International Symposium on Environmental Endocrine Disrupters 2000

Saturday, December 16 - Monday, December 18, 2000

セッション4
2000年12月18日（月）

Session 4
Monday, December 18, 2000

作用メカニズム

Mechanisms of Action

Gonadal Sex Differentiation in Fish and the Effects of Environment Endocrine Disrupters

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In fish, as well as in other vertebrates, gonadal primordia are formed during ontogenesis. Subsequent gonadal sex differentiation results in a developed ovary or testis. Treatments with extremely small quantities of sex hormones around the time of sex differentiation, however, have profound effects on gonadal development including sex reversal, hermaphroditism and sterilization. The gonad is, therefore, extremely sensitive to environmental stimuli around the time of sex differentiation, often with irreversible effects. As a result, environmental endocrine disrupters (EEDs) likely affect sex differentiation in wild fish.

In this study, I will describe the mechanism of normal sex differentiation (tilapia), and the effects of sex hormones on the expression of steroidogenic enzymes in fish, and the effects of EEDs on sex differentiation (amago salmon).

Gonadal sex differentiation in fish

To understand the effects of EEDs on sex differentiation in fish, the normal mechanism of sex differentiation must find be elucidated, paticularly, the role of endogenous steroid hormones. Four specific antibodies against steroidogenic enzymes (cholesterol side chaincleavage cytochrome P450, 3β-hydroxysteroid dehydrogenase, cytochrome P45017α-hydroxylase/17,20 lyase, and cytochrome P450 aromatase) essential for the biosynthesis of all major sex steroid hormones, were generated. Aromatase is the critical enzyme for the biosynthesis of estradiol-17β from testosterone.

Using these antibodies, differentiating gonads of genetically controlled all-female and all-male tilapia *Oreochromis niloticus* were stained immunohistochemically. Immunopositive cells against all antibodies appeared first in the undifferentiated gonads of genetic females. Immunopositive steroid-producing cells increased in number throughout ovarian differentiation and increased further in the developed ovaries. In contrast, immunopositive reactions were not observed in the gonads of undifferentiated gonads in genetic males. Weakly positive cells first appeared after testicular differentiation, while stronger reactions, except for aromatase, were seen in the testes just before the onset of spermatogenesis. From these results, estradiol-17β is apparently produced in the gonads around the time of ovarian differentiation. Consequently this estrogen may have an important role in ovarian differentiation. It is less likely, however, that steroid hormones, including androgen, have an important role in testicular differentiation.

To further clarify the role of endogenous estrogen in sex differentiation, I examined the effects of aromatase inhibitor (AI, Fadrozole) on genetically controlled all-female tilapia. All fish treated with AI at 200 and 500 μg/g deits from 8 DAH for 22 DAH had well developed testes. In contrast, all fish that received a diet containing both AI 500 and estradiol-17β 250 μg/g diet had normal ovaries, suggesting that AI masculinizes genetic female by inhibiting aromatase activity.

Taken together, these results strongly suggest that endogenous estrogen have an important role in ovarian differentiation, while the lack of estrogen may be an important for testicular differentiation. Consequently, it is

highly possible that EEDs disrupt this estrogen dependent or independent mechanism of sex determination in fish.

Effect of steoid hormone on the expression of steroidogenic enzymes.

Exogenous sex hormones or EED treatments around the time of sex differentiation bring about irreversible effects. To clarify these mechanisms of action, expression of steroidogenic enzymes was examined immunohistochemically during sex reversal from genetic female to phenotypic male by androgen treatment. Positive reactions against steroidogenic enzymes were not observed in the gonads of treated fish, though strong reactions appeared in the gonads of control fish. Androgen treatment in the immature ovary also suppressed at least the expression of aromatase, indicating that exogenous androgen suppress the expression of steroidogenic enzymes. The decrease of estrogen biosynthesis due to the inhibition of the expression of steroidogenic enzymes may then be responsible for the profound effects on sex differentiation. As EEDs are thought to act agonists or antagonists of estrogen, it is highly possible that EEDs also effect on the expression of steroidogenic enzymes. Currently, I am studying the role of EEDs in the expression of steroidogenic enzymes during sex differentiation.

Effects of EEDs on sex differentiation

I examined effects of EEDs on sex differentiation in male amago salmon *Oncorhynchus rhodurus*. First, I determined the susceptible period for feminization by estradiol-17β (E2) (250 ng/l) treatment at various times and durations around the time of sex differentiation. From these experiments, I concluded that the critical period for amago salmon feminization by exogenous estrogen is from 5 to 25 days after hatching (DAH). Treatment with extremely low doses (20 ng/l) of E2 during this period induced a single oocyte in the testicular tissue. Ethynylestradiol, a common synthetic estrogen in oral contraceptive pills, at a dose of 10 ng/l induced sex reversal of most treated fish. Nonylphenol (NP), a representative EED, induced a single oocyte at a dose of 20 μg/ l. Most of fish receiving NP at dose of 100 μg/l were either sex-reversed females or hermaphrodites with both testicular and ovarian tissues. Bisphenole A, an EED found in plastics, also induced feminization at dose of 1000 μg/l.

From these results, EEDs appear to have clear feminizing effect which induces complete sex reversal in salmonid fish, although their potency is lower than that of natural or synthesized estrogens.

Acknowledgement

This work was supported by Integrated Research Program for Effects of Endocrine Disrupters on Agriculture, Forestry and Fisheries and Their Action Mechanisms on Domestic Animals and Fishes.

Transcription Factors supporting Gonad Sex Differentiation

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Sex of many higher animals is determined by combination of the sex chromosomes. However, sex of wildlife in the natural environment can be determined by other factors in addition to sex chromosomes. It is thought that sex determination in mammals is under a strong genetic regulation and not influenced by extrinsic factors, while some kind of disease is also known to induce sex reversal. These facts indicates that sexually indifferent gonads essentially have sexual bipotentiality that is clearly seen in non-mammalian species. The gene involved in the sex determination must be expressed during the process of sex determination, therefore it is considered that the regulating mechanism of the gene represents the molecular basis supporting sex differentiation. When sex difference is observed morphologically in indifferent gonads, sexually dimorphic expression of genes also appear both in male and female glands. This sex difference can be observed in not only the amount but the distribution of the transcription factors required for the gonad development. Therefore, it can be considered that the sex dependent expression of these transcription factors is likely to define sex differentiation. From this aspect, the regulating mechanism of the sex dependent expressions of the transcription factor genes, which are indispensable for the gonad development, and the regulating mechanisms of transcription by these transcription factors are critical points to understand the fundamental process of sex determination. Analyses have revealed genetic cascade among Ad4BP/SF-1, Dax-1, GATA4, and WT-1, during the gonad differentiation. Involvement of various growth factors in the gonad differentiation is also important and we are obtaining interesting results that present relationship relevant to the regulating mechanism of the transcription factor gene. Such research is essential to understand the mechanism of the gonad differentiation and provides us important information to reveal the influence of the exogenous compounds.

The Effects of Endocrine Disruptors on Steroidogenesis and StAR Protein

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Environmental pollutants have adverse affects on the reproductive capacity of both wildlife and humans and these affects are often a result of decreased testosterone biosynthesis. Indeed, alterations in serum steroid hormone levels are typical findings in humans and animals following environmental toxin exposure. Since the synthesis of adequate levels of testosterone by testicular Leydig cells is required for maintaining spermatogenesis, male secondary sex characteristics and thus, normal fertility in males, pesticides or herbicides which disrupt steroidogenesis can ultimately cause infertility. While such observations are numerous, the mechanism(s) involved in steroid inhibition by these compounds is not well understood. We have focused our current studies on the hypothesis that endocrine disruptors might inhibit steroid hormone biosynthesis by inhibiting the expression of the Steroidogenic Acute Regulatory (StAR) protein. StAR protein mediates the rate-limiting and acutely-regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. The importance of StAR protein in the regulation of steroidogenesis was dramatically demonstrated when it was found that mutations in the StAR gene are the only known cause of the potentially lethal condition known as lipoid congenital adrenal hyperplasia. This disease is characterized by an almost complete inability of the newborn to synthesize steroids. Since StAR protein plays a key regulatory role in steroidogenesis, we reasoned that it may be a target for environmental pollutants. We have performed studies with several classes of such pollutants to determine the level at which steroid biosynthesis is inhibited. These studies include lindane, the γ-isomer of hexachlorocyclohexane (HCH), an environmentally persistant insecticide, the widely used pesticide Dimethoate and the herbicide Roundup. The affects of these compounds on StAR and the first two enzymes in the steroidogenic pathway, cytochrome P450scc and 3β-hydroxysteroid dehydrogenase were evaluated using the MA-10 mouse Leydig tumor cell line as a model system.

Lindane, the γ-isomer of hexachlorocyclohexane (HCH), is a one of the oldest synthetic pesticides that is still used today worldwide. Numerous reports have shown that this organochlorine pesticide adversely affects reproductive function in animals, although the mechanisms by which it influences reproductive function remain unclear. However, recent reports indicate that it can directly inhibit adrenal and gonadal steroidogenesis. MA-10 cells were used to assess the potential effects of γ-HCH and its isomers, α- and δ-HCH, on steroid production, P450scc and 3β-HSD steroidogenic enzyme expression/activity, and StAR expression. Our studies demonstrate that α,- δ,-and γ-HCH inhibited $(Bu)_2$cAMP-stimulated progesterone production in MA-10 cells in a dose-dependent manner without affecting general protein synthesis, protein kinase A or steroidogenic enzyme expression/activity. In contrast, each of these isomers dramatically reduced $(Bu)_2$cAMP-stimulated StAR protein levels in a dose-dependent manner. Therefore, our results indicate that α,- δ,-and γ-HCH directly inhibited steroidogenesis by reducing StAR protein expression, an action which likely contributes to the pathogenesis of lindane-induced reproductive dysfunction.

Dimethoate is a widely used organophosphate insecticide that has also been shown to disrupt reproductive function in animals. As with lindane, the pathogenesis of Dimethoate-induced reproductive toxicity remains to be determined, however, a reduction in serum testosterone levels is thought to play an important role in Dimethoate-

induced infertility. This pesticide decreased progesterone production in a dosage-dependent manner without producing a concomitant decrease in total protein synthesis, thus excluding acute toxicity and a general disruption in translation as mechanisms of steroidogenic inhibition. These studies demonstrated that Dimethoate inhibited (Bu) 2cAMP stimulated steroidogenesis by 76%. To determine if the inhibitory effect of Dimethoate on $(Bu)_2$cAMP-stimulated progesterone production might be due to an inhibition of the activities of the steroidogenic enzymes, P450scc and/or 3β-HSD, 22R-hydroxycholesterol (22R-HC) was provided as a substrate to the cells. Dimethoate significantly reduced 22R-HC-driven steroidogenesis by 63%, indicating that it inhibited P450scc and/or 3β-HSD enzyme activity. However, as $(Bu)_2$cAMP+22R-HC-stimulated steroid production was only reduced by 43%, this indicated that 22R-HC could partially reverse the inhibition of (Bu)2cAMP-stimulated steroidogenesis. Further studies indicated that Dimethoate significantly reduced P450scc activity, but did not alter 3β-HSD enzyme activity, indicating that the herbicide was not acutely toxic to cells or mitochondria. Western blot analysis of mitochondrial protein revealed that it did not alter P450scc or 3β-HSD enzyme levels while Northern blot analysis revealed that Dimethoate did not affect P450scc mRNA levels but significantly reduced 3β-HSD mRNA levels. Most dramatically, Western blot analysis revealed that this pesticide reduced StAR protein levels by 83% while Northern blot analysis revealed that it reduced total StAR mRNA levels by 81%. To determine if Dimethoate reduced StAR protein levels by blocking *StAR* transcription, nuclear run-on analysis was performed. Bu2cAMP increased the rate of *StAR* gene transcription 5-fold. However, Dimethoate decreased *StAR* transcription by 55%, indicating that it primarily reduced StAR protein expression and steroidogenesis at the level of transcription.

Studies using Roundup showed that this herbicide also decreased steroidogenesis in a dose-dependent manner and apparently did so by disrupting StAR expression post-transcriptionally. In addition to reducing StAR protein levels, acute exposure to Roundup also lowered P450scc activity and 3β-HSD mRNA levels. However, a reduction in StAR protein levels alone could account for Roundup's effects on steroidogenesis, since StAR acts upstream of the P450scc and 3β-HSD enzymes and also because Roundup decreased StAR levels in proportion to the observed inhibition in steroidogenesis. Furthermore, while Roundup reduced 3β-HSD mRNA levels, it did not alter the activity of this enzyme. These findings suggest that the reduction in StAR levels may be more physiologically relevant in explaining the effects of Roundup on steroidogenesis than the reductions in P450scc activity or 3β-HSD mRNA levels.

Taken together, these results indicate that the observed reductions in reproductive capacity noted in wildlife and humans following exposure to environmental endocrine disruptors may be a result of decreased steroid hormone biosynthesis. We have shown that some of these toxins can inhibit steroid biosynthesis at different and in some cases at multiple levels in the steroidogenic pathway. We have further shown that a particularly vulnerable locus is at the level of the StAR protein suggesting that StAR may be useful as a biomarker in studies of this type.

Transcription Factors and Cofactors with Endocrine Disruptors

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The transcriptional control of the target gene is carried out by transcription factors including steroid hormone receptors forming the complex with cofactors (coactivators and corepressors) and basic transcription factors. We have clarified that endocrine disruptor disturbs the communication of the complexes of steroid hormone receptors and cofactors on the target gene.

We established screening methods of endocrine disruptors using the co-transfections of reporter gene of MMTV-luciferase and steroid receptor gene and using image analyzer of laser confocal microscope with three dimensional analysis system. By using these two screening methods, we have screened new chemicals which disturbs androgen action. And the mechanism of action and classification of new chemicals with anti-androgen activity were determined.

Fifty-one chemicals were examined. The combination with the transcription activation measurement using reporter gene and image analyzer system which we established, confirmed to be very effective for the screening of the endocrine disruptors.

We identified several new anti-androgenic chemicals and clarified the mechanism of action of these anti-androgenic chemicals via the complex of androgen receptor and cofactors. Dihydrotestosterone binds to androgen receptor and this ligand-receptor complex translocates into the nucleus and recruit coactivators (SRC1, TIF-2). These complexes are localized in the euchromatin along heterochromatin with dot formation. Several anti-androgenic chemicals bind to androgen receptor and the complexes of the chemicals and androgen receptor translocate to the nucleus, but these complexes are not localized in the euchromation and can not make dot formation. Therefore transcription activity is suppressed. Another chemicals suppress the transcription activity of androgen receptor. But the complex of chemical and androgen receptor does not translocate into the nucleus. Another mechanism may exist.

Endocrine Disrupter Action and Toxicology: Studies in ER Knock-Out Mice

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Estrogen receptors (ER) are thought to play a crucial role in development, reproduction, carcinogenesis and normal physiology. Using gene targeting techniques, we have produced lines of transgenic mice homozygous for the disrupted ERα gene (αERKO) and ERβ genes (βERKO). Comparable levels of ER-β mRNA were found in tissues of αERKO mice suggesting that ER-β expression is not dependent on ERα. αERKO mice were totally unresponsive to uterotropic assays with estradiol, Genistein, hydroxy TAM, DES or growth factors such as EGF or IGF-1 treatment. Progesterone receptor (PR) mRNA was detected in αERKO mice, but not stimulated by estrogen in the uterus, mammary gland and ovary. Pituitary hormones are elevated in αERKO females but same as WT in β ERKO mice indicating that ERα plays a major role in peptide hormone regulation. Genistien was equally effective in αERKO as WT mice in suppressing serum LH levels suggesting its action in this estrogen target response involved a non-ER mediated mechanism compared to the uterine genistein activity. Analysis of the mammary glands of adult αERKO mice, showed a primitive ductal rudiment while the BERKO had the fully developed ductarl tree seen in WT siblings. The influence of ERα activity on mammary tumorgenicity was evaluated by crossing αERKO with an oncogenic WNT-1 transgenic mouse line having an increased incidence of mammary tumors. Mice with WT ER levels and WNT-1 transgene show 98% tumor incidence at 27 weeks of age. α ERKO/WNT-1 mice showed a delayed onset (54 weeks). These results indicate that mammary tumors can develop from ER negative tissues, but with a delayed onset. A second cross with erB2 (Neu) transgenics shows that WT and hetero Neu mice have a tumor incidence at 54 wks of age. αERKO/Neu have an extremely low incidence past 90 wks. Compared to the WNT/ERKO the ERKO/Neu have a poorer tumor rate suggesting that ERα plays a more prominent role in this oncogenic tumor induction. Since the initial description of DES carcinogenesis in both humans and mice, a major question has been aspect of the DES action was ER mediated or non-receptor mediated. Perinatal DES treatment to WT mice showed numerous phenotypic toxicological and carcinogenic effects including, persistent vaginal cornification, uterine hyperplasia, oviductal hypertrophy in females. In similarly treated males, DES induced seminal vesicle atrophy and prostatic hyperplasia. α ERKO treated males and females showed none of the observed toxic effects indicating that ERα was involved in mediating these DES effects and not ERβ. Uterine Hoxa10 gene expression is reduced by DES in WT mice associated with female reproductive tract anomalies. α ERKO females do not exhibit this reduction in Hoxa10 indicating this early response related to the toxicity is meditated by ERα.

Further characterization of the mice and comparison of the DES effects in βERKO mice and double ER gene KO mice will be needed to more fully the specific roles of the two different forms of ER in estrogen hormone toxicology and hormonal carcinogenesis.