Endocrine Disruptors and the Thyroid Gland—A Combined *in Vitro* and *in Vivo* Analysis of Potential New Biomarkers

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Background: There is growing evidence that, in addition to the reproductive system, the hypothalamic–pituitary–thyroid axis is a target of endocrine-disrupting compounds (EDCs). However, this is not reflected adequately in current screening and assessment procedures for endocrine activity that to date determine only general parameters of thyroid function.

Objective and Methods: We used several *in vitro* and *ex vivo* assays in an attempt to identify suitable biomarkers for antithyroid action testing a selected panel of putative EDCs.

Results: *In vitro* we detected stimulation or inhibition of iodide uptake into FRTL-5 rat thyroid cells, inhibition of thyroid hormone binding to transthyretin, agonistic or antagonistic effects in a thyroid hormone receptor–dependent reporter assay, and inhibition of thyroid peroxidase using a novel assay system based on human recombinant thyroperoxidase that might be suitable for routine screening for potential EDCs. In rats, chronic application of several EDCs led to changes in thyroid morphology, alterations of thyrotropin and thyroid hormone serum levels as well as alterations in peripheral thyroid hormone–regulated end points such as malic enzyme and type I 5′-deiodinase activity.

Conclusions: As the effects of EDCs do not reflect classic mechanisms of hormone-dependent regulation and feedback, we believe multitarget and multimodal actions of EDCs affect the hypothalamic–pituitary–thyroid axis. These complex effects require a diverse approach for screening, evaluation, and risk assessment of potential antithyroid compounds. This approach involves novel *in vitro* or cell-based screening assays in order to assess thyroid hormone synthesis, transport, metabolism, and action as well as *in vivo* assays to measure thyroid hormone–regulated tissue-specific and developmental end points in animals.

Key words: deiodinase, flavonoids, malic enzyme, pituitary, sodium iodide symporter, thyroid gland, thyroid peroxidase, transthyretin, UV filters. *Environ Health Perspect* 115(suppl 1):77–83 (2007). doi:10.1289/ehp.9369 available via *http://dx.doi.org/* [Online 8 June 2007]

Endocrine-disrupting chemicals (EDCs) became the focus of both public and scientific interest when defects in sexual behavior and reproductive ability of wild-living animals were ascribed to their steroid-like or anti-steroid androgenic properties (Colborn et al. 1993). In the aftermath, these observations were reproduced in many laboratory animal models. Whether human reproduction is also affected by the action of EDCs is currently a topic of controversial discussion (Safe 2004; Toppari 2002; Waring and Harris 2005). Furthermore, there is growing evidence that, in addition to the reproductive, other endocrine systems such as the hypothalamus–pituitary–thyroid (HPT) axis may be targets of endocrine disruption. In particular, polyhalogenated phenolic compounds such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), probably because of their structural resemblance to thyroid hormones (Cody et al. 1986), may cause disturbance of thyroid hormone homeostasis, hypothyroidism, thyroid hyperplasia, and neoplasia (Hagmar 2003; Siddiqi et al. 2003), and developmental defects of the central nervous system (CNS) in experimental animals and humans (Koopman-Esseboom et al. 1994; Meerts et al. 2004).

It now has been shown that there may be multiple targets for interference by various EDCs with the complex regulatory network of thyroid hormone synthesis, metabolism, distribution, and action on the various levels of endocrine regulation and feedback control. These targets include thyrotropin receptor (Auf'mkolk et al. 1985a, 1985b; Santini et al. 2003); iodide uptake by the sodium iodide symporter (NIS; Schröder-van der Elst et al. 2004); type I 5′-deiodinase (5′DI) (Auf'mkolk et al. 1986; Ferreira et al. 2002; Schmutzler et al. 2004); transthyretin (TTR) (Köhrle et al. 1988; van den Berg 1990; Yamauchi et al. 2003); thyroid hormone receptor (TR) (Bogazzi et al. 2003; Moriyama et al. 2002); and thyroid hormone-dependent growth of pituitary cells (Ghisari and Bonefeld-Jorgensen 2005). In the brain, PCBs may disrupt normal differentiation regulated by thyroid hormones, although they do not act as thyroid hormone-like ligands (Zoeller 2005). Moreover, complex biological developmental programs controlled by thyroid hormones may be disturbed by EDC action such as metamorphosis in the amphibian *Xenopus laevis* (Kloas 2002). Yet, data on EDCs affecting the HPT axis are comparatively scarce.

Within the CREDO (cluster of research into endocrine disruption in Europe) Cluster, which is part of the Sixth European Union framework program, the EURISKED (multiorganic risk assessment of selected endocrine disruptors) consortium worked on a group of projects assessing multiorganic effects of selected endocrine disruptors. These projects included a focus on the thyroid gland and its target organs. In this article we summarize the first results. Chosen for analysis was an environmentally and nutritionally relevant collection of substances suspected to have endocrine-disrupting activity because of their steroid-like or anti-steroid effects. These substances included genistein as well as glycitein and daidzein (isoflavones from soybean); resveratrol (phytoalexin from grapes); silymarin (flavonol–lignane mixture from the milk thistle); xanthohumol (XN; prenylated chalcone from hops, *Humulus lupulus* L.); 8-prenylnaringenin (8-PN; prenylated flavonone from hops); benzophenone-2, benzophenone-3, 4-methylbenzylidene camphor, and octyl-methoxycinnamate [BP2, BP3, OMC, 4-MBC, respectively; ultraviolet (UV) filters]; F21388 (synthetic, halogenated flavonoid); 4-nonylphenol (4-NP; e.g., emulgator); bisphenol A (BPA; building block for, e.g., polycarbonate plastics); dibutylphthalate (e.g., plasticizer); linuron and procymidon (pesticides); 5α-androstane-3β,17β-diol [adiol; proposed to be an endogenous ligand

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of estrogen receptor β (ER-β)] and 17β-estradiol benzoate (E_2). We show that several of these substances also interfere with multiple targets at various levels of the HPT axis in a tissue-specific manner. These targets included weight and morphology of the thyroid gland, iodide uptake by the NIS, iodide organification by thyroid peroxidase (TPO), binding of the thyroid hormone thyroxine (T_4) to TTR, the metabolism of thyroid hormones by deiodinases, and the action of the biologically active thyroid hormone triiodothyronine (T_3) mediated by TRs functioning as ligand-dependent transcription factors.

Current Protocols for Assessing Effects on the HPT Axis

Assays currently being used to address thyroid-disrupting effects were developed from existing protocols for detecting general toxicologic effects of substances. These assays measure biochemical, clinical, hematologic, histologic, neurologic, behavioral, reproductive, and developmental end points. To address thyroid toxicity, the ability to determine several thyroid-relevant parameters was incorporated into these tests. Examples are the male and female pubertal assays as developed by the U.S. Environmental Protection Agency [Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) 1998] and the enhanced Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (Gelbke et al. 2004). Currently, end points describing thyroid function, that is, serum T_4, T_3, and thyrotropin (TSH) as well as thyroid weight and histology, are being assessed according to these protocols.

An amphibian assay (XEMA) based on the T_3-dependent metamorphosis of tadpoles using the model *X. laevis* has been developed and is now being validated (Degitz et al. 2005; Opitz et al. 2006). This assay examines, for example, tail resorption, forelimb emergence, histologic alterations in the thyroid gland, and TSH expression. No sufficiently developed assay or standardized, validated protocol exists for the assessment of thyroid function in birds or fish nor is there any functional *in vitro* screening test for antithyroid action. Whether EDCs affect thyroid hormone economy and action in invertebrates, which do not have thyroid tissue organized as a gland composed of follicles (amphibians, birds, mammals) or at least single thyroid hormone–producing follicles (fish), has not been determined. An extensive overview of testing protocols used to determine thyroid toxicants is published by the OECD Environment Directorate and is available on the OECD website (BATTELLE 2006).

In Vitro Assays

Iodide uptake. Iodide accumulation in the epithelial cells of the thyroid gland, the thyrocytes, is the first step in thyroid hormone biosynthesis. This process is catalyzed by the NIS, a member of the SGLT-1 (sodium glucose cotransporter type 1) family of sodium-dependent transporters. By coupling the transport of iodide against its gradient to the influx of sodium along its gradient across the cytoplasmic membrane of the thyrocyte, NIS concentrates the trace element iodide by a factor of up to 50-fold compared with serum levels (Dohan et al. 2003).

The influence of EDCs on iodide uptake (Table 1) was analyzed in FRTL-5 cells, a model of normal, nontransformed rat thyrocytes (Weiss et al. 1984). One compound tested was xanthohumol (XN/ Radovic et al. 2005). A time-dependent stimulation of iodide uptake by NIS was observed with nanomolar concentrations of XN. Acute treatment did not have any effect; however, a rise in iodide accumulation was observed after 2 and 3 days of culture in the presence of XN. The increase was maximal (about 50% compared with the untreated control) after 3 days of stimulation with 1 nM XN. Northern blot analysis did not reveal any difference in NIS mRNA transcript levels between control cells and those treated with 0.1 nM–1 μM XN for 3 days, indicating that posttranscriptional events are probably responsible for the effects. Alternatively, there may also be an influence on "auxiliary" proteins such as Na^+/K^+-ATPase, which is responsible for building up the sodium gradient across the thyrocyte membrane. Recently it was reported that several dialkyl phthalates both increase iodide uptake and stimulate NIS promoter activity in FRTL-5 cells (Breous et al. 2005; Wenzel et al. 2005).

In contrast, the soy isoflavone genistein decreased iodide accumulation. After 5 days of incubation with 10 μM genistein, iodide uptake into FTRL-5 cells was reduced to 52% of the untreated control. Iodide uptake was also inhibited, by the UV filters OMC and 4-MBC at concentrations of 0.1 and 1.0 μmol/L and by 4-NP at 10 μmol/L after 5 days in the presence of these EDCs (Schmutzler et al. 2006). No acute effects were observed for any of the substances. Interestingly, *in vivo* administration of low doses of 4-MBC (7–47 mg/kg body weight/day) caused goiter in the treated rats as well as in the F_1 generation of their offspring (Schlumpf et al. 2004). If this is indeed a consequence of reduced thyroid hormone synthesis due to lower iodide availability, this must be clarified in future investigations.

Together, these results indicate that exposure of rat thyrocytes to EDCs in nanomolar to micromolar concentrations may either increase or decrease iodide uptake by NIS after one or two cell cycles, whereas in our experiments, no acute effects were observed.

Iodide organification. The heme protein TPO plays a central role during thyroid hormone synthesis, as it catalyzes all the essential steps involved in this process (Taurog 2005). The oxidation of iodide, the iodination of tyrosyl residues of thyroglobulin (Tg), and the coupling of two iodotyrosyls to give Tg-coupled thyroid hormones T_4 and T_3 which, after hydrolysis, are released by the thyroid gland in response to an appropriate stimulus by the pituitary hormone TSH. Hydrogen peroxide (H_2O_2), generated by the thyrooxidase enzymes ThOX1 and ThOX2, is an essential cosubstrate in this reaction sequence because it serves as a source for oxidative equivalents. TPO is a target for the inhibiting activity of propylthiouracil (PTU) and methimazole (MMI), currently the only antithyroid drugs with known therapeutic relevance for the treatment of hyperthyroidism (Cooper 2005). TPO also seems to be the target of goitrogens from nutritive sources such as the isoflavone

Table 1. Effects of selected EDC tested in thyroid-relevant *in vitro* assays.

Compound	Iodide uptake	TPO activity	TR agonist	TR antagonist	T_4 binding to TTR
4-MBC	↓	—	ND	ND	ND
4-NP	↓	↓	↑	↓	ND
Adiol	↓	—	ND	ND	ND
BPA	—	↓	ND	ND	ND
BP2	—	↓	↑	—	ND
BP3	—	—	↑	—	ND
Daidzein	ND	ND	ND	ND	↓
Dibutylphthalate	—	—	ND	ND	ND
E_2	↓	—	ND	ND	ND
F21388	ND	↓	ND	ND	ND
Genistein	↓	↓	↑	—	↓
Glycitein	ND	ND	ND	ND	↓
Linuron	—	—	↑	—	ND
OMC	↓	—	↑	—	ND
Procymidon	—	—	ND	ND	ND
Resveratrol	—	↓	↑	—	ND
Silymarin	—	↓	↑	—	ND
Xanthohumol	↑	—	ND	ND	ND

Abbreviations: ↑, stimulatory or agonistic effect; ↓, inhibitory or antagonistic effect; —, no effect; ND, not determined.

genistein from soybeans or related environmental or nutritional flavonoids (Cody et al. 1986). It was already known more than four decades ago that feeding infants with soy milk caused goiter in those with inadequate iodine (Hydovitz 1960; Shepard et al. 1960; Van Wyk et al. 1959). As demonstrated later, genistein, an isoflavone component of soy, could be responsible for the pathogenesis of this type of childhood goiter, as this compound, well known for its endocrine activity exerted via interaction with estrogen and other nuclear receptors (Cos et al. 2003; Divi et al. 1997; Ricketts et al. 2005), also inhibits TPO *in vitro* and *in vivo* (Chang and Doerge 2000).

We therefore looked for the effects of other suspected EDCs on TPO (Table 1) using as a source for enzyme activity FTC-238 human thyroid carcinoma cells stably transfected with an expression clone coding for human recombinant (hr) TPO (data not shown). Functional hrTPO was prepared by digitonin extraction of the cell membranes and used in the guaiacol oxidation assay (Hosoya 1963), a classic test for the determination of peroxidase activities. In this system, several suspected EDCs from plant sources inhibited TPO activity, namely genistein (used as a positive control), but also resveratrol, silymarin, and the synthetic flavonoid F21388. Furthermore, the industrial chemicals 4-NP and BPA also inhibited TPO. IC_{50} (median inhibitory concentration) values ranged from 0.83 to 174 µmol/L. 4-MBC, OMC, procymidon, linuron, BP3, 4-NP, E_2, and adiol had no effect. As reported for genistein (Divi et al. 1997), preincubation of TPO in the presence of BP2 or F21388 in combination with H_2O_2, but without the substrate guaiacol, inactivated TPO. This effect, however, was prevented by adequate (micromolar) concentrations of iodide in the preincubation mixture. BP2-treated rats exhibited decreased T_4 and increased TSH serum levels (Jarry et al. 2004). These results indicate that the inhibitory effects of EDCs on TPO may add to and aggravate the consequences of an inadequate iodide supply (Schmutzler et al. 2006).

TR-mediated transcriptional activation. To assess interference with the action of thyroid hormone receptors, an *in vitro* reporter system was established to screen for the binding and transcriptional activity of EDCs. A duplicated thyroid hormone response element of the DR4 type was cloned into the luciferase vector pGL3 upstream of a SV40 promoter. This construct responded to T_3 with a > 50-fold induction of luciferase activity and an EC_{50} (median effective concentration) value of 1.0 nmol/L in the human hepatocarcinoma cell line HepG2. Our data indicate that BP2, BP3, genistein, resveratrol, silymarin, linuron, OMC, and 4-NP behave as TR agonists in this reporter assay. 4-NP also displayed antagonistic activity if co-applied with 1 nM T_3 (Table 1). In our experiments, TR-agonistic or -antagonistic effects are observed at rather high micromolar concentrations compared with those of the natural ligand T_3 acting at picomolar or nanomolar concentrations. Whether they have any physiologic or pathophysiologic relevance must be tested *in vivo* in animal models.

Thyroid hormone distribution. There are three thyroid hormone binding proteins in human plasma: albumin, with high capacity and low affinity; thyroxine-binding globulin (TBG), with low capacity and high affinity; and TTR with an intermediate capacity and affinity (Köhrle 2000). T_4 binding to these distributor proteins, especially to TTR, is known to be highly sensitive for interference by polyhalogenated phenolic compounds or by synthetic flavonoids (Köhrle et al. 1988; van den Berg 1990; Yamauchi et al. 2003). Therefore, the influence of soy isoflavones on T_4 binding to these three distribution proteins was analyzed by nondenaturing polyacrylamide gel electrophoresis direct and binding assays using purified TTR (Radovic et al. 2006). Soy flavonoids competed with thyroid hormone binding to TTR (Table 1) but not to albumin and TBG. Complete displacement of $[^{125}I]T_4$ binding to TTR was observed in human serum incubated with > 10 µM genistein; interference started at approximately 0.1 µM genistein. Glycitein showed decreased and daidzein the lowest displacement potency compared with genistein. $[^{125}I]T_4$ was displaced to albumin in rat and to TBG in human serum. Soy isoflavones also obstruct $[^{125}I]T_4$ binding to TTR in human cerebrospinal fluid. IC_{50} values were 0.07 µM for genistein, 0.2 µM for glycitein, and 1.8 µM for daidzein. Thus, isoflavones might alter free thyroid hormone concentrations, resulting in altered tissue availability, metabolism, renal excretion (Schröder-van der Elst et al. 2003), and, as a consequence, disturbed feedback to the pituitary.

Ex Vivo Assays

Animals. Female Sprague-Dawley rats (Winkelmann, Borchen, Germany) were ovariectomized at 14 weeks of age and afterwards treated for 12 weeks by oral application of the following compounds in a specially prepared rat chow (n = 8–11 animals/group): 2.5 or 12.5 g/kg OMC, 2.5 or 12.5 g/kg 4-MBC, 20 or 80 mg/kg 4-NP, 150 mg/kg adiol and 34.2 mg/kg E_2 as a positive control. Because soy and particularly its flavonoid compound genistein have a major effect on thyroid function, food completely free from soy was compared with food containing soy, alone or in combination with EDC (12.5 g/kg OMC, 12.5 g/kg 4-MBC, 80 mg/kg 4-NP, 150 mg/kg adiol and 34.2 mg/kg E_2). In a second feeding experiment, animals were fed 0.1 or 1 g/kg genistein, 84 or 840 mg/kg resveratrol, 0.126 or 1.26 g/kg 8-PN, 75 or 300 mg/kg adiol and 4.3 and 17.3 mg/kg E_2. After the treatment, animals were sacrificed; organs were removed and frozen in liquid nitrogen and kept at –80°C until use. The animals were treated humanely and with regard for alleviation of suffering, and all studies have been approved by the local University Ethical Committee.

Histology and serum hormone levels. From feeding experiment 1, a small number of thyroid samples (n = 2–3/treatment group) were examined histologically. In the 4-NP–treated animals, a significant decrease in the volume of epithelial compartments resulted in a decrease of the epithelium versus colloid ratio by about 50% (Table 2). We observed effects at both high and low concentrations compared with those of untreated control. 4-NP-treated animals showed a significant increase in both serum T_3 and T_4 levels; however, TSH was not decreased as might have been expected from the morphologic data. The other animals showed no significant alterations in the morphology of their thyroid glands.

In the 4-MBC-treated animals, T_4 serum levels were significantly decreased without

Table 2. Effects of selected EDCs tested in female ovariectomized rats.

					ME		5´DI	
Compound	Histology	tT_4	tT_3	TSH	Liver	Kidney	Liver	Kidney
E_2	—	—	—	—	↑	—	↓	↑
Adiol	—	—	—	—	↑	↑	—	↑
4-Nonylphenol	⇑	↑	↑	—	—	—	—	—
OMC	—	↓	—	—	—	↑	↓	↓
4-MBC	—	↓	↑	↑	—	—	—	↓
Genistein	ND	↑	—	—	↓	—	—	↑
8-PN	ND	—	↑	—	↓	—	—	—
Resveratrol	ND	—	↑	—	↓	—	—	↑
Soy	—	↓	—	—	↓	—	—	↑
Soy + E_2	—	—	—	—	↑	↑	↓	↑
Soy + 4-nonylphenol	—	↓	—	—	—	—	—	↑
Soy + OMC	—	↓	—	—	↑	—	↓	↓
Soy + 4-MBC	—	↓	↓	—	—	—	—	↓

Abbreviations: ⇑, 50% increase in epithelium vs. colloid ratio; ↑, stimulation; ↓, inhibition; —, no or insignificant effect; ND, not determined; tT_3 and tT_4, total T_3 and T_4, respectively.

parallel alterations in T_3 levels, and TSH was significantly increased. This is a combination of symptoms characteristic for a hypothyroid condition. In rats, administration of low doses of 4-MBC caused goiter in the treated rats and in the F_1 generation of their offspring (Schlumpf et al. 2004). If this is a consequence of reduced thyroid hormone synthesis due to lower iodide availability, this must be clarified in future investigations. This might be a consequence of the inhibition of iodide uptake via the NIS as mentioned above, which is compatible with a well-known increase of the T_3/T_4 ratio in the serum of individuals with inadequate iodine.

In the OMC-treated rats, T_4 was also decreased, but there was no significant alteration in T_3 or in TSH levels. In the animals fed soy-containing chow, T_4 levels were reduced compared with those of untreated controls. This might be due to the inhibitory action of genistein and other soy isoflavones on TPO activity. Changes in T_3 and TSH levels were not significant. In feeding experiment 2, total serum T_3 was significantly increased by both low and high concentrations of 8-PN and resveratrol (1.5- and 1.3-fold, respectively). Only small and nonsignificant alterations in serum TSH were observed. As for total T_4 levels, only the 1.15-fold increase elicited by the high genistein concentration was significant. This represents a certain contradiction to feeding experiment 1, where soy-containing chow led to a decrease in total serum T_4. On the other hand, this is in line with data published by Chang and Doerge (2000), who did not observe hypothyroid effects in genistein-treated rats. Also, one has to consider that genistein alone might not be able to mimic all the effects exerted by the various components contained in soy food, as soy also contains other antithyroid isoflavones (glycitein and daidzein) in addition to genistein. Furthermore, soy proteins were reported to have metabolic as well as endocrine effects, probably including the thyroid gland, although it has not been determined if these effects are caused by nonprotein compounds present in the soy protein isolates (Badger et al. 2001; Goldin et al. 2005; Messina and Redmond 2006; Zhuo et al. 2004).

Malic enzyme. One of the best-characterized thyroid hormone-regulated end points is malic enzyme (ME). This protein is expressed at high levels in the liver and is involved in lipid metabolism by providing NADPH for fatty acid biosynthesis (Mariash et al. 1981). In the livers of T_3-treated versus untreated rats, ME mRNA levels were stimulated 15.5-fold and enzyme activities 10.3-fold. In other organs, T_3-triggered stimulation was less pronounced; the increases in mRNA levels and enzyme activities were 1.7- and 2.6-fold in the heart and 1.7- and 3.4-fold in the kidney, respectively (Dozin et al. 1985). T_3-responsive elements were characterized in the ME promoters of several vertebrate species, including rat and human (Gonzalez-Manchon et al. 1997; Petty et al. 1990). We thus tested if ME may be used as an appropriate marker for thyroid hormone–disrupting action.

For the rats examined in feeding experiment 1 described above, basal ME activities were highest in the heart (18.3 μmol NADPH generated per minute and per milligram extract protein) compared with the liver (10.4 μmol NADPH) and the kidney (5.9 μmol NADPH). Among the EDCs tested, 4-NP, OMC, and 4-MBC slightly increased ME activity in the liver up to 1.2-fold, although these differences were not significant. The increases of about 1.5- to 2-fold triggered by E_2, adiol, E_2 plus soy, and OMC plus soy, however, were significant. Only slight differences (up to 1.25-fold) were observed in the kidney; however, they were significant in the case of adiol, OMC, and E_2 plus soy (Kovacs et al. 2004; Schmutzler et al. 2004). In feeding experiment 2, liver ME activity was significantly reduced by genistein, resveratrol, and 8-PN to 76–50% of control values; in kidney, only minimal and nonsignificant alterations were observed. A complete overview over all observed changes in ME activities is given in Table 2.

The most prominent and significant change in ME activity, a 2.3-fold increase, was elicited by E_2, accompanied by an 8-fold increase in mRNA levels as determined by real-time polymerase chain reaction (PCR) (Kovacs et al. 2004). On the other hand, ME enzyme activities did not correlate with serum T_4 levels in the EDC-treated rats. This demonstrates a disadvantage of ME as a biomarker for HPT axis disruption. Although it is one of the best-characterized metabolic end points of T_3 in the liver, there is also E_2-dependent regulation of lipid metabolism, including the activity of ME (Cho and Park 1990; Sissan and Leelamma 1996). This dual responsiveness does not allow discrimination between a possible estrogenic and a T_3-agonistic action of an EDC under investigation. Although this may be without consequences for the general classification of a substance as an EDC that disrupts metabolic end points of hormonal regulation, it may complicate clarifying its mechanism of action with the aim to design other chemicals as adequate substitutes without endocrine-disrupting properties.

Type I 5′-deiodinase. Three deiodinase isoenzymes are involved in thyroid hormone metabolism. Type II 5′-deiodinase activates the "precursor" T_4 to the biologically active T_3 by removing an iodide atom from the 5′ position or the "outer ring," whereas type III 5-deiodinase has an inactivating function as it removes iodide from the 5 position or the "inner ring." Type I 5′-deiodinase can catalyze both of these steps. Hepatic 5′DI is another well-characterized T_3-regulated end point in the liver. The enzyme is stimulated by T_3 and a high carbohydrate diet (Köhrle 2002), and thyroid hormone responsive elements have been described in the 5′ regulatory region of the 5′DI gene (Jakobs et al. 1997; Toyoda et al. 1995). Furthermore, various xenobiotics are known to inhibit hepatic 5′DI activity (Auf'mkolk et al. 1986; Ferreira et al. 2002).

In EDC-treated rats from feeding experiment 1, 5′DI activity was significantly reduced by E_2 and OMC, both alone and in combination with soy-containing food, whereas adiol and 4-MBC also caused a small but insignificant reduction in 5′DI activity (Schmutzler et al. 2004). In feeding experiment 2, genistein and resveratrol induced increases in liver 5′DI activity of about 33 and 59%, whereas 8-PN reduced enzyme activity by 24%; however, none of these changes was significant.

In the kidney (Hamann et al. 2005), there was a significant increase in 5′DI activity triggered by E_2 (about 1.5-fold) in contrast to the liver. OMC and 4-MBC both reduced 5′DI activity significantly (to < 50%). Whereas in the liver the action of OMC and 4-MBC might be interpreted as E_2-like, this is not the case in the kidney, as effects occur in the opposite direction. Some of these findings are compatible with the sex-specific difference of hepatic 5′DI activity, which is higher in male than in female rats (Miyashita et al. 1995; Ogawa et al. 1999). It must also be considered that the accessibility for EDCs may vary in different tissues and cell types. For a summary of these and other changes in 5′DI activities triggered by the various EDC, see Table 2.

Parameters of Thyroid Hormone Action in the Heart

As the heart is the organ that shows the most sensitive reaction to a hyperthyroid status, we determined several thyroid hormone–regulated end points in this organ using hearts of rats from feeding experiment 1. Effects on ME activity were small, with the only significant difference being an approximately 16% reduction in the animals fed with soy-containing food compared with the control (Schmutzler et al. 2004). We also determined the expression of the thyroid hormone–regulated genes coding for myosin heavy chain α and β and glycerol-3-phosphate dehydrogenase α by real-time PCR. However, no effect was detected, indicating that there are no thyromimetic effects of these substances tested in feeding experiment 1

or that they are detectable only at higher concentrations.

EDCs and Iodine Deficiency

The essential trace element iodine plays a central role as a constituent of the thyroid hormones T_4 and its hormonally active form T_3, which controls many aspects of vertebrate physiology, including growth, especially of the skeleton, differentiation and maturation of the central nervous system as well as energy and lipid metabolism, thermoregulation, and heart rate and output. Iodine deficiency and consequent deficiency in thyroid hormones result in goiter. If this shortage is severe during development, this may lead to critical growth defects combined with mental retardation known as cretinism. However, mild or moderate iodine shortage causes significantly lower intelligence quotient values in school children (Santiago-Fernandez et al. 2004; Vermiglio et al. 2004).

According to data from the World Health Organization, in 1999, 30% of the world's population was at risk of iodine deficiency disorders; 740 million people suffered from goiter; 43 million people had iodine deficiency disorder–related brain damage and mental retardation, and 5.7 million patients were afflicted by cretinism (International Council for the Control of Iodine Deficiency Disorders 1999). Iodine deficiency is not necessarily a result of malnutrition found in developing countries but may occur also in industrial countries such as Germany (Andersson et al. 2005). Certain risk groups show a greater tendency to suffer from the consequences of an insufficient iodine supply, including pregnant and breastfeeding women (Ares et al. 2005; Darcan and Goksen 2003), as well as vegans and vegetarians (Borak 2005; Krajcovicova-Kudlackova et al. 2003).

There is evidence for a mutual enhancement of the effects of iodine deficiency and nutritional or environmental goitrogens. Use of soy milk in the diet can lead to the development of (reversible) goiter in iodine-deficient children, probably via the inhibition of TPO by genistein (Hydovitz 1960; Van Wyk et al. 1959). Tobacco smoke contains several goitrogenic substances, among them cyanide, which is metabolized to thiocyanate, a potent inhibitor of the NIS. Smoking, as well as increased thiocyanate levels, is associated with an increased thyroid volume; with a higher risk for goiter development (Brauer et al. 2006; Galanti et al. 2005; Völzke et al. 2005); with higher prevalence, increased severity, and poorer outcome of Hashimoto's thyroiditis; and with a higher prevalence of thyroid multinodularity (Ericsson and Lindgarde 1991; Knudsen et al. 2002). Additional sources for goitrogenic cyanide may be industrial pollution (Brauer et al. 2006) or consumption of cyanogenic vegetables (Biassoni et al. 1998; Chandra et al. 2004; Laurberg et al. 2002).

These literature data suggest that an increased burden of EDCs with effects on the HPT axis might, similar to goitrogens, aggravate adverse effects of inadequate iodine supply or exacerbate thyroid disease status.

Summary and Conclusions

Our results, which are summarized in Tables 1 and 2, show that the HPT axis is a relevant target of EDC action, which has to be considered in forthcoming screening and assessment protocols that test for endocrine activity. Furthermore, effects of EDCs on the HPT axis must be considered in connection with special dependency of the thyroid gland on the essential trace element iodine. There may be an enormous capacity of the thyroid gland to adapt to adverse effects of EDCs, but probably only as long as the iodine supply is adequate. If this is not the case, as iodine deficiency still occurs in many parts of the world, a further challenge by EDCs in addition to their own effects may unmask or aggravate effects elicited by iodine deficiency.

We detected interference by EDCs on several levels of the HPT axis. However, they did not conform to classic "textbook" mechanisms of endocrine regulation and feedback. *a*) Hormone levels did not show "concerted" alterations, that is, lower T_4 was not always accompanied by higher TSH, for example, in the case of 4-NP and OMC. *b*) Activities of T_3-sensitive enzymes did not always correlate with concentrations of circulating thyroid hormone levels, as although 4-NP-treatment increased T_4 levels, there was no parallel increase in 5′DI or ME activity in the liver or in the kidney of treated rats. Although total T_4 serum levels did not change significantly and total T_3 levels were even increased, ME activity was reduced by up to 59% and 5′DI activity was slightly but nonsignificantly decreased by resveratrol in the liver. *c*) Thyroid histology did not necessarily reflect hormone levels, as only effects of 4-NP on morphology were observed, although 4-MBC also altered T_4 levels. *d*) Furthermore, biomarkers such as ME and 5′DI reacted to EDC action with tissue-specific effects. Thus, thyroid function, thyroid hormone action in the periphery, and feedback regulation could be disrupted at the same time and effects would occurr in an organ-specific manner. The reason for this disruption, apart from a different availability of certain EDCs to the various tissues, might be that one compound affects more than one target in the HPT axis or even more than one endocrine axis. A striking example is genistein, which on one hand inhibits TPO enzyme activity and thyroid hormone binding to TTR and on the other hand also displays estrogenic and antiestrogenic effects by interacting with ERβ. Additionally, estrogenic action of a compound may enhance or mask an antithyroid action. Taken together, there seems to be synergistic as well as antagonistic interference at several levels of thyroid hormone synthesis, action, and regulation.

These complex actions also imply that current testing protocols may not be adequate, as they focus on parameters of thyroid function such as serum TSH, T_4, and T_3 as well as thyroid weight and morphology (EDSTAC 1998; Gelbke et al. 2004). Assessment of the function of thyroid hormones and their availability to organs, considering cell type- and tissue-specific effects, is also required. Our data on TPO-inhibition by certain EDCs strongly suggest that substances that are easily oxidized will inhibit TPO and should, therefore, be tested accordingly. Given the strong effect of thyroid hormones on growth and development, especially on the differentiation and maturation of the central nervous system, developmental end points also should be included. This inclusion is further stressed by the recent finding that EDCs can act in a transgenerational manner by epigenetic modification of genes (Anway et al. 2005). Furthermore, as shown for body weight regulation and obesity but might also apply to other endocrine axes, the set points for endocrine axes are modulated by the maternal "environment" of the fetus (Plagemann 2005); this process could be disturbed by the influence of EDCs. Thus, novel assays that evaluate tissue and cell typical biomarkers of thyroid hormone and EDC action in development and maintenance of healthy organisms are urgently needed. Our data suggest some possibilities, and future experiments will show if they can be developed into sensitive, significant, robust and efficient screening and risk assessment assays.

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