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**"Multi-organic Risk Assessment of
Endocrine Disrupters"**

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1. DEVELOPMENT OF IN VITRO CELLULAR BIOASSAY TO MONITOR THE OESTROGENIC ACTIVITY OF ENVIRONMENTAL ENDOCRINE DISRUPTERS (ED'S)	5
Sophie Bernichtein¹, Heli Salminen^{1,2}, and Ilpo T. Huhtaniemi^{1,2}	5
2. ANTIANDROGENIC EFFECTS IN VITRO AND IN VIVO OF THE FUNGICIDE PROCHLORAZ	6
Anne Marie Vinggaard*, Christine Nellemann*, Kirsten Jarfeldt*, Majken Dalgaard*, Helle Raun Andersen§, Eva Bonfeld-Jørgensen# and Ulla Hass*	6
3. EFFECTS OF ENDOCRINE DISRUPTERS ON SEX STEROID SYNTHESIS AND METABOLISM PATHWAYS IN FISH	7
Rémi Thibaut and Cinta Porte	7
4. IDENTIFICATION OF VERTEBRATE-TYPE STEROID METABOLISM IN INVERTEBRATES	8
Janer G¹, LeBlanc G.A², Porte C¹.	8
5. EVALUATION OF THE SENSITIVITY OF MORPHOLOGICAL ENDPOINTS IN MALE OFFSPRING AFTER PERINATAL EXPOSURE TO ANTIANDROGENS	9
Majken Dalgaard, Thuri Kledal, Anne Marie Vinggaard and Ulla Hass	9
6. A MARKET BASKET APPROACH TO ESTIMATE PP'DDE DIETARY INTAKE BY THE ITALIAN POPULATION DURING 2003	10
Galassi, S. and Ciceri, F.	10
7. ANALYSIS OF ENDOCRINE DISRUPTORS USING LUCIFERASE REPORTER SYSTEMS IN CELL LINES AND PRIMARY CULTURES OF HUMAN OVARIAN SURFACE EPITHELIAL (OSE) CELLS	11
O Gubbay , PT Saunders* and SG Hillier.	11
8. POLYBROMINATED DIPHENYL ETHERS IN BREAST MILK COLLECTED IN THE CZECH REPUBLIC	12
J. Hajšlová, J. Poustka, R. Kazda, T. Cajka	12
9. THE EFFECT OF ENDOCRINE DISRUPTERS ON T3-REGULATED ENDPOINTS IN LIVER, KIDNEY AND HEART IN FEMALE RATS	13
Hamann I¹, Hofmann P¹, Kovacs G^{1,2}, Stemmler L¹, Schmutzler C¹, Jarry H³, Seidlova-Wuttke D³, Wuttke W³ and Köhrle J¹	13

10. POLYBROMINATED DIPHENYL ETHERS IN BREAST MILK COLLECTED IN THE CZECH REPUBLIC	14
R. Kazda, J. Hajšlová, J. Poustka, T. Cajka	14
11. POLYBROMINATED DIPHENYLEETHERS INHIBIT TCDD-INDUCED EROD-ACTIVITY IN CARP HEPATOCYTES	15
R.V. Kuiper^{1,2,3}, Å. Bergman⁴, J.G. Vos^{2,3} and M. van den Berg¹.	15
12. POLAR BEAR CASE STUDY	16
Elisabeth Lie^{1,2}, Hans Jørgen S. Larsen², Erik Ropstad², Irma Oskam², Stig Larsen², Andrew E. Derocher³, Nick Lunn⁴, Ross Norstrom⁵, Øystein Wiig⁶, Bjørn M. Jenssen⁷, Janneche Utne Skaare^{1,2}	16
13. SOY ISOFLAVONES INFLUENCE THYROID HORMONE SYNTHESIS AND TRANSPORT	17
Branislav Radovic, Birgit Mentrup, Cornelia Schmutzler and Josef Köhrle	17
14. THE GERMAN ENVIRONMENTAL SPECIMEN BANK AS A TOOL FOR THE RETROSPECTIVE MONITORING OF EXPOSURE (AND EFFECTS?) OF ENDOCRINE DISRUPTERS	18
Christa Schröter-Kermani	18
15. BROMINATED FLAME RETARDANTS (BFRS) IN THE HIGH ARTIC MARINE FOOD CHAIN	19
Eugen G. Sørmo^{1*}, Maria P. Salmer¹, Kine Bæk², Janneche U. Skaare² & Bjørn M. Jenssen¹	19
16. TESTOSTERONE CONJUGATING ACTIVITIES IN INVERTEBRATES: ARE THEY TARGETS FOR ENDOCRINE DISRUPTORS?	20
Janer G¹., LeBlanc G.A²., Porte C¹.	20
17. EFFECT OF ORAL INTAKE OF DIBUTYLPHthalate ON THE METABOLISM OF ADULT LONG EVANS RATS.	21
Castillo C., Salazar V., Ariznavarreta C., Tresguerres JAF.	21
18. EFFECT OF ORAL INTAKE OF DIBUTYLPHthalate ON REPRODUCTIVE PARAMETERS OF LONG EVANS RATS AND PRE-PUBERTAL DEVELOPMENT OF THEIR OFFSPRING.	22
Salazar V., Castillo C., Ariznavarreta C., Tresguerres JAF.	22

19. EFFECTS OF BROMINATED FLAME RETARDANTS ON THE ACTIVITY OF THE STEROIDOGENIC ENZYME AROMATASE (CYP19) IN H295R HUMAN ADRENOCORTICAL CARCINOMA CELLS IN CULTURE 23

Rocío F.Cantón¹, Thomas Sanderson¹, Robert Letcher², Åke Bergman³ and Martin van den Berg¹. 23

20. DIFFERENTIAL EFFECTS OF BENZOPHENONE-2 (BP2), BISPHENOL-A (BPA) AND DIBUTYLPHTHALATE (DBP) IN THE UTERUS, VAGINA AND THE METAPHYSIS OF THE TIBIA: COMPARISON WITH CHRONIC E₂ TREATMENT IN OVX RATS 24

D. Seidlová-Wuttke, W. Wuttke 24

21. EFFECTS OF ESTRADIOL, GENISTEIN AND RESVERATROL ON THE HYPOTHALAMO-PITUITARY-GONADAL (HPG) AND HYPOTHALAMO-PITUITARY-THYROID (HPT) AXES UNDER ACUTE AND LONG TERM TREATMENT 25

J. Christoffel, G. Rimoldi, H. Jarry, W. Wuttke 25

22. ORGAN-SELECTIVE EFFECTS OF ESTRADIOL (E2), BENZOPHENONE-2 (BP2) AND BENZOPHENONE-3 (BP3) ON THE EXPRESSION PATTERN OF THE ARYL HYDROCARBON RECEPTOR (AHR) AND THE ESTROGEN RECEPTORS (ER) 26

H. Klammer, C. Schlecht, H. Jarry, W. Wuttke 26

23. EFFECTS OF ESTRADIOL (E2), BENZOPHENONE-2 (BP2) AND BENZOPHENONE-3 (BP3) ON THE TISSUE-SPECIFIC EXPRESSION OF THE ESTROGEN RECEPTOR RELATED RECEPTOR 1 (ERR1). 27

C. Schlecht, H. Klammer, H. Jarry, W. Wuttke 27

24. HISTOLOGICAL FINDINGS ON UTERUS AND MAMMARY GLAND OF RATS TREATED WITH THE PHYTOESTROGENS GENISTEIN AND RESVERATOL 28

G. Rimoldi, J. Christoffel, W. Wuttke 28

25. PERSISTENT AND PERVASIVE TISSUE-SPECIFIC ACTION OF ORGANOCHLORINE COMPOUNDS IN ESTROGEN-REPORTER MALE MICE 29

Villa R*, Bonett E*, Penza ML*, Ganzerla S*, Biasiotto G°, Iacobello D*, Bugari G*, Apostoli P§, Caimi L^, Ciana P#, Maggi A', Di Lorenzo D*

1. DEVELOPMENT OF IN VITRO CELLULAR BIOASSAY TO MONITOR THE OESTROGENIC ACTIVITY OF ENVIRONMENTAL ENDOCRINE DISRUPTERS (ED'S)

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The ovarian hormone oestrogen (E) plays a well-known role in the establishment and maintenance of reproductive functions in both females and males, via specific receptors (R), related as ER α and ER β . These receptors are not only found in reproductive organs but also in most other cells of the organism. Therefore, the EDs, which can bind to ER, do not only interfere with the reproductive system but can also putatively act in ubiquitous manner, and must be considered to exert non-reproductive effects yet to be explored.

In the present study our final aim is to develop strong and sensitive tools such as genetically modified animal models and cell lines enabling to monitor reproductive as well as non-reproductive oestrogenic bioactivity of different EDs. We have first generated different gene constructs leading to ubiquitous oestrogen-regulated gene expression of a reporter transgene both in vitro and in vivo models. The construct consists of the luciferase reporter gene driven by a consensus oestrogen responsive element (2xERE) linked to a thymidine kinase (tk) minimal promoter. This construct (ERE-tk-luc) was then transiently co-transfected in vitro with either ER α or ER β in 293 HEK cells, and luciferase activity induced by several EDs was monitored. The next step is the development of cell lines stably co-transfected with both ER and ERE-tk-luc genes. The first results on ED effects on ER activation in these cell lines will be presented.

2. ANTIANDROGENIC EFFECTS *IN VITRO* AND *IN VIVO* OF THE FUNGICIDE PROCHLORAZ

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Chemicals with antiandrogenic action are suspected of being involved in adverse effects on human male reproductive health giving rise to poor semen quality, malformed sex organs and testis cancer. The fungicide prochloraz that is commonly used within horticulture and agriculture was dosed orally (200 mg/kg/day) for 7 days to both 70-days old intact and to 42-days old testosterone-treated (0.5 mg/kg/day) castrated male Wistar rats. Flutamide was included as a positive control. Body weights were unaffected, whereas liver weights increased for both intact and castrated prochloraz-treated animals. In the intact prochloraz-treated males the absolute and relative weight of the seminal vesicles were significantly decreased (by approx. 30%) and the LH level was significantly increased (by 75%). Prochloraz exposure to castrated testosterone-treated males gave rise to statistically significant reductions in absolute and relative weights of the following tissues: ventral prostate (46%), seminal vesicles (37%), musc. levator ani/bulbocavernosus (31%), and bulbourethral gland (35%). These effects were accompanied by an increase in LH (440%) and by decreased levels of ornithin decarboxylase mRNA (52%) and PBP C3 (41 %) in the prostate as determined by real-time PCR using LightCycler technology. However, no effects were observed on TRPM-II mRNA. Antiandrogenic effects *in vitro* were tested in a reporter gene assay based on transient co-transfection of CHO cells with a plasmid encoding for the human androgen receptor and an MMTV-luciferase plasmid. A weak inhibition of the R1881-induced response was observed (IC₅₀ 3.6 µM). These results point to the conclusion that prochloraz antagonizes the peripheral androgen receptors resulting in decreased growth of androgen-dependent tissues and that it antagonizes central androgen receptors blocking the negative feed-back mechanism of testosterone resulting in increased LH secretion from the pituitary. To further study the antiandrogenic effects of prochloraz, an *in utero* animal experiment is in progress. Pregnant rats were gavaged with 30 mg/kg prochloraz during gestation and lactation and results show that prochloraz reduces testosterone levels in male fetal testis and causes nipple formation in the male offspring. These results further point to the conclusion that prochloraz is a newly identified antiandrogenic pesticide.

3. EFFECTS OF ENDOCRINE DISRUPTERS ON SEX STEROID SYNTHESIS AND METABOLISM PATHWAYS IN FISH

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The interaction of estrogenic (nonylphenol, dicofol, atrazin), androgenic (organotins, phthalates, fenarimol), and anti-androgenic compounds (vinclozolin, diuron, *pp'*-DDE) with key enzymatic activities involved in both synthesis and metabolism of sex hormones was investigated in-vitro by looking at androstenedione testicular metabolism (17 β -hydroxysteroid dehydrogenase -17 β -HSD- and 5 α -reductase activities), ovarian synthesis of maturation-inducing hormone (20 α - and 20 β -hydroxysteroid dehydrogenase activities), and glucuronidation and sulfation pathways. Carp testicular microsomal fractions incubated in the presence of different xenobiotics evidenced higher sensitivity of 5 α -reductase than 17 β -HSD to different chemicals. Dicofol, organotins, and phthalates were among the most effective inhibitors. In contrast, 20 α - and 20 β -HSD activities were enhanced by nonylphenol (NP), dicofol, fenarimol and *pp'*-DDE. Metabolic clearance pathways of hormones were also affected. Fenarimol, nonylphenol, and TPT inhibited the glucuronidation of testosterone and estradiol at a concentration as low as 10, 50 and 100 μ M, respectively. TPT, TBT, and NP were also inhibitors of estradiol sulfation, with IC₅₀ values of 17, 18, and 41 μ M, respectively. Overall, the data indicates the interaction of selected chemicals with key enzymatic pathways involved in both synthesis and metabolism of sex hormones. This interference might be one of the underlying mechanisms for the reported hormonal disrupting properties of the tested compounds, and might finally affect physiological processes, such as gamete growth and maturation.

4. IDENTIFICATION OF VERTEBRATE-TYPE STEROID METABOLISM IN INVERTEBRATES

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Investigations into the steroid metabolic pathways of three invertebrate species, the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus*, demonstrated the capacity of all three species to convert androstenedione into testosterone, and a variety of 5 α -reduced androgens, including 5 α -androstenedione, 5 α -androstane-3 β ,17 β -diol, and 5 α -dihydrotestosterone. The synthesis of those steroids varies with sex (*M. cornuarietis*) and tissue (*P. lividus*), suggesting that they are involved in the reproductive process. Inter-species differences were also evidenced. Thus, androstenedione was primarily converted to 5 α -androstenedione in *M. cornuarietis*, while it was metabolized to testosterone in *P. lividus* and *H. azteca*. The activation of testosterone to DHT was only observed in gonads of *P. lividus*, whereas in digestive tube the inactivation to 4-androstene-3 β ,17 β -diol was recorded. Overall, the work reports on the existence of 17 β -, 3 β -hydroxysteroid dehydrogenase, and 5 α -reductase catalyzed reactions in invertebrates, evidences similarities and differences with vertebrate studies, and contributes to the better knowledge of invertebrate endocrinology.

5. EVALUATION OF THE SENSITIVITY OF MORPHOLOGICAL ENDPOINTS IN MALE OFFSPRING AFTER PERINATAL EXPOSURE TO ANTIANDROGENS

Majken Dalgaard, Thuri Kledal, Anne Marie Vinggaard and Ulla Hass

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The dose-response relationships of single compounds were investigated in male Wistar rats used in our lab in order to select dose levels for mixture-studies of similarly acting antiandrogens.

Dams were dosed from gestational day 7 to postnatal day (PND) 16 with 6 doses of either vinclozolin, or flutamide, or 2 doses of prochloraz. In male offspring, anogenital distance (AGD) was measured, nipples/areolas were counted, the degree of external genitalia-malformation was scored, and reproductive organs were weighted. The well-known effects of flutamide and vinclozolin were reproduced in our Wistar rats. Prochloraz, a herbicide used within agri- and horticulture, changed AGD and nipple retention, increased the incidence of malformations of external male genitalia and induced agenesis of several androgen-dependent tissues. Compared to flutamide and vinclozolin the effect of prochloraz was around 100-fold and 10-fold lower, respectively.

We found LOAELs (Vin: 5 mg/kg, Flu: 0.5 mg/kg, Pro: 50 mg/kg) for the three compounds, but a NOAEL was not obtained for any of the compounds.

AGD and areola/nipple retention are early and sensitive endpoints for antiandrogenic activity. Compared to malformations of external genitalia and reproductive organ weights, these endpoints seem more suitable for detecting antiandrogenic effects especially at low doses.

6. A MARKET BASKET APPROACH TO ESTIMATE PP'DDE DIETARY INTAKE BY THE ITALIAN POPULATION DURING 2003

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This study was undertaken in the framework of COMPRENDO project with the aim to evaluate the exposure of European population to some well known endocrine disruptors through the diet.

We present the preliminary results dealing with pp'DDE levels in the Italian diet. This compound is one of the most ubiquitous pollutants, whose risk to human health is difficult to estimate mostly due to uncertainties associated with endocrine disruption and other long-term effects.

Foods to include in the market basket were selected according to the composition of the total diet, allowing to estimate the pp'DDE daily intake with a reasonable approximation. Seasonal variability was also evaluated performing three sampling campaigns in the town of Milan during February, May and September 2003. The daily intake values resulted 0.26; 0.33 and 0.97?g/day, respectively. Similar results were obtained in Basque Country (Spain) during 1990/91, while higher values (1.8 ?g/day) are reported in a previous study performed in Italy during 1997. In this last study, however, data refers to total DDT concentrations in foods from all the Italian Regions.

7. ANALYSIS OF ENDOCRINE DISRUPTORS USING LUCIFERASE REPORTER SYSTEMS IN CELL LINES AND PRIMARY CULTURES OF HUMAN OVARIAN SURFACE EPITHELIAL (OSE) CELLS

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Outline

In this study endocrine disruptors (EDs) are tested *in vitro* using a luciferase gene reporter system. EDs have initially been examined using conventional cell lines such as Hek293 or HepG2 cells, but will be further examined in primary cultures of human ovarian surface epithelial (OSE) cells.

To examine the effect of EDs on estrogen-responsive genes, the luciferase reporter gene is examined under the control of either the *Xenopus vitellogenin* (containing ERE) or human collagenase (containing AP1 sites) gene promoter regions. To examine the effect of EDs on androgen-responsive genes, the luciferase reporter gene is also examined under the control of the human prostate specific antigen (containing ARE) gene promoter region.

Cell lines

For analysis in Hek293 and HepG2 cell lines, a plasmid reporter system is employed. However, since these cells do not express estrogen receptors, cells are co-transfected with plasmids that express either ER α or ER β . So far the greatest effects are observed with 17 β -Estradiol 3-benzoate, 5 α -Androstane-3 β -17 β -diol and bisphenol A. The compounds 4-MBC and Dibutyl phthalate exhibited the weakest effects.

Primary human OSE cells

Normal human OSE cells are obtained, with informed consent and local ethical committee approval, from premenopausal women during laparotomy for benign gynaecological conditions. Since these cells are not easily transfected, recombinant adenoviruses are currently being prepared that contain the luciferase reporter gene constructs. Infection with these recombinant adenoviruses will therefore allow us to examine the effect of EDs on endogenous receptors. In addition to the reporter gene assay, gene expression will also be examined in human OSE cells using the EUROSTERONE microarray chip that contains 250 steroidogenic and steroid-responsive genes. By comparing EDs that exhibit the greatest effect using the reporter system with estrogen, we hope to provide some insight into the effect EDs have on cellular function.

This research was supported by The European Commission EU Contract EUK1-CT-2002-00128 (EURISKED).

8. POLYBROMINATED DIPHENYL ETHERS IN BREAST MILK COLLECTED IN THE CZECH REPUBLIC

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Polybrominated diphenyl ethers (PBDEs) represent chemicals widely used as additives to polymeric materials for the fire safety. PBDEs make their way to human populations primarily via food intake, nevertheless other ways (e.g. inhalation) are also possible. PBDEs are suspected to be transferred via placenta and breast milk from the mother to the offspring in mammals. Available data suggest that PBDEs are potential thyroid disruptors and developmental neurotoxicants.

In the Czech Republic no study on the levels of PBDEs in human milk has been conducted yet. Within the pilot study 103 breast milk samples, obtained from mothers living in Olomouc region have been examined (GC/MS-NCI method used). Altogether 10 PBDE congeners were monitored (No. 28, 47, 49, 66, 85, 99, 100, 153, 154 and 183). All samples examined till now contained PBDEs residues (LOD were in the range 0.02 – 0.05 ng.g⁻¹ lipid weight), the dominating contaminant representing this group was congener BDE 47. In most of analysed samples levels of this compound ranged from 0.2 to 2 ng.g⁻¹ lipid weight. This tetrabromo congener which was found in all samples at concentration level exceeding LOD typically contributes with about 40–70% to the total of PBDEs content.

Other relatively abundant congeners present in human milk samples were BDE 99, 100 and 153. Concentrations of these contaminants were above detection limit in more than 60% of the samples. Congeners BDE 49, 66, 85, 154 and 183 were detected only in approximately 20% of the samples. BDE 28 was above detected only in seven samples. Three exceptionally contaminated samples, containing levels of PBDEs 5–10 times higher than other samples, were found. The source of mothers' exposure has not been revealed.

No correlation was observed both for the lipid content ($r=0.149$, $a=0.05$) or age of mothers ($r=0.059$, $a=0.05$) and the levels of PBDEs in breast milk samples. The absence of the mathematical relationship between the PBDEs content and age of the donors indicates that individual habits rather than a general exposure determine the body burden by PBDEs.

The results from second set of samples (130 human milks) will be also shown in the presented poster. These samples are currently being analysed.

This study has been carried out within the EU project QLRT-2001-00596 FIRE (Flame retardant Integrated Risk assessment for Endocrine disruption). Breast milk samples were collected by University Hospital Olomouc.

9. THE EFFECT OF ENDOCRINE DISRUPTERS ON T3-REGULATED ENDPOINTS IN LIVER, KIDNEY AND HEART IN FEMALE RATS

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Endocrine disrupters (ED), natural or synthetic compounds, were initially found to impair endocrine regulation of the reproductive system, but some of them are suspected to exert anti-thyroidal actions, too. Among others the soy-component genistein exerts estrogenic and antiestrogenic effects, but also interferes with thyroid hormone biosynthesis. To assess effects on the thyroid axis, female ovariectomized rats were treated for 12 weeks with substances used as plasticizers (4-nonylphenol, NPH, 20 or 80 mg/kg), UV absorbers in sunscreens (octyl-methoxycinnamate, OMC, 12.5 g/kg; 4-methylbenzylidene-camphor, 4-MBC, 12.5 g/kg), the estrogen agonist 5 α -androstane-3 β ,17 β -diol (Adiol, 150 mg/kg) and estradiol benzoate (E2, 342 mg/kg) alone or in combination with soy-containing food. T3, T4 and TSH serum levels were measured. T4 was significantly increased by NPH but decreased by OMC and MBC. Activities of the known T3-regulated endpoints malic enzyme (ME) and type I 5'-deiodinase (5'DI) were analyzed. In the liver, ME activity was ~2-fold elevated by E2 and Adiol, but reduced ~2-fold by 4-MBC. Similar results were obtained for ME mRNA-levels using Real Time PCR and Northern blot. Soy-containing chow reduced ME activity in combination with any ED. Only minor effects on these endpoints were detected in kidney and heart. These results strongly support the notion that effects of EDs are tissue-specific and also concern endocrine regulation via the thyroid hormone axis.

Supported by "Multi-Organic Risk Assessment of Selected Endocrine Disrupters" (EU grant)

10. POLYBROMINATED DIPHENYL ETHERS IN BREAST MILK COLLECTED IN THE CZECH REPUBLIC

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Polybrominated diphenyl ethers (PBDEs) represent chemicals widely used as additives to polymeric materials for the fire safety. PBDEs make their way to human populations primarily via food intake, nevertheless other ways (e.g. inhalation) are also possible. PBDEs are suspected to be transferred via placenta and breast milk from the mother to the offspring in mammals. Available data suggest that PBDEs are potential thyroid disruptors and developmental neurotoxicants.

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11. POLYBROMINATED DIPHENYLEETHERS INHIBIT TCDD-INDUCED EROD-ACTIVITY IN CARP HEPATOCYTES

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Ethoxyresorufin-O-deethylase (EROD) activity is a popular biomarker for exposure to AhR agonists. In environmental studies, complex mixtures of xenobiotics are subject to investigation where a number of pollutants inhibiting EROD activity may hamper adequate exposure assessment. This study investigates the effect of the increasingly environmentally detected polybrominated diphenylethers (PBDEs) on EROD activity in carp hepatocytes. BDEs -47, -99, -100 and -153 were selected based on their environmental abundance. Commercial penta-BDE (DE-71, Great Lakes Chemical Corp.) untreated and after clean up to remove planar impurities, a BDE-47 metabolite, 6OH-BDE-47, and PCB-153, a known inhibitor of EROD activity, were included. Freshly isolated carp hepatocytes were co-exposed for 5 days to 2,3,7,8-TCDD (0, 1, 3, 10, 30 and 100 pM), and one of the pure PBDE/PCB congeners (0, 0.25 and 2.5µM) or either DE-71 fraction (0, 0.1 and 10 µM), and EROD activity was determined. Exposure to TCDD alone resulted in dose-dependent increase of EROD activity. This induction was significantly reduced in the presence of BDEs-47, -99, -153, and both DE-71 fractions, dependent on PBDE dose, and generally not paralleled by reduced catalytic conversion of MTT as viability parameter. Particularly strong inhibition was observed for the environmentally most abundant congener BDE-47 (down to 6% of the corresponding control at 2.5µM BDE), and cleaned-up DE-71 (to 4% of the control at 1.0 µM DE-71). BDEs-47 and -153 added shortly prior to measurement did not reduce EROD activity, indicating that inhibition is not catalytic. PCB-153 did not affect EROD activity in this study. Thus, environmentally relevant PBDEs may interfere with EROD measurement *in vitro*, resulting in underestimation of toxic hazard in environmental samples. Supported by FIRE, EU contract number QLK4-CT-2002-00896.

12. POLAR BEAR CASE STUDY

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The most prominent and persistent organic pollutants (POPs) that are associated or even causally linked with endocrine disruption in wildlife are the organohalogen compounds (OCs) e.g. DDTs and polychlorinated biphenyls (PCBs). At Svalbard, alarmingly high PCB levels have been found in polar bears and the present study has investigated possible endocrine disruption and immunotoxic effects caused by this high PCB exposure. We found significant relationships between blood levels of PCBs and the endocrinological parameters testosterone, cortisol, retinol, progesterone and thyroid hormones. These results are indications of alteration of the endocrine system and suggest that perinatal exposure to OCs may affect normal reproductive development. Furthermore, we have demonstrated that high PCB exposure affects IgG blood levels, the ability to produce antibodies following immunization with certain microbes, and lymphocyte function after in vitro stimulation with mitogens and antigens. These results indicate that PCBs are associated with decreased resistance to infections. Thus, it is reasonable to conclude that the population and health of polar bears with high levels of OCs are at risk. A question to be raised is how additional stressors such as the brominated flame retardants (BFRs) will affect these polar bears.

13. SOY ISOFLAVONES INFLUENCE THYROID HORMONE SYNTHESIS AND TRANSPORT

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Flavonoids, phenolic secondary metabolites of plants, are common dietary components which, apart from their action as phytosteroids, exhibit several other biological effects. They influence feedback regulation of hormonal networks, including the pituitary-thyroid-periphery axis. In this project we analysed the interference of soy isoflavones genistein, daidzein and glycitein with key components of the thyroxine (T₄) biosynthesis pathway and T₄ binding to serum transport proteins.

Competition for serum binding of ¹²⁵I-labeled L-T₄ was determined in the absence or presence of increasing concentrations of isoflavones using non-denaturing PAGE gels. A phosphoimager-based analysis quantified the binding and distribution of T₄ among the individual binding proteins. The concentration-dependent inhibitory effect of isoflavones (IC-50 about 10⁻⁷ M) was confirmed by direct binding assays using purified transthyretin (TTR) and dextran-coated charcoal for separation of free from bound T₄.

Interference of soy isoflavones with T₄ biosynthesis was analyzed by inhibition of 1) the Na⁺/I⁻ symporter (NIS), an integral plasma membrane glycoprotein that mediates active I⁻ transport into the thyroid follicular cells and 2) the human recombinantly expressed thyroperoxidase (TPO) activity.

Our study confirmed inhibitory effects of soy isoflavones on the thyroid hormone synthesis machinery and emphasized a potent competition for T₄ binding to TTR, pointing on the existence of several non-steroidal targets susceptible to the action of endocrine disruptors.

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14. THE GERMAN ENVIRONMENTAL SPECIMEN BANK AS A TOOL FOR THE RETROSPECTIVE MONITORING OF EXPOSURE (AND EFFECTS?) OF ENDOCRINE DISRUPTERS

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The German environmental specimen bank (ESB) was established in 1985 as a permanent institution for the systematic collection, processing, characterization and storage of environmental samples from marine, fresh water and terrestrial ecosystems as well as human samples. The ESB offers the opportunity to monitor the exposure of man and wildlife with endocrine disrupting substances not only in current but also in retrospective studies.

Sampling strategy in the framework of the ESB allows general statements regarding the enrichment and concentration of endocrine disrupters in various levels of the food web.

In addition to the chemical analyses detailed biometric characterizations of environmental samples and substantial questionnaires by contributors of human samples are surveyed and used to interpret changes in exposure.

Several efforts are in progress to enhance the convenience of the ESB: the existing set of human specimen (blood, urine, hair) will be completed by perinatal samples (cord blood, placenta, milk). In case of environmental samples processing will be partly modified to allow additional histo- and biochemical as well as genetic studies.

15. BROMINATED FLAME RETARDANTS (BFRS) IN THE HIGH ARTIC MARINE FOOD CHAIN

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BFRs such as the high production volume chemicals polybrominated diphenyl ethers (PBDEs) and hexabromocyclodecane (HBCDD) are lipophilic and persistent against degradation causing them to biomagnify in the marine food chains. Due to their semi-volatile properties long-range transport and bioaccumulation in food chains of pristine waters such as the high-artic is anticipated. We therefore analysed for HBCDD and PBDEs (BDE-28, -47, -99, -100, -153, -154, -183, -209) in representative species of the different trophic levels of the polar bear food chain (i.e., zooplankton, polar cod, ringed seals). BDE-28, -47 and -99 were the only BFRs quantified in the in lower pelagic zooplankton species *C. glacialis*, *T. inermis* and *T. libellula*, and the sum concentration of these compounds were in the lower range of $\sim 1 \text{ ng g}^{-1}$ (lipid weight basis [lw]) in these species. Concentrations of most BFRs increased with increasing trophic level, with the noticeable exception of the polar bear which appeared capable of metabolising several of the compounds. HBCDD and SPBDEs (BDE-28, -47, -99, -100, -153) ranged from 5 – 25 ng g^{-1} and 10-15 ng g^{-1} lw, respectively in the polar cod. SPBDEs (BDE-28, -47, -99, -100, -153, -154) ranged from 50-100 ng g^{-1} lw in the ringed seal and from 15-30 ng g^{-1} lw in the polar bear, whereas HBCDD ranged from 15-35 ng g^{-1} in the ringed seal and from 5-15 ng g^{-1} lw in the polar bear. All compounds except for BDE-183 were detected in the polar bear. BDE-47 was by far the most abundant PBDE congener in all species, whereas BDE-153 appeared to be most persistent PBDE congener in the food chain. The concentrations reported in present study were one order of magnitude lower compared to in food chains from the North Sea.

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16. TESTOSTERONE CONJUGATING ACTIVITIES IN INVERTEBRATES: ARE THEY TARGETS FOR ENDOCRINE DISRUPTORS?

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Testosterone conjugation pathways -microsomal acyltransferases and cytosolic sulfotransferases -, were investigated in three invertebrate species, the gastropod *Marisa cornuarietis*, the amphipod *Hyalella azteca*, and the echinoderm *Paracentrotus lividus*. All three species exhibited high acyl-CoA:testosterone acyltransferase (ATAT) activity, in the range of 100 to 216 pmol/min/mg protein. Enzyme kinetics and specific activities –very similar to the ones reported in rats- indicated that ATAT is a highly conserved steroid conjugating pathway in evolution. In contrast, the activity of testosterone sulfotransferase was rather low (0.05 to 0.18 pmol/min/mg protein) in *M. cornuarietis* and *H. azteca*, when compared to *P. lividus* digestive tube (50-170 pmol/min/mg protein). The interference of model pollutants (triphenyltin –TPT-, tributyltin –TBT-, and fenarimol) with those conjugation pathways was further investigated in-vitro. Both, 100 µM TPT and TBT, were found to inhibit ATAT in *P. lividus* (68 and 42% inhibition, respectively); while only TBT had an effect in *M. cornuarietis* (20% inhibition at 100 µM). Fenarimol did not affect ATAT in any of the species tested. As regards to the sulfation of testosterone, a strong inhibitory effect of TBT and fenarimol (54-56%), but also TPT (28%) was detected in the *P. lividus*. The results evidenced the existence of interphyla differences in testosterone conjugation, and the susceptibility of those hormone clearance pathways to inhibition by model pollutants.

17. EFFECT OF ORAL INTAKE OF DIBUTYLPHthalate ON THE METABOLISM OF ADULT LONG EVANS RATS.

Castillo C., Salazar V., Ariznavarreta C., Tresguerres JAF.

In order to investigate the role of dibutylphthalate (DP) as a possible endocrine disrupter, 80 Long Evans rats were obtained from the mating of mothers that had been fed in the previous 2 months experimental chows with different DP doses. Animals were divided in 3 experimental groups according to the diet of their mothers: (1) control; (2) DP 0.61 g/Kg chow (12 mg/Kg rat/day); (3) DP 2.5 g/Kg chow (50 mg/Kg rat/day). At 12 weeks of age animals were sacrificed by decapitation. Heart, thoracic aorta, tibia, liver, kidney, skin, brain, adrenal glands, bladder, pituitary gland, thyroid gland, uterus, vagina, mammary gland, periuterine fat, seminal vesicles and prostate were collected. Plasma was also obtained for the determination of Estradiol, Testosterone. RESULTS: pituitary gland weight showed a significant increase ($p < 0.05$) in both female and male rats from groups 2 and 3. Kidney relative weight presented an evident increase ($p < 0.01$) in both sexes of rats belonging to groups 2 and 3. Absolute weight of animals and heart weight were not statistically different in the 3 groups. Relative weight of adrenal glands, spleen and liver seemed to be not affected either. Ovarian and uterine relative weights showed a slight increase in groups 2 and 3. Testis relative weight was significantly ($p < 0.05$) higher in group 3. Plasma levels of Testosterone were evidently ($p < 0.001$) lower in group 2, while group 3 showed intermediate values. Plasma Estradiol levels were significantly higher in group 3. CONCLUSION: oral intake of dibutylphthalate during pregnancy exerts alterations on the reproductive system of adult offspring.

18. EFFECT OF ORAL INTAKE OF DIBUTYLPHTHALATE ON REPRODUCTIVE PARAMETERS OF LONG EVANS RATS AND PRE-PUBERTAL DEVELOPMENT OF THEIR OFFSPRING.

Salazar V., Castillo C., Ariznavarreta C., Tresguerres JAF.

To investigate the influence of dibutylphthalate (DP) given in a soya-free rat chow on pre-pubertal development, 46 Long Evans female rats 2 months-old were divided into 3 experimental groups and fed 3 different chows: (1) control; (2) DP 0.61 g/Kg chow (12 mg/Kg rat/day); (3) DP 2.5 g/Kg chow (50 mg/Kg rat/day) for two months. They were later mated and their offspring studied. Litter size and female:male ratio was recorded. At 14 days of age 6 male pups of each group were sacrificed and testis and thymus were excised and weighed. Pups were weaned at 22 days of age and continued into 3 experimental groups according to diet. From day 22 onwards, vaginal opening, occurrence of 1st estrous and preputial separation were recorded. RESULTS: the % of pregnancies showed a marked decrease in group 3, while no difference was observed between group 1 and 2. Sex prevalence and litter size were not affected by the different diets. Pups survival showed a decrease when mothers were fed diet 2, but it was similar in diet 1 and 3. Pups weight in Day 2 showed an evident ($p < 0.05$) reduction in groups 2 and 3, being the decrease more marked ($p < 0.001$) in group 3. On Day 6, pups of group 2 showed lower weights ($p < 0.01$) as compared with the other groups. Weight gain was significantly higher in pups of group 3. Eyes opening was not affected by the different diets. 14 days-old male pups' relative weight of thymus and testis showed a decrease in animals whose mothers had been fed diets 2 and 3. Vaginal opening and occurrence of first estrous showed an evident delay ($p < 0.05$; $p < 0.01$) in females fed diets 2 and 3. No significant differences in preputial separation of the different groups were observed. CONCLUSION: offspring pre-pubertal development seems to be affected by oral intake of DP by their mothers during pregnancy, being the effects more evident in the reproductive development of female pups.

19. EFFECTS OF BROMINATED FLAME RETARDANTS ON THE ACTIVITY OF THE STEROIDOGENIC ENZYME AROMATASE (CYP19) IN H295R HUMAN ADRENOCORTICAL CARCINOMA CELLS IN CULTURE

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Flame retardants are substances used in plastics, textiles, electronic circuitry and other materials to prevent fire ignition processes. Brominated flame retardants (BFRs) are ubiquitous chemicals with large and global industrial use. Due to this fact, several BFRs are found in quantifiable levels in wildlife and humans. Several in vitro studies have shown that these chemical have endocrinological effects. Our study focuses on the potential interactions of a wide range of these BFRs with aromatase (CYP19). We used the human H295R adrenocortical carcinoma cell line to assess the effects on this steroidogenic enzyme activity after a 24h exposure to twenty different BFRs. These included polybrominated diphenyl ethers (PBDEs) and several of their hydroxylated metabolites (OH-BDEs, MeO-BDEs), tetrabromobisphenol-A (TBBPA) and brominated phenols (BP). Effects were studied in the concentration range from 0.5 to 7.5 μM . Aromatase activity was measured using the tritiated water-release assay. MTT and LDH assays were used to measure cytotoxicity of these BFRs. Exposure of H295R cells to 6OH-BDE47 and 6OH-BDE99 showed an inhibitory effect on aromatase activity at concentrations $> 2.5\mu\text{M}$ and $> 5\mu\text{M}$, respectively, although 6OH-BDE47 caused a decrease in cell viability at concentrations $> 2.5\mu\text{M}$ possibly explaining the aromatase inhibition. Replacement of the 6OH group by a methoxy group eliminated cytotoxicity, while significant aromatase inhibition remained. A concentration-dependent induction of aromatase activity was caused by 2,4,6-tribromophenol(TBP) between 0.5 and $7.5\mu\text{M}$. These inductive properties were lost when the hydroxy-group was replaced by a methoxy-group or adjacent bromines were removed. The presented in vitro results give a first indication of possible structure activity relationships for interaction of BFRs with the enzyme aromatase.

20. DIFFERENTIAL EFFECTS OF BENZOPHENONE-2 (BP2), BISPHEENOL-A (BPA) AND DIBUTYLPHTHALATE (DBP) IN THE UTERUS, VAGINA AND THE METAPHYSIS OF THE TIBIA: COMPARISON WITH CHRONIC E₂ TREATMENT IN OVX RATS

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BP2, BPA and DBP are endocrine disrupters with known estrogenic and the latter two substances with anti-androgenic activities as tested in the uterus or prostate respectively. As estrogen and androgen receptors (ER and AR) are also expressed in many other organs we tested whether these substances have effects in the vagina and bone of ovariectomized (ovx) Sprague Dawley rats and compared them with effects in the uterus. In ligand binding assays BP2 proved to bind equipotently to ER α and ER β , in comparison to E2 while DBP and BPA did also bind but much weaker and with a higher affinity to ER β . Following 3 months of treatment BP2 at 2 doses (185mg or 925 mg/kgBW/day) proved to have identical effects as E2 in the uterus, the vagina and in the bone as determined by weight, morphological and molecular parameters. Differential in part antagonistic effects of BPA (92,5mg and 462,5mg/kgBW/day) and DBP (37 μ g and 370 μ g /kgBW/day) were observed in the endo- and myometrium and in the bone, where ovariectomy resulted in substantial loss bone mineral density of the metaphysis of the tibia. This osteoporosis could be largely prevented by E2 and BP2 but was further augmented by BPA and DBP.

These results demonstrate that Benzophenonenon-2 act like estradiol in the uterus, the vagina and the bone, while, despite binding to ER α and ER β , BPA and DBP had effects which are dissimilar to those of E2 and which are possibly exerted by repressors of ER α activity such as the aryl hydrocarbhone/aryl hydrocarbhone nuclear translocator complex.

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21. EFFECTS OF ESTRADIOL, GENISTEIN AND RESVERATROL ON THE HYPOTHALAMO-PITUITARY-GONADAL (HPG) AND HYPOTHALAMO-PITUITARY-THYROID (HPT) AXES UNDER ACUTE AND LONG TERM TREATMENT

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The phytoestrogens Genistein (GEN) and Resveratrol (RES) are increasingly consumed as nutritional supplements in artificially high doses. Both compounds bind to the estrogen receptor subtypes ER α and ER β . Since Estradiol (E2) also has an impact on the HPT-axis, we studied the effects of these phytoestrogens and E2 in ovariectomized rats after acute administration of a single high dose per gavage, in comparison to a 3 months exposure to two dosages of each test compound via food. In the acute experiment blood samples were withdrawn through a previously implanted jugular vein catheter at time points -1, -0.5, 0, 0.5, 1, 2, 3, 4 and 6 hours. In both experiments decapitation blood was collected, uteri removed and weighed. Pituitary and thyroid hormones in serum were determined by RIA.

Uterine weights were stimulated by E2 and GEN under acute and long term treatment. LH concentrations were decreased by E2 under both regimens, by GEN only under acute conditions. E2 and GEN increased TSH concentrations after the single dose, but showed no significant effects after 3 months. RES showed no effect on uterine weight, LH and TSH levels in either study. All three substances had varying effects on T3 and T4. Especially RES reduced thyroid hormones acutely, but increased them after 3 months.

It is concluded that GEN but not RES has estrogenic effects on the HPG-axis, while the effects of the phytoestrogens on the HPT-axis were different from those of E2. Other than estrogenic mechanisms may become functional there.

This work was supported by "Multi-Organic Risk Assessment of Selected Endocrine Disrupters" (EU grant EVK1-CT-2002-00128)

22. ORGAN-SELECTIVE EFFECTS OF ESTRADIOL (E2), BENZOPHENONE-2 (BP2) AND BENZOPHENONE-3 (BP3) ON THE EXPRESSION PATTERN OF THE ARYL HYDROCARBON RECEPTOR (AHR) AND THE ESTROGEN RECEPTORS (ER)

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The AhR, first described as dioxin receptor, is a ligand-inducible transcription factor of which the endogenous ligand is unknown. It was shown to interact with the estrogen receptor signalling pathway. A wide variety of environmental toxicants and/or industrial pollutants as well as possibly endogenous ligands like tryptophan derivatives can cause endocrine effects through activation of the AhR. The relative abundance of the AhR in a variety of organs may elucidate the importance of this receptor in these organs. Furthermore the regulation of the AhR by E2 may also be critical for its modulating effects in estrogen regulated organs.

To investigate a possible mediation of the estrogenic effects of BP2 and BP3, widely used UV filters in cosmetics and plastics, by the AhR and the ER's, adult ovariectomized rats were treated orally for 5 days with BP2 or BP3. Estradiol served as positive control. Rats were sacrificed 6 hrs after the last treatment and organs were collected. Gene expression was determined using RT-PCR. The relative abundance of AhR was highest in the uterus > pituitary > thyroid. E2 and BP2 but not BP3 stimulated uterine weight and decreased the expression of IGF1 significantly, indicating an estrogen-like activity for BP2 while BP3 exerts no estrogen-like activity at all. The effects on the expression pattern of the AhR and the ER's are quite diverse. Here, E2, BP2 and BP3 show an organ-selective effect. This indicates that BP2 and BP3 exert their effects through different mechanisms than Estradiol does.

23. EFFECTS OF ESTRADIOL (E2), BENZOPHENONE-2 (BP2) AND BENZOPHENONE-3 (BP3) ON THE TISSUE-SPECIFIC EXPRESSION OF THE ESTROGEN RECEPTOR RELATED RECEPTOR 1 (ERR1).

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The almost ubiquitously expressed orphan receptor ERR1 shows close structural relationship to the estrogen receptors. ERR1 is supposed to be a constitutionally active transcription factor which is inactivated by synthetic ligands like diethylstilbestrol. An endogenous ligand of ERR1 is unknown. ERR1 exerts its regulatory effect by binding to ERR1-, and ER response elements. Additionally ERR1 may compete with the estrogen receptors for cofactors, indicating an important role in modulating estrogen related responses, which may explain effects of substances with estrogenic action in some but not all organs. Hence, abundance and regulation of ERR1 may help to explain such organ specificity. Benzophenones are used in cosmetics and BP2 but not BP3 is known to exert estrogenic effect in the uterus. Whether these substances exert effects in other organs is unknown. Adult ovariectomized rats were treated orally with BP2 or BP3 for 5 days, estradiol-valerate served as positive control. The rats were sacrificed and organs were collected. A possible involvement of the ERR1 in the mediation of estrogenic effects of BP2 and BP3 was investigated by measuring gene expression of the ERR1 using RT-PCR. Highest expression of ERR1 mRNA was found in thyroid > adrenal gland > uterus. E2 and BP2 increased uterine weight significantly while no effect was seen in the BP3 group. E2 increased ERR1 gene expression significantly in the uterus and decreased it significantly in the thyroid gland. BP2 showed a dose-independent increase of ERR1 mRNA in the uterus while BP3 had no effect. Consistent with this BP2 but not BP3 elevated uterine expression of C3 significantly. Both BP2 and BP3 exerted a significant dose dependent E2 like decrease of ERR1 mRNA in the thyroid. BP2 alone showed a significant decrease of the ERR1 mRNA levels in the adrenal gland. These data suggest that ERR1 may be involved in modulating the estrogenic effects of BP2 and BP3 in a tissue specific manner

24. HISTOLOGICAL FINDINGS ON UTERUS AND MAMMARY GLAND OF RATS TREATED WITH THE PHYTOESTROGENS GENISTEIN AND RESVERATOL

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Introduction: Genistein and Resveratol are two phytoestrogens with binding affinities to estrogen receptors, (mainly beta), but their effects in the uterus and mammary gland after binding to these receptors are still unclear.

Materials and Methods: Groups of 12 Sprague Dawley rats bred under soy free food were ovariectomized at the age of three months, and immediately switched to a chow containing E₂, different doses of Genistein or Resveratol. After three months animals were sacrificed, the uterus and fifth mammary gland removed, and prepared for routine histological analyses and immunohistochemistry with antiserum against proliferation cell nuclear antigen (PCNA) to explore the proliferation stimulating effects of the test substances.

Results: Uterus: Control animals showed an atrophic uterus.

Estradiol treated: All structures (epithelium, endometrium and myometrium) were hypertrophic and hyperplastic at both doses.

Genistein: At high dose uterine weight was significantly higher ($p < 0,05$) than control group. No differences were seen in the epithelium at low dose, but the lamina propia stromal cells showed the typical spindle appearance from estrus, and an extremely complex mosaic appearance was seen in the high dose.

Resveratol: Almost no changes in comparison to controls were detected at the low dose, at the higher some spindle shaped lamina propia cells were found.

Mammary gland: Control: Atrophy of epithelial structures, densely packed in multilayered buds, showing mainly inactive nuclei.

Estradiol high: Actively secreting epithelial mammary cells, with clear nuclear activity, cytoplasmatic vacuolization, and well formed duct acinar structures.

Estradiol low: No stimulation or acinar formation and milk production.

Genistein: No clear morphological differences were observed between controls and low dose treated animals. At high doses acinar formation, was increased and lumen formation was more evident than in control groups.

Resveratol: Mild morphological acine appearance was detected at both doses.

Cell proliferation detected by PCNA immunohistochemistry, was extremely variable in the E₂ high group and was higher in the E₂ low ($p < 0.05$ vs. control). The test substances did not show significant differences from control group.

Conclusions: Resveratol had negligible estrogenic effects in the uterus and mammary gland, while Genistein at the high dose stimulated morphological appearance of both organs clearly, but not proliferation on mammary gland.

25. PERSISTENT AND PERVASIVE TISSUE-SPECIFIC ACTION OF ORGANOCHLORINE COMPOUNDS IN ESTROGEN-REPORTER MALE MICE.

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Abstract

Organochlorines are lipophilic molecules that accumulate in fat where they remain for years. During weight loss they are mobilized and their concentration increases in blood. The present work tests, in transgenic estrogen-reporter mice (ERE-tk-LUC), whether this increase is sufficient to modulate the ERs (estrogen receptors) in the whole body. Three weak estrogens were studied: p,p'-DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane], p,p'-DDE [1,1-Dichloro-2,2-bis(p-chlorophenyl)ethylene], and BBHC [β -Benzene-hexachloride]. Dose-dependent analysis of reporter expression (luciferase) were performed in tissues of acutely treated mice. A body map of ER activation was obtained. All these chemicals activated the ERs, although to a different efficacy and depending upon the tissue analyzed. After experimental accumulation in fat tissue, followed by a 48 hours period of fasting, we tested whether these compounds could reach a sufficient level to activate the ERs in the same tissues. In loaded mice, BBHC mobilization resulted in a strong ER activation in the liver, lung, prostate and hypothalamus, while p,p'-DDT mobilization had no effect in the liver and acted very weakly in most of the tissues, except the testis (1.5 fold increase). During fasting BBHC, p,p'-DDT and the metabolite p,p'-DDE increased in blood concentration, from 225±25, 51±9 and 38±6 ng/ml to 824±95, 452±68 and 506±57 ng/ml respectively. The effect produced by these organochlorines in the liver correlates with a positive modulation of the ER α protein. We conclude that these organochlorines specifically modulate the estrogen receptors in male mice, depending upon the compound and tissue analyzed.