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**Associations between CB-153 and p,p'-DDE and hormone levels
in serum in middle-aged and elderly men**

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Abstract

Background: Animal and epidemiologic data indicate that exposure to persistent organochlorine pollutants (POPs) may disrupt the hypothalamus-pituitary-thyroid (HPT) and the hypothalamus-pituitary-gonadal (HPG) axes. We have assessed whether the POP-biomarkers 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethene (p,p'-DDE) affect thyrotropin (TSH), thyroid hormones, gonadotropins or sex hormone concentrations in men.

Methods: Lipid adjusted serum concentrations of CB-153, and p,p'-DDE, were determined in 196 men (median age 59 years, range 48-82). Hormone analyses in serum were performed with immunoassays. The effect of CB-153 and p,p'-DDE (as continuous or categorized variables) were evaluated by linear regression models, adjusting for potential confounders.

Results: There was a significant positive association between p,p'-DDE and TSH. An increase of 100 ng/g lipid of p,p'-DDE corresponded to an increase of 0.03 mU/L (95% Confidence Interval [CI] 0.01, 0.05) in TSH level. The explanatory value (R^2) of the multivariate model was only 7 %. Moreover, there was a significant negative association between p,p'-DDE and estradiol. An increase of 100 ng/g lipid of p,p'-DDE corresponded to a decrease of 0.57 pmol/L (95% CI -1.0, -0.12) in estradiol level. The R^2 -value was only 4 %. No associations were observed between any of the POP biomarkers and the other hormones.

Conclusions: The positive association between p,p'-DDE and TSH and the negative association between p,p'-DDE and estradiol, among middle-aged and elderly men, were not accompanied by associations between the POP-markers and thyroxin, testosterone, and gonadotropins, respectively. The results gives some additional support for that POP exposure may affect HPT- and HPG-axes also in humans, but the overall epidemiological data are still not coherent enough to allow any firm conclusions.

1. Introduction

Persistent organochlorine pollutants (POP) such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans (dioxins), polychlorinated biphenyls (PCB), and 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethene (p,p'-DDE), which is the major metabolite of DDT, are detected in tissues from almost every individual in Western societies. Some POPs have been claimed to possess endocrine-disrupting potency (Peterson et al., 1993, Brouwer et al., 1995, Brouwer et al., 1998), with a direct relevance for e.g. neurodevelopment and reproductive function. The interest has mainly been directed towards possible disruption of the hypothalamus-pituitary-thyroid (HPT) and the hypothalamus-pituitary-gonadal (HPG) axes. We will in this paper focus on possible effects of POPs on hormone levels in middle-aged and elderly human males.

Animal data show that POPs may directly interfere with the thyroid gland, with thyroid hormone metabolizing enzymes, and with the plasma transport system of thyroid hormones (Brouwer et al., 1998). In rats and monkeys, dioxins and PCB cause a reduction of plasma thyroxin concentrations and a concomitant increase in thyrotropin (TSH) concentrations (Bastomsky 1977; Brewster et al., 1988; van den Berg et al., 1988; Ness et al., 1993).

Previous epidemiological studies give an ambiguous picture of whether POP exposure might affect human thyroid hormone homeostasis (cf Hagmar et al., 2003). The strongest data supporting such an association came from studies on school children showing increased TSH levels (Osius et al., 1999; Schell et al., 2004), while the results for infants and adults were more contradictory.

In animal studies POP exposure has caused disruption of the HPG-axis. Exposure to rats with the anti-androgenic compound p,p-DDE led to reduced testosterone production, while serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were

increased, indicating an impairment of the steroid negative feedback (Ben Rhouma et al., 2001).

Environmental exposure to POPs has been shown to adversely interfere with the sexual maturation of boys during the foetal and pubertal stages (Den Hond et al 2002). However, data on the effect of POP exposure on the HPG-axis in adult men are conflicting. Exposure to both dioxins, DDT/DDE and PCB have in several studies (Egeland et al., 1994; Henriksen et al., 1996; Ayotte et al., 2001; Persky et al., 2001; Martin et al., 2002; Richthoff et al., 2003; Dalvie et al., 2004), but not all (Hagmar et al., 2001a, Cocco et al., 2004; Rignell-Hydbom et al., 2004) been associated with a decrease in testosterone levels, and with an increase of gonadotropins in a single study (Egeland et al., 1994), while no convincing association with gonadotropin levels were observed in any of the other studies (Henriksen et al., 1996; Ayotte et al., 2001; Hagmar et al., 2001; Persky et al., 2001; Richthoff et al., 2003; Dalvie et al., 2004; Cocco et al., 2004; Rignell-Hydbom et al., 2004).

Among malaria vector control workers highly exposed to DDT abnormally high basal estradiol levels and consistent positive associations of these levels with DDT compounds, were observed (Dalvie et al., 2004). Estrone sulphate was inversely and significantly associated with PCBs in serum but only in men with low BMI (Persky et al., 2001). In other studies of adult men no associations between POP exposure and estradiol were observed (Richthoff et al., 2003; Cocco et al., 2004; Rignell-Hydbom et al., 2004).

For the population living in the coastal areas around the Baltic Sea, consumption of locally caught fatty fish is the most important source of exposure for POPs (Svensson et al., 1991; Asplund et al., 1994; Svensson et al., 1995). If a high dietary intake of POPs will result in endocrine disruption, subjects with a high consumption of contaminated fatty fish from the Baltic Sea may constitute a risk population.

We have used 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) as a biomarker for POP exposure, because it correlates very well with both total PCB concentration in plasma and serum (Grimvall et al., 1997; Glynn et al., 2000), with the PCB derived dioxin-like effect as well as the total POP derived dioxin-like effect (Gladen et al., 1999). Another relevant biomarker is the antiandrogenic compound p,p'-DDE, which is still present in relatively high serum concentrations in men consuming fatty fish from the Baltic Sea (Sjödin et al., 2000).

The aim of the study was to assess whether serum levels of CB-153 and p,p'-DDE are associated with TSH, thyroid hormones, gonadotropins or sex hormone concentrations in middle-aged and elderly men.

2. Subjects and methods

2.1. Study population and interview

A postal questionnaire, primarily aimed to assess the fracture incidence, was sent in year 2000 to 1500 fishermen from the Swedish east coast, off the Baltic Sea, born between 1920 and 1954 (Wallin et al., 2004). The fishermen were part of a previously established cohort of Swedish fishermen (Svensson et al., 1995). A fraction of these fishermen were recruited for an assessment of the association between CB-153 and p,p'-DDE in serum and bone mineral density. Details of the recruitment and selection process have been given elsewhere (Wallin et al., in press). The participants were interviewed about their current weight and height and life-long smoking habits. A non-participant analysis showed that those who participated did not differ from those who were initially invited but did not participate with respect to age, body mass index (BMI) and smoking habits (Wallin et al., in press). Totally 196 men with a median age of 59 years (range 48-82) were examined and we have utilized this group for assessing the association between the POP biomarkers and hormone levels in serum. Only one of the men

was currently on Levaxin treatment. No other case of thyroid disease had occurred in the population.

2.2. Blood sampling

Venous blood samples were drawn between 7.30 and 10.00 A.M., after 12 h fasting, into sterile Vacutainer glass tubes. Serum was separated by centrifugation (4000 rpm for 10 min) and transferred to glass bottles and special tubes. All samples were coded and stored frozen at -80°C before they were analyzed.

2.3. Hormone analyses

Follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol and testosterone were measured on an automated immunoassay system with chemiluminescent detection (Modular, Roche Diagnostics, Mannheim, Germany). The coefficients of variations (CV) were 1.8% for FSH at 15 IU/L and 2.1% for LH at 16 IU/L. The CV was 8.5% for estradiol at 227 pmol/L and 6.8% for testosterone at 3.9 nmol/L. TSH and free thyroxin were measured on another automated immunoassay system with chemiluminescent detection (Advia Centaur, Bayer Corp., East Walpole, MA, USA). The CV for TSH was 5.0% at 4 mU/L and for free thyroxin 5.3% at 15 pmol/L. Sex hormone binding globuline (SHBG) was measured on an automated immunoassay system with fluorescence detection (Autodelfia, Wallac Oy, Turku, Finland), showing a CV of 3.3% at 54 nmol/L. All analyses were performed on the routine Clinical Chemistry Laboratory at the University Hospital in Uppsala, Sweden.

2.4. Determination of CB-153 and p, p'-DDE in serum

The serum levels of CB-153 and p, p'-DDE were determined as previously described (Richthoff et al., 2003, Rignell Hydbom et al., 2004). CB-153 and p, p'-DDE were extracted

from serum by solid phase extraction (Isolute ENV+; IST, Hengoed, UK) using on-column degradation of the lipids and analysis by gas chromatography mass spectrometry. $^{13}\text{C}_{12}$ -labeled CB-153 and $^{13}\text{C}_{12}$ -labeled p, p'-DDE were used as internal standards. The relative standard deviations, calculated from samples analyzed in duplicate at different days, was 8% at 0.8 ng/mL and 7% at 2.7 ng/mL for CB-153 and 7% at 1.3 ng/mL and 9% at 7.0 ng/mL for p, p'-DDE. The quantification limits were 0.05 ng/mL for CB-153 and 0.1 ng/mL for p, p'-DDE, and the compounds could be quantified in samples from all subjects. The analyses of CB-153 and p, p'-DDE are part of the Round Robin inter-comparison program (Professor Dr. med. Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg) with analysis results within the tolerance limits.

2.5. Determination of serum lipids by enzymatic methods

Serum concentrations of triglycerides and cholesterol were determined by enzymatic methods using reagents from Boehringer-Mannheim (Mannheim, Germany). The inter-assay CVs for cholesterol and triglyceride determinations were 1.5-2.0%. The average molecular weights of triglycerides were assumed to be 807. For cholesterol we used an average molecular weight of 571, assuming that the proportion of free and esterified cholesterol in plasma was 1:2. Based on a paper by Rylander et al the total lipid concentration in serum (g/L) was calculated by the following equation (Rylander et al., 2006): $\text{Total} = 0.96 + 1.28 * (\text{triglycerides} + \text{cholesterol})$

2.6. Statistics

The effect of the exposure variables CB-153 and p,p'-DDE, respectively, on the outcome variables (given in Table 1) were evaluated by linear regression models. Serum levels of CB-153 and p,p'-DDE correlated strongly ($r=0.64$) and were therefore not included in the models

at the same time. This decision was supported by the fact that when the two exposure measures were divided at the medians, less than 10 % of the individuals had both high p,p'-DDE and low CB-153 concentrations. The exposure variables were treated as continuous variables (untransformed and log transformed) as well as categorized into four groups with equal numbers of subjects in each. The categorized variables were entered as dummy variables in the models. As potential confounders we considered the variables presented in Table 1. These variables were included in the regression models, one at a time, together with the exposure variables. The confounders were kept in the model if the effect estimate (i.e. the β coefficients) was changed more than 15% (i.e. from the univariate effect of the exposure variable on the outcome variable). We did, however, for significant associations also show the crude estimates, i.e. with no confounders in the model. Model assumptions were checked by means of residual analysis. *A priori*, the most obvious confounder was age. In the present data set there were significant correlations between age and CB-153 ($r=0.31$, $p<0.001$) and p,p'-DDE ($r=0.35$, $p<0.001$), respectively. In addition, age was the only variable that fulfilled the inclusion criteria and, accordingly, was kept in the final models.

3. Results

The serum concentrations of CB-153 as well as p,p'-DDE did in the univariate analyses show a positive association with TSH (Table 2). The association was still present for p,p'-DDE, but not for CB-153, when age was taken into account. None of the other potential confounders fulfilled the inclusion criteria. An increase of 100 ng/g lipid of p,p'-DDE corresponded to an increase of 0.03 mU/L (95% CI; 0.01, 0.05) in TSH level. The explanatory value (R^2) of the multivariate model was 7 %. The analysis using categorical p,p'-DDE values did not give any convincing support for a threshold level for effect. The POP markers showed no association with free thyroxin.

Neither CB-153 or p,p'-DDE were in the univariate analyses associated with estradiol. However, when age was included in the models, there was a significant negative association between p,p'-DDE and estradiol level (Table 3). None of the other potential confounders fulfilled the inclusion criteria. An increase of 100 ng/g lipid of p,p'-DDE corresponded to a decrease of 0.57 pmol/L (95% CI -1.0, -0.12) in estradiol level. The R²-value of the multivariate model was 4 %. The analysis using categorical p,p'-DDE values gave some support for an exposure response association and for a threshold level for effect of about 600 ng/g of p,p'-DDE. The association between CB-153 as a continuous variable and estradiol was not significant (p=0.08, Table 3), and the analysis using categorical CB-153 levels did not give any firm support for an exposure-response association.

There were no associations between serum concentrations of CB-153 and p,p'-DDE, respectively, and gonadotropins or total testosterone, SHBG or total testosterone/SHBG ratio.

The model assumptions were not better fulfilled when the exposure variables were log transformed, and we do therefore only present the results for untransformed exposure variables.

4. Discussion

The main results of the present study were the observed positive association between p,p'-DDE and TSH and the negative association between p,p'-DDE and estradiol. However, no associations were observed for free thyroxine, gonadotropins or testosterone. The biological impact of the associations with TSH and estradiol might be considered to be low (measured as explanatory values of only 7 and 4 %, respectively), but the associations are anyhow of mechanistic interest. Due to the high inter-correlation between p,p'-DDE and CB-153 levels in the present cohort, it was not possible to disentangle whether the associations observed for both compounds with estradiol in serum (significant for p,p'-DDE, but

significant for only some exposure categories for CB-153), were due to independent effects of the two POP compounds or the effect of only one of them.

It is always important in cross-sectional studies to be aware of potential selection bias. A comparison between subjects who declined participation, and the participants showed no obvious differences with factors that might affect the hormone levels such as age, BMI, smoking habits, or alcohol consumption. We do therefore not believe that selection bias is of major concern in the present study.

For the biomarkers of exposure, CB-153 and p,p'-DDE, the analytic accuracies and precisions were good. Therefore, we don't consider non-differential misclassification of exposure to be of any importance in the present study.

A thorough and systematic analysis of the effect of including a number of potential confounders in the multivariate models showed that only age was of importance for assessing the impact of the exposure variables. We had information on a number of relevant potential confounders. Therefore, we believe that residual confounding is probably not an issue of great concern. However, we cannot exclude that imperfect measurements of the confounders have caused some residual confounding.

It is in different animal strains clarified that high doses of POPs causes a certain hypothyroidism with reduction of plasma thyroxin concentrations and a concomitant increase in TSH concentrations (Bastomsky 1977; Brewster et al., 1988; van den Berg et al., 1988; Ness et al., 1993). Various mechanisms seem to be involved such as a direct effect on the thyroid gland, effects on thyroid hormone metabolizing enzymes, and on the plasma transport system of thyroid hormones (Brouwer et al., 1998). In humans, with considerably lower POP concentrations in blood from environmental exposure sources, the effects on thyroid hormone homeostasis have not been as obvious or consistent. However, the present positive association between p,p'-DDE and TSH adds to previous

studies showing positive associations between POP exposure and TSH in infants and children (Koopman-Essebom et al., 1994; Nagayama et al., 1997; Osius et al., 1999; Schell et al., 2004). On the other hand, other previous studies on infants and children (Fiolet et al., 1997; Longnecker et al., 2000; Steuerwald et al., 2000; Matsuura et al., 2001) or adults (Koopman-Essebom et al., 1994; Persky et al., 2001; Hagmar et al., 2001a; Hagmar et al., 2001b) did not show any such associations. However, it should be noted that there are no previous study indicating a negative association between POP exposure and TSH (cf Hagmar et al., 2003). We did not observe any concurrent increase in thyroxin levels together with increased TSH levels. This is in conflict with some (Nagayama et al., 1997; Schell et al., 2002) but not other (Koopman-Essebom et al., 1994; Osius et al., 1999) previous studies. The interpretation of human data is more complex than of animal data as congener mixtures, doses and age ranges differs between studies. A direct comparison between current POP concentrations in serum may be misleading, as the age range differs between studies, but it can be noted that the exposure levels in the present study were higher than in all previous studies evaluating the association between POP exposure and thyroid hormone homeostasis, with the exception of the study by Steuerwald et al. on Faroe Island women with comparable exposure levels (2000). Based on data available up to now, it cannot be concluded that POP exposure has affected thyroid homeostasis in humans. On the other hand, available data do not exclude such an association.

Animal data support that at least certain POPs may cause disruption of the HPG-axis (Ben Rhouma et al., 2001), whereas the results from human studies are much more ambiguous. Among South African men highly exposed to DDT, high basal estradiol levels and positive exposure-response associations were observed (Dalvie et al., 2004), whereas in the present group of considerably less p,p'-DDE exposed men the opposite association was

observed. Our findings were more in concordance with that of low exposed US males for whom a negative association between PCB and estrone sulphate in serum was seen (Persky et al., 2001). A caveat with the latter study was, however, that this association did only appear in men with low BMI, which make it possible to be a spurious finding. It should be mentioned that there are other studies of adult men with relatively low POP exposure in which no associations with estradiol levels were observed (Richthoff et al., 2003; Cocco et al., 2004; Rignell-Hydbom et al., 2004). We have no biological explanation to why high DDT exposure should have another biological impact on peripheral estradiol levels than low exposure, except that the ratio DDT/DDE is very high among highly exposed malaria control workers, while it is very low (much more DDE than DDT) among subjects in the Western world mainly exposed through diet. Swedish east coast fishermen, from whom the present study group was recruited, have extremely low DDT/DDE ratios (Hagmar et al., 2001).

We did not find any associations between the POP biomarkers and gonadotropins or testosterone, which adds to the conflicting results in the literature. Occupational, accidental and dietary exposure of adult men to various POPs has in several studies (Egeland et al., 1994; Henriksen et al., 1996; Ayotte et al., 2001, Persky et al., 2001; Martin et al., 2002; Richthoff et al., 2003; Dalvie et al., 2004), but not in all (Hagmar et al., 2001a; Cocco et al., 2004; Rignell-Hydbom et al., 2004), been associated with a decrease in testosterone levels. In a single study, dioxin exposure was associated with an increase of gonadotropins (Egeland et al., 1994), while no convincing association between POPs and gonadotropin levels were observed in any of the other studies (Henriksen et al., 1996; Ayotte et al., 2001; Hagmar et al., 2001; Persky et al., 2001; Richthoff et al., 2003; Cocco et al., 2004; Dalvie et al., 2004; Rignell-Hydbom et al., 2004).

To conclude, we have among middle-aged and elderly men observed a positive association between p,p'-DDE and TSH and a negative association between p,p'-DDE and estradiol, but

no effects on free thyroxin, testosterone or gonadotropins. The results gives some additional support for that POP exposure may affect HPT- and HPG-axes also in humans, but the overall epidemiological data are still not coherent enough to allow any firm conclusions

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Table 1. Distributions of exposure variables, potential confounders and hormone levels in 196 fishermen from the Swedish east coast off the Baltic Sea.

	n	%	Median	(5 th , 95 th perc.)
<i>Exposure variables</i>				
S-CB-153 (ng/g lipid)			370	(110, 1010)
S-p,p'-DDE (ng/g lipid)			580	(110, 2140)
<i>Potential confounders</i>				
Age (yr)			59	(49, 75)
BMI (kg/m ²)			28.3	(23.5-35.5)
Current smoking	39	20		
Cumulative cigarette smoking (pack-years)			13	(0, 65)
Current alcohol consumption (g/month)			250	(0, 1300)
<i>Outcome variables</i>				
S-TSH (mU/L)			1.5	(0.7, 3.7)
S-free thyroxin (pmol/L)			15.6	(12.1, 21.0)
S-FSH (IU/L)			6.1	(2.6, 28.0)
S-LH (IU/L)			5.0	(2.4, 11.7)
S-Estradiol (pmol/L)			99	(65, 142)
S-Total testosterone (nmol/L)			14.9	(7.2, 25)
S-SHBG (nmol/L)			41	(21, 78)
Testosterone/SHBG			0.36	(0.19, 0.58)

Table 2. The effect of serum concentrations of CB-153 and p,p'-DDE, respectively, on TSH in 196 men, obtained from linear regression models. The exposure variables were treated as continuous (estimated effects [β] on TSH by 100 ng/g lipids increase of exposure concentrations) as well as categorized variables. Crude and adjusted effects are showed with 95 % confidence intervals (CI). In addition, the p-values are shown.

Exposure variable	TSH (mU/L)					
	Crude			Adjusted ^a		
	β	95% CI	p	β	95% CI	p
CB-153 (ng/g lipid)						
<i>Continuous</i>						
↑ 100	0.06	(0.01, 0.10)	0.02	0.04	(-0.01, 0.09)	0.10
<i>Categorical</i>						
-220 ^b	Ref			Ref		
>220 – 370	-0.16	(-0.55, 0.24)	0.44	-0.25	(-0.65, 0.15)	0.21
>370 - 560	-0.14	(-0.53, 0.26)	0.48	-0.29	(-0.70, 0.12)	0.16
>560	0.33	(-0.07, 0.72)	0.11	0.14	(-0.28, 0.56)	0.50
p,p'-DDE (ng/g lipid)						
<i>Continuous</i>						
↑ 100	0.04	(0.02, 0.06)	<0.001	0.03	(0.01, 0.05)	0.002 ^c
<i>Categorical</i>						
-300 ^b	Ref			Ref		
>300 – 600	-0.13	(-0.52, 0.26)	0.52	-0.18	(-0.57, 0.21)	0.36
>600 – 1100	-0.20	(-0.59, 183)	0.30	-0.28	(-0.67, 0.11)	0.15
>1100	0.47	(0.08, 0.86)	0.02	0.33	(-0.08, 0.73)	0.12

^a Adjusted for age.

^b Reference category.

^c Explanatory value (R^2) for TSH due to age and p,p'-DDE was 7 %.

Table 3. The effect of serum concentrations of CB-153 and p,p'-DDE, respectively, on estradiol among 196 men, obtained from linear regression models. The exposure variables were treated as continuous (estimated effects [β] on estradiol by 100 ng/g lipids increase of exposure concentrations) as well as categorized variables. Crude and adjusted effects are showed with 95 % confidence intervals (CI). In addition, the p-values are shown.

Exposure variable	Estradiol (pmol/L)					
	Crude			Adjusted ^a		
	β	95% CI	p	β	95% CI	p
CB-153 (ng/g lipid)						
<i>Continuous</i>						
↑ 100	-0.56	(-1.6, 0.50)	0.30	-0.96	(-2.1, 0.13)	0.08
<i>Categorical</i>						
-220 ^b	Ref			Ref		
>220 – 370	-9.1	(-18, -0.28)	0.04	-12	(-20, -2.6)	0.01
>370 - 560	-2.2	(-11, 6.6)	0.62	-5.9	(-15, 3.2)	0.21
>560	-9.9	(-19, -0.99)	0.03	-14	(-24, -5.0)	0.003 ^c
p,p'-DDE (ng/g lipid)						
<i>Continuous</i>						
↑ 100	-0.35	(-0.78, 0.07)	0.10	-0.57	(-1.0, -0.12)	0.01 ^d
<i>Categorical</i>						
-300 ^b	Ref			Ref		
>300 – 600	-2.4	(-11, 6.6)	0.60	-4.0	(-13, 4.9)	0.37
>600 – 1100	-7.8	(-17, 1.0)	0.08	-10	(-19, -1.2)	0.03
>1100	-8.0	(-17, 0.90)	0.08	-12	(-21, -2.8)	0.01

^a Adjusted for age.

^b Reference category.

^c Explanatory value (R^2) for estradiol due to age and CB-153 was 3 %.

^d Explanatory value (R^2) for estradiol due to age and p,p'-DDE was 4 %.