sulfatase-catalyzed iodothyronine sulfate hydrolysis was negligible. These findings suggest, besides the important inner ring deiodination by D3, an additional role for sulfation in the inactivation of thyroid hormone during fetal development. Desulfation seems not to be important in thyroid hormone reactivation in the rat. In the human this situation may be different, since in human fetal serum and amniotic fluid high concentrations of iodothyronine sulfates have been demonstrated (1-5), and besides considerable sulfotransferase activities also significant iodothyronine sulfatase activities were found in fetal human tissues such as the liver (6).

So far, arylsulfatase C (ARSC, also called steroid sulfatase) is the only member of the arylsulfatase family known to catalyze iodothyronine sulfate hydrolysis (Chapter 4). Human ARSC is predominantly expressed in tissues such as placenta, liver and brain (7). In the placenta the enzyme is principally involved in estrogen biosynthesis (8). We characterized iodothyronine sulfatase activities of human ARSC and of human placenta and liver, and demonstrated that ARSC is the main sulfatase for iodothyronine sulfate hydrolysis in the placenta, whereas in the liver, at least in the adult, also other, still unidentified sulfatases are involved.

Iodothyronine sulfation is catalyzed by various cytosolic phenol sulfotransferases, which are located in tissues such as liver, kidney, brain and intestine (9,10). In contrast to the human SULT1A isoenzymes, rat SULT1A1 does not catalyze the sulfation of iodothyronines (11). However, iodothyronines are sulfated by both rat SULT1B1 and 1C1. Since expression of these isoenzymes is low before birth (12-14), whereas considerable iodothyronine sulfotransferase activities are found in fetal tissues such as the liver (Chapter 2), additional isoenzymes seem to be involved in iodothyronine sulfation in the fetal rat, which remain to be identified. Richard et al. studied the ontogeny of the human sulfotransferases hSULT1A1 and 1A3 (6). They found higher hSULT1A1 expression in the fetal than in the postnatal liver and lung. Also in the fetal human brain high hSULT1A1 expression was observed, which was localized in the choroid plexus (6). Highest hSULT1A3 expression was found in the liver early in development, decreasing in the late fetal and early neonatal period (6). The different tissues were also tested for 3,3'-T2 sulfation, and strong correlations between hSULT1A1 expression and 3,3'-T2 sulfation were found, indicating that in these tissues hSULT1A1 is largely responsible for the sulfation of 3,3'-T2 (6). The ontogeny of other human phenolic sulfotransferases catalyzing iodothyronine sulfation remains to be investigated.

In humans, all members of the phenol sulfotransferase family were found to catalyze the sulfation of iodothyronines (15, Chapters 5 and 6). For most of these enzymes 3,3'-T2 is by far the preferred iodothyronine substrate (i.e. 3,3'-T2>>T3~rT3>T4). However, human SULT1E1 equally prefers 3,3'-T2 and rT3 (i.e. 3,3'-T2~rT3>T3~T4). Biochemical characterization of the different sulfotransferases was usually done on crude cytosols of recombinant sulfotransferase-expressing cells (Chapters 3, 5 and 6). When purified recombinant hSULT1A1, 1A3, 1B1 and 1E1 are compared, the receptor-active T3 appears to be sulfated at similar rates by the different isoenzymes (Chapter 6: Fig. 2). Further kinetic analysis of purified enzymes, e.g. hSULT1C1, is necessary to elucidate their relative importance for iodothyronine sulfation.

Although iodothyronines are metabolized importantly by hSULT1E1, it should be noted that the estrogens estrone (E1) and estradiol (E2) are clearly the preferred substrates for this isoenzyme, also called human estrogen sulfotransferase (hEST). K_m values of iodothyronines are in the µM range for the different iodothyronine sulfotransferases, including hSULT1E1, the K_m values of E1 and E2 for hSULT1E1 are around 5 nM (Chapter 6). The higher ratio of sulfated *vs* free estrogens compared to iodothyronines also reflect this enzyme's preference for estrogens (16-18). Nevertheless, given the facile sulfation of iodothyronines by hSULT1E1, in addition to its principal role in reversible estrogen inactivation, a physiological role for the enzyme in thyroid hormone metabolism cannot be excluded.

Is the uterus important in thyroid hormone metabolism during fetal development?

The high D3 activities in the placenta have been demonstrated to limit transplacental passage of maternal T4 and T3 to the fetus, and to contribute to the low T3 and high rT3 concentrations during fetal life (19-21). In addition to the placenta, the role of the uterus in the regulation of thyroid hormone bioactivity during fetal development is intriguing. Nothing is known about expression of D3 in the normal and pregnant human uterus, but it has recently been demonstrated that in the pregnant rat uterus expression of D3 is extremely high (22). During pregnancy, D3 is initially localized at the implantation site in uterine decidual tissue (E9) and later (after E12 and E13) in the single epithelial cell layer lining the uterine lumen (22). Although SULT1E1 expression in reproductive tissues of the pregnant rat is low to undetectable, SULT1E1 is highly expressed in pregnant mouse uterus, localized in the decidua basalis (23). SULT1E1 has also been observed in the human endometrium, where it is up-regulated by progesterone (24,25). Therefore, besides the high D3 activity in placenta and fetal tissues and the iodothyronine sulfotransferase activity in fetal tissues (Chapter 2), also D3 and SULT1E1 in the pregnant uterus may contribute to the low plasma T3 and high plasma rT3 and iodothyronine sulfate levels in the human fetus (Fig. 1). Possibly, the pregnant uterus protects the fetus from excessive thyroid hormone by catalyzing the reversible (SULT1E1) and irreversible (D3) inactivation of the hormone. However, in addition to placental transfer, a role for the uterus in the supply of thyroid hormone (sulfates) from mother to fetus, via the fetal membranes and the amniotic fluid, is not excluded. It is remarkable that the products of thyroid hormone deiodination by D3, i.e. rT3 and 3,3'-T2, are also the preferred substrates for hSULT1E1, suggesting that T4 and T3 are metabolized in the uterus by successive deiodination and sulfation. Since rT3, but not T3, has profound and acute effects on the cytoskeleton in brain cells (26), it is possible that rT3 has an important function in fetal brain development. The production of rT3 sulfate by the uterus may thus be an important means to provide the developing brain with (reversibly inactivated) rT3, from which active rT3 can be formed by action of sulfatases expressed in a tissue-specific and development stage-dependent manner (27). Further studies are needed to explore the role and expression of hSULT1E1 and D3 in the human endometrium throughout gestation.

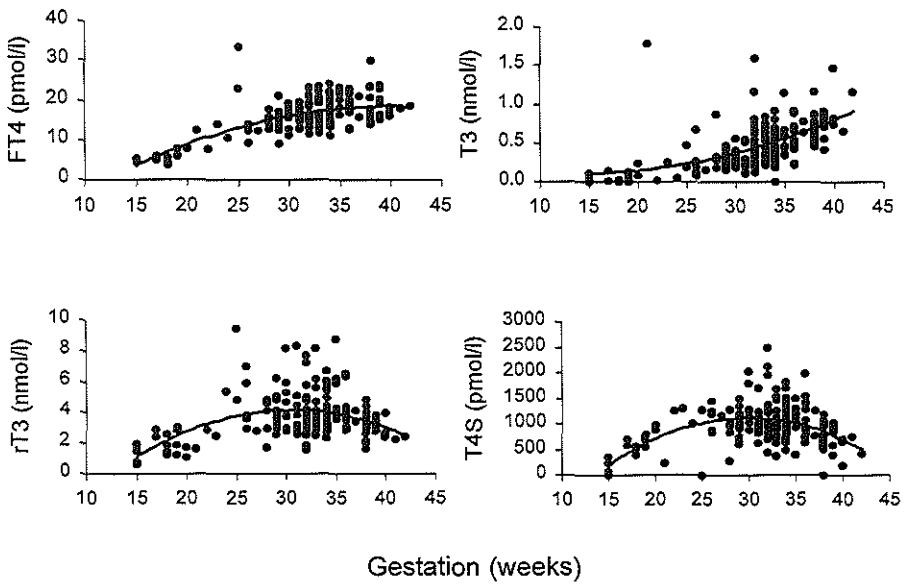

Fig.1. Human fetal serum thyroid hormone levels during gestation (courtesy of Prof.dr. R. Hume).

Importance of type III iodothyronine deiodinase (D3) during fetal development

Apart from sulfation, type III iodothyronine deiodinase (D3) obviously plays an essential role in the regulation of fetal thyroid hormone status. As mentioned before, D3 activity is high in placenta and pregnant uterus, and although no significant D3 activity was found in rat fetal liver (Chapter 2), considerable D3 activity is found in human fetal liver (28). Figure 1 shows human fetal serum levels of FT4, T3, rT3 and T4S throughout gestation. FT4 levels steadily increase during fetal development, until similar levels are reached as in the maternal circulation at the end of gestation. Serum T3 level is very low in the human fetus compared to the adult (mean normal adult level ~2 nM), whereas rT3 and T4S levels are >10-fold higher than in the adult (mean normal adult levels ~0.25 nM and <100 pM, respectively). In contrast to normal adults, in the fetus the rT3 level is higher than the level of T3. This combination of high rT3 versus low T3 has long been considered to be due not only to the high placental D3 activity, but also to a low fetal hepatic D1 activity. Indeed, hepatic D1 activity starts to be expressed in late gestational

stages in the rat (Chapter 2). However, considerable D1 activity (i.e. about 25% of that in the normal adult) is already expressed in the human fetal liver after the first trimester (28). Therefore, in addition to the high D3 activity in placenta and pregnant uterus it is rather the high hepatic D3 activity than a low hepatic D1 activity that may contribute to the low T3 and the high rT3 levels in the human fetal serum. In agreement with this, studies in the embryonic chicken have clearly established a negative correlation between liver D3 expression and serum T3 levels (27,29).

Future studies on iodothyronine deiodinase D3 during fetal development

Future studies are addressed to delineating the role of D3 in the regulation of thyroid hormone bioavailability in the fetus. We will study the ontogeny of D3 activity, protein and mRNA expression in the pregnant uterus, placenta and fetal tissues, such as the fetal brain.

Furthermore, we will use human brain, endometrium and placenta cell lines to study the mechanisms of regulation of D3 expression, and the functionality and tissue-specificity of potential regulatory factors. *In vitro* and *in vivo* studies show tissue-specific regulation of D3 expression by different factors. E.g., D3 activity is induced in cultured astroglial cells and preadipocytes by growth factors, hormones as T3 and retinoids as well as the second messenger cAMP (30-32). Activation of protein kinase C by the phorbol ester TPA also induces D3 activity in astroglial cells (30). *In vivo* studies demonstrated that in rat brain and skin D3 activity is under positive control of thyroid state (33,34), whereas D3 activity in rat placenta is unaffected by hyper- and hypothyroidism (35). In the embryonic chicken an acute pretranslational down-regulation of D3 by dexamethasone and growth hormone was demonstrated in the liver, but not in the brain (27,29).

As, at least in the rat, uterine D3 is up-regulated during pregnancy (22), estrogens and/or progestins may stimulate uterine D3 expression. Although expression levels of D3 in the normal or pregnant human uterus are unknown, we have recently observed high and regulated D3 expression in ECC-1 human endometrial carcinoma cells (36).

Recently, the mouse D3 gene has been cloned and characterized (37). The availability of the human D3 (hD3) gene sequence (BAC clones R-1029J19 and R-796G6) allows us to clone the putative hD3 gene promoter, and study its regulation. The *in vitro* and *in vivo* characterization of the hD3 promoter region, and the identification of functional transcription factor-binding sites therein are essential for understanding the tissue-specific regulation of D3 expression. Again, this is subject of my future research.

Does the potent inhibition of estrogen sulfotransferase by PHAH-OHs *in vitro* contribute to PHAH-related endocrine disruption *in vivo*?

Polyhalogenated aromatic hydrocarbons (PHAHs) are environmental chemicals which are well known for their endocrine disrupting effects. Most PHAHs and their hydroxylated metabolites (PHAH-OHs) show low affinity for both estrogen receptors α and β (38-40). In the studies on the effects of hydroxylated PHAH metabolites on hSULT1E1 reported in the chapters 7 and 8 it was demonstrated that the estrogen sulfotransferase hSULT1E1 is very potently inhibited by various hydroxylated PHAH metabolites *in vitro* (Chapters 7 and 8). This inhibition of hSULT1E1 by PHAH-OHs forms an alternative mechanism explaining the estrogenic effects of PHAHs, based on the increase of receptor-active estrogens, rather than on agonistic estrogen receptor binding (Fig. 2).

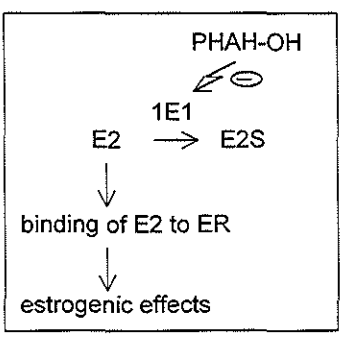

Fig. 2. Putative mechanism explaining pseudo-estrogenic effects of hydroxylated PHAH metabolites.

We also studied the effects of various PHAH-OHs on E2 sulfation by endogenous EST-expressing Ishikawa endometrial carcinoma cells. E2 sulfation was inhibited, but the inhibition was >100-fold less potent than the inhibition of the recombinant hSULT1E1. This difference in potency of the compounds in the different *in vitro* systems may be explained by the only partial cellular uptake of the various compounds. In chapter 8 it was shown that hSULT1A1 and 1E1 both catalyze the sulfation of different hydroxylated PHAHs. The metabolism of the compounds by different enzymes, such as hSULT1A1 and 1E1, present in the Ishikawa cells, forms an additional explanation for the difference in potencies.

We believe that at least part of the estrogenic effects of hydroxylated PHAH metabolites are due to the increase of E2 bioavailability by inhibition of hSULT1E1, also because the PHAH-OHs have very low affinity for the estrogen receptors (38-40). Still, this alternative mechanism remains to be tested in *in vitro* and *in vivo* model systems. In preliminary experiments we did not detect E2-potentiating effects of different PHAH-OHs on alkaline phosphatase activity in the

Ishikawa cell system (unpublished results). However, to increase the sensitivity of the E2 response, potential pseudo-estrogenic effects could also be tested in Ishikawa cells cotransfected with an hSULT1E1-expression vector and an estrogen response element (ERE)-reporter construct. Furthermore, Falany et al. developed a mouse xenograft system in which hSULT1E1-expressing and control MCF-7 tumors are grown in athymic mice. They will use this system to study the *in vivo* significance of hSULT1E1-inhibition by PHAH-OHs. Tumor growth will be determined of hSULT1E1-expressing and control MCF-7 tumors, grown in the athymic nude mice exposed to E2 and PHAH-OHs (C.N. Falany, personal communication).

Apart from disrupting the estrogen system, PHAHs disrupt the thyroid hormone system in several ways (Chapter 1). As we have demonstrated that hSULT1E1 also efficiently sulfates iodothyronines, inhibition of hSULT1E1 by hydroxylated PHAHs might also have thyroid hormone disrupting effects. Further *in vivo* experiments should determine to what extent estrogen and possibly also thyroid hormone levels are disrupted by inhibition of hSULT1E1.

The nitrofen model of congenital diaphragmatic hernia

The administration of the herbicide nitrofen to pregnant rats induces congenital diaphragmatic hernia in the offspring. Recent findings suggest that thyroid hormone as well as vitamin A-disrupting mechanisms contribute to the impaired lung development associated with nitrofen-induced congenital diaphragmatic hernia. Serum thyroid hormone levels are reduced in fetuses of nitrofen-exposed pregnant rats (41,42), and the congenital diaphragmatic hernia-inducing effect of nitrofen is counteracted by administration of thyroid hormone or vitamin A (42,43). In addition, in the Hyt/Hyt mice, which are hypothyroid due to a TSH-receptor mutation, fetal and neonatal lung maturation is delayed (44). Vitamin A deprivation during development also leads to impaired lung growth (45), and compound retinoic acid receptor (RAR) mutant mice show various defects characteristic for fetal vitamin A deprivation, including congenital diaphragmatic hernia (46). Moreover, 50% reduced retinol and retinol-binding protein (RBP) levels are found in human babies with congenital diaphragmatic hernia (47).

As was described before, PHAHs and their hydroxylated metabolites interfere with thyroid hormone-binding proteins such as the thyroid hormone receptors, the sulfotransferases SULT1A1 and 1E1, type I deiodinase, and the plasma transport protein transthyretin (TTR) (48-53, Chapters 7 and 8). Furthermore, PHAHs are known to induce uridine diphosphate (UDP)-glucuronyltransferase expression in tissues such as the liver, thus dramatically increasing the hepatic clearance of thyroid hormone and retinol (48). Regarding thyroid hormone plasma transport, Meerts et al. found that especially polybrominated diphenylethers (PBDEs) potently

inhibit the binding of T4 to TTR. Various PBDEs have an even higher affinity for TTR than T4 (53). Also vitamin A transport is likely to be affected by competitive binding of PHAH-OHs to TTR, as the retinol-RBP complex binds to a specific site on TTR, and retinoic acid binds with a relatively high affinity to the T4 binding site of TTR (54). As a special polyhalogenated diphenylether nitrofen or its metabolites possibly interfere with the thyroid hormone and vitamin A system in a manner similar to other PHAH(-OHs). Because of the strong indications that in the nitrofen-induced congenital diaphragmatic hernia model disruption of the thyroid hormone and vitamin A system contribute to abnormal fetal lung development, future research on the etiology of congenital diaphragmatic hernia should address putative effects of hydroxylated nitrofen metabolites on the various thyroid hormone and vitamin A-regulating mechanisms.

Concluding remarks

Given the detrimental effects of exposure to insufficient or excessive levels of thyroid hormone during fetal development, strictly regulated thyroid hormone levels during fetal development are of crucial importance. The ontogenic patterns of deiodinase, sulfotransferase and sulfatase expression suggest important roles for D3-catalyzed inner ring deiodination and for thyroid hormone sulfation in the regulation of thyroid hormone bioactivity during fetal development (6,28, Chapter 2). Whereas D3 prevents the exposure of fetal tissues to excessive T3, in the human fetus inactivation of thyroid hormone by sulfation seems reversible (6, Chapter 4).

In addition to the placenta, the uterus may also be important for the supply of maternal thyroid hormone to the fetus, since the iodothyronine deiodinase D3 and the estrogen and iodothyronine sulfotransferase hSULT1E1 are expressed in the uterus (Chapter 6, 22,23,25), and thyroid hormone sulfation and inner ring deiodination activities are high in the pregnant uterus (22,55). This putative role for the uterus in the regulation of fetal thyroid hormone status needs to be further investigated.

The potent inhibition of hSULT1E1-catalyzed thyroid hormone and estrogen sulfation by hydroxylated polyhalogenated aromatic hydrocarbons such as PCB-OHs is intriguing (Chapters 7 and 8). These findings suggest that PHAH-OHs induce indirect estrogenic effects, by increasing estradiol bioavailability in target tissues. Because of the possible function of this enzyme in thyroid hormone metabolism, inhibition of hSULT1E1 by these environmental chemicals may also disrupt thyroid hormone levels during fetal development. This potential, novel mode of action of endocrine-disrupting chemicals needs to be investigated in *in vivo* model systems.

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Summary

Strictly regulated thyroid hormone levels are required for normal organ development; a disrupted fetal thyroid hormone status may lead to congenital anomalies. Thyroid hormone metabolism is a key process in the regulation of thyroid hormone homeostasis. The prohormone thyroxine (T4), which is the major secretory product of the thyroid gland, is deiodinated by type I deiodinase (D1) to the receptor-active 3,3',5-triiodothyronine (T3), and by type III deiodinase (D3) to the metabolite reverse T3 (rT3). T3 and rT3 are further metabolized by D1 and D3 to 3,3'-diiodothyronine (T2). Apart from deiodination, iodothyronines are metabolized by glucuronidation or sulfation of the phenolic hydroxyl group. Whereas iodothyronine-glucuronidating uridine diphosphate glucuronyltransferases are not expressed before birth, enzymes catalyzing iodothyronine sulfation (sulfotransferases) are present in fetal tissues such as liver, kidney and brain. We hypothesized that during fetal development sulfation is a reversible thyroid hormone-inactivation pathway. Iodothyronine sulfates might serve as a reservoir of inactive thyroid hormone, from which active T3 is formed by sulfatases in a tissue-specific and development stage-dependent manner.

First, the importance of thyroid hormone sulfation during fetal development was investigated. Different developmental profiles of deiodinase and sulfotransferase activities were found in the various tissues. However, no sulfatase activities were detected in rat tissues, in contrast to fetal human tissues such as the liver, where significant sulfatase activities were found. Furthermore, unlike the elevated iodothyronine sulfate levels in fetal human serum, iodothyronine sulfate levels in fetal rat serum at gestational age E20 were very low. We concluded from these studies that reversible iodothyronine sulfation is not important in the fetal rat (Chapter 2).

The characterization of sulfatase activities in human and rat liver and placenta is described in chapter 4. In general, sulfatase activities in adult human liver and placenta were higher than in adult rat liver and placenta. Human steroid sulfatase arylsulfatase C (ARSC) is the only arylsulfatase known to catalyze iodothyronine sulfate hydrolysis. By comparing sulfatase activities in human liver and placenta with activities in homogenates of ARSC-expressing V79 cells, it was demonstrated that ARSC is the main enzyme in iodothyronine sulfate hydrolysis in human placenta; in the liver additional sulfatases seem to be involved.

To further assess the importance of thyroid hormone sulfation, we identified and characterized the sulfotransferases involved in iodothyronine sulfation. Whereas the rat phenol sulfotransferases rSULT1A1 and 1E1 and hydroxysteroid sulfotransferases rSULT2A1, 2A2

and 2A3 failed to catalyze iodothyronine sulfation, the phenol sulfotransferases rSULT1B1 and 1C1 catalyzed sulfation of iodothyronines in the order 3,3'-T2 >> T3 ~ rT3 > T4 (Chapter 3). We compared K_m values and substrate specificities for rat liver, kidney and brain cytosol with those for rSULT1B1 and 1C1, and found that rSULT1B1 and 1C1 may be responsible for iodothyronine sulfation in rat liver and kidney, whereas other sulfotransferases are involved in iodothyronine sulfation in rat brain (Chapter 3). In the studies described in chapters 5 and 6 it was found that all human phenol sulfotransferases, including the estrogen sulfotransferase hSULT1E1, catalyze iodothyronine sulfation. Interestingly, although estrone and estradiol are clearly the preferred substrates for hSULT1E1, rT3 and T4 are sulfated much better by this isoenzyme than by any other iodothyronine sulfotransferase. Whereas hSULT1E1 equally prefers 3,3'-T2 and rT3, hSULT1A1, 1A3 and 1B1 prefer 3,3'-T2 over rT3, T3 and T4.

It is intriguing that high levels of D3 expression have recently been demonstrated in the pregnant rat uterus, and also hSULT1E1 is expressed in the uterus. D3 catalyzes the deiodination of T4 and T3 to rT3 and 3,3'-T2, respectively. Since these products are the preferred substrates for hSULT1E1, T4 and T3 are possibly metabolized in the uterus by successive deiodination and sulfation. As iodothyronine sulfate levels are high in the fetal serum and amniotic fluid, the uterus may serve as an alternative pathway for the supply of thyroid hormone sulfates from mother to fetus. The possible role of the uterus in the regulation of fetal thyroid hormone status needs further research.

PCBs and other polyhalogenated aromatic hydrocarbons (PHAHs) are known for their endocrine-disrupting effects. To assess the potential role of hydroxylated metabolites of these chemicals in disruption of thyroid hormone and estrogen sulfation, we studied the effects of a wide range of PHAH-OHs, on E2 and iodothyronine sulfation by human SULT1E1 (Chapters 7 and 8). We found that hSULT1E1 is very potently inhibited by various hydroxylated PHAH metabolites. This inhibition of hSULT1E1 by PHAH-OHs forms an alternative mechanism explaining the estrogenic effects of these compounds, based on the increase of estrogen concentrations, rather than on agonistic estrogen receptor binding. The significance *in vivo* of inhibition of hSULT1E1 in estrogen and thyroid hormone disruption remains to be investigated.

In conclusion, apart from the important inner ring deiodination by D3, sulfation is a regulatory pathway of thyroid hormone metabolism during fetal development. We identified different iodothyronine sulfotransferases catalyzing the sulfation of thyroid hormones in humans and in the rat, and found that thyroid hormone and estrogen sulfation are potently inhibited by various PHAH-OHs.

Samenvatting

Nauwkeuring gereguleerde schildklierhormoonspiegels zijn vereist voor een normale orgaanontwikkeling; een verstoorde schildklierhormoonstatus zou kunnen leiden tot aangeboren afwijkingen. Het schildklierhormoonmetabolisme is een belangrijk mechanisme in de regulatie van schildklierhormoonhomeostase. Het prohormoon thyroxine (T4), dat het belangrijkste uitscheidingsprodukt van de schildklier is, wordt gedejodeerd door type I dejodase D1 naar het receptor-aktieve 3,3',5-trijodothyronine (T3) en door type III dejodase (D3) naar de metaboliet reverse T3 (rT3). T3 en rT3 worden verder gemetaboliseerd door glucuronidering of sulfatering van de fenolische hydroxylgroep. Jodothyronine-glucuroniderende uridinedifosfaat-glucuronyltransferases komen niet tot expressie voor de geboorte, enzymen die sulfatering katalyseren (sulfotransferases) zijn daarentegen aanwezig in foetale weefsels zoals lever, nier en hersenen. Een te onderzoeken hypothese was dat gedurende de foetale ontwikkeling sulfatering een reversibele route van inaktivering van schildklierhormoon is. Jodothyronine-sulfaten zouden kunnen dienen als reservoir van inaktief schildklierhormoon, waaruit aktief T3 wordt gevormd op een manier, die weefselspecifiek is en afhankelijk van het ontwikkelingsstadium.

Allereerst werd het belang van schildklierhormoonsulfatering gedurende de foetale ontwikkeling onderzocht. Verschillende ontwikkelingspatronen van dejodase- en sulfotransferase aktiviteiten werden gevonden in de verschillende weefsels. Er werden echter geen sulfatase aktiviteiten waargenomen in rattenweefsels, in tegenstelling tot foetale humane weefsels zoals de lever, waarin significante sulfatase aktiviteiten zijn gevonden. Bovendien waren jodothyroninesulfaatspiegels in rattenserum op foetale leeftijd E20 (twee dagen voor de geboorte) erg laag, in tegenstelling tot de verhoogde jodothyroninesulfaatspiegels in foetaal humaan serum. We concludeerden uit deze studies dat reversibele jodothyroninesulfatering niet belangrijk is in de foetale rat (Hoofdstuk 2).

De karakterisering van sulfatase aktiviteiten in humane en rattenlever en -placenta is beschreven in hoofdstuk 4. In het algemeen waren sulfatase aktiviteiten in volwassen humane lever en -placenta hoger dan in volwassen rattenlever en -placenta. Humane steroidsulfatase arylsulfatase C (ARSC) is het enige sulfatase waarvan bekend is dat het jodothyroninesulfaat-hydrolyse katalyseert. Door sulfatase aktiviteiten in humane lever en placenta te vergelijken met aktiviteiten in homogenaten van ARSC-expresserende V79-cellen, werd aangetoond dat ARSC het belangrijkste enzym is in jodothyroninesulfaathydrolyse in humane placenta, terwijl in de lever ook andere sulfatases een rol lijken te spelen.

Om het belang van schildklierhormoonsulfatering verder uit te zoeken hebben we de sulfotransferases die betrokken zijn bij jodothyroninesulfatering geïdentificeerd en gekarakteriseerd. Terwijl de rattenfenolsulfotransferases rSULT1A1 en 1E1 en hydroxysteroid-sulfotransferases rSULT2A1, 2A2 en 2A3 jodothyroninesulfatering niet bleken te katalyseren, katalyseerden de fenolsulfotransferases rSULT1B1 en 1C1 de sulfatering van jodothyronines in de volgorde 3,3'-T2 >> rT3 ~ T3 > T4 (Hoofdstuk 3). We vergeleken schijnbare K_m waarden en substraatspecificiteiten voor rattenlever-, rattennier- en rattenhersencytosol met die voor rSULT1B1 en 1C1, en vonden dat rSULT1B1 en 1C1 verantwoordelijk zouden kunnen zijn voor jodothyroninesulfatering in rattenlever en -nier, terwijl waarschijnlijk andere sulfotransferases betrokken zijn bij jodothyroninesulfatering in rattenhersenen (Hoofdstuk 3). In de studies beschreven in de hoofdstukken 5 en 6 is gevonden dat alle humane fenolsulfotransferases, inclusief de oestrogeensulfotransferase hSULT1E1, jodothyroninesulfatering katalyseren. Hoewel oestron en oestradiol duidelijk de geprefereerde substraten zijn voor hSULT1E1, worden rT3 en T4 veel beter gesulfateerd door dit isoenzym dan door enig ander jodothyroninesulfotransferase. Terwijl hSULT1E1 een voorkeur heeft voor zowel 3,3'-T2 en rT3, prefereren hSULT1A1, 1A3 en 1B1 3,3'-T2 boven rT3, T3 en T4.

Opmerkelijk is dat hoge niveaus van D3-expressie zijn aangetoond in de zwangere rattenuterus, terwijl ook hSULT1E1 tot expressie komt in de uterus. D3 katalyseert de dejodering van T4 en T3 naar respectievelijk rT3 en 3,3'-T2. Aangezien deze produkten de geprefereerde substraten zijn voor hSULT1E1, worden T4 en T3 mogelijk gemetaboliseerd in de uterus door opeenvolgend dejodering en sulfatering. Omdat jodothyroninesulfaatspiegels hoog zijn in foetaal serum en amnionvloeistof, zou de uterus kunnen dienen als een alternatieve route voor de voorziening van schildklierhormoonsulfaten van moeder naar foetus. De mogelijke rol van de uterus in de regulatie van de foetale schildklierhormoonstatus vereist verder onderzoek.

PCBs en andere polygehalogeneerde aromatische koolwaterstoffen (PHAKs) zijn bekend om hun hormoon-verstorende effecten. Om de potentiële rol van gehydroxyleerde metabolieten van deze chemicaliën in de verstoring van schildklierhormoon- en oestrogeensulfatering te onderzoeken, hebben we de effecten van een grote groep PHAK-OHs en verwante stoffen, zoals gehydroxyleerde nitrofen-metabolieten, op E2- en jodothyroninesulfatering door humaan SULT1E1 bestudeerd (Hoofdstukken 7 en 8). We vonden dat hSULT1E1 zeer sterk wordt geremd door verschillende gehydroxyleerde PHAK-metabolieten. De remming van hSULT1E1 door PHAK-OHs vormt een alternatieve verklaring voor de oestrogene effecten van deze stoffen, gebaseerd op de verhoging van concentraties van oestrogenen, in plaats van op agonistische oestrogeenreceptor-binding. Het belang *in vivo* van remming van hSULT1E1 voor

verstoring van oestrogeen- en schildklierhormoonspiegels moet nog onderzocht worden.

Concluderend: naast de belangrijke binnenring-dejodering door D3 is ook sulfatering een regulerende route binnen het schildklierhormoonmetabolisme tijdens de foetale ontwikkeling. We identificeerden verschillende jodothyroninesulfotransferases die de sulfatering van schildklierhormonen bij de mens en de rat katalyseren, en vonden dat schildklierhormoon- en oestrogeensulfatering sterk geremd worden door verschillende gehydroxyleerde PHAK-OHs.

Curriculum Vitae

Monique H.A. Kester werd op 3 april 1973 geboren te Sint-Michielsgestel. Na het behalen van het VWO-diploma aan het RK Gymnasium Beekvliet te Sint-Michielsgestel in 1991, studeerde zij Medische Biologie aan de Universiteit Utrecht. Tijdens de doctoraalfase deed zij onderzoek naar de rol van prostaglandine E2 en zijn second messenger cAMP in de adhesie van neutrofiele granulocyten aan humaan bronchial epitheel, in een bijvakstage op de afdeling Farmacologie van de faculteit Farmacie van de Universiteit Utrecht, onder begeleiding van Dr. P.G.M. Bloemen en Dr. P.A.J. Henricks. Hierna volgde zij haar hoofdvakstage, getiteld 'The cloning of a second thrombin receptor on MEG-01 megakaryoblasts' op de afdeling Hematologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van Dr. G. Van Willigen en Prof.dr. J.W.N. Akkerman. In augustus 1996 behaalde zij het doctoraal examen (met genoegen). Van november 1996 tot en met december 2000 was Monique Kester werkzaam als Assistent in Opleiding bij de afdelingen Inwendige Geneeskunde en Kinderheelkunde van de Erasmus Universiteit Rotterdam. Als onderdeel van het promotieonderzoek werkte zij in oktober en november 1999 als visiting scientist onder begeleiding van Prof.dr. C.N. Falany op de Department of Pharmacology and Toxicology van de University of Alabama at Birmingham, Birmingham, USA. Het in dit proefschrift beschreven onderzoek werd verricht onder begeleiding van Prof.dr.ir. T.J. Visser en Prof.dr. D. Tibboel, en is gesubsidieerd door de Sophia Stichting voor Wetenschappelijk Onderzoek. Vanaf 1 januari 2001 is Monique Kester werkzaam als wetenschappelijk medewerker aan de vakgroep Inwendige Geneeskunde, vanaf 1 juli 2001 als post-doc op dezelfde afdeling op het project 'Importance of type III iodothyronine deiodinase (D3) for human fetal development', waarvoor een NWO fellowship is toegekend.

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