Pregnenolone Metabolism in the Ovarian Thecal Layers of the Rainbow Trout, *Oncorhynchus mykiss: in vitro* Inhibitory Effects of Cyanoketone and Trilostane

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The effects of specific inhibitors of 3β-hydroxy-△^{5}-steroid dehydrogenase (3β-HSD; an enzyme catalyzing conversion of △^{5} steroids to △^{4} steroids), cyanoketone and trilostane, on ^{3}H-pregnenolone metabolism in isolated ovarian thecal layers have been investigated *in vitro*. At all doses of cyanoketone (10^{-6} and 10^{-5} M) and trilostane (10^{-5} and 10^{-4} M), 3β-HSD enzyme activity that transforms pregnenolone to 17α-hydroxyprogesterone was inhibited in the thecal layers. Trilostane appeared to be more efficient than cyanoketone. Trilostane at doses of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M/ml caused a dose-response inhibition of △^{4} steroids accumulation in the medium from pregnenolone, but not completely blocked the conversion of △^{5} to △^{4} steroids.

Key words : pregnenolone metabolism, cyanoketone, trilostane, 3β-hydroxy-△^{5}-steroid dehydrogenase, steroid biosynthesis

Introduction

The steroid metabolism in the ovarian thecal and granulosa cells of the rainbow trout was previously studied in the presence of labeled or unlabeled precursors (△^{5}-steroids, △^{4}-steroids), which showed that the activity of 3β-hydroxy-△^{5}-steroid dehydrogenase (or 20β-hydroxy steroid dehydrogenase), a key enzyme in 17α-hydroxyprogesterone (or 17α-hydroxy, 20β-dihydroprogesterone) biosynthesis (Baek, 1990; Baek and Lee, 1993), appeared to be under trophic hormone regulation. The various intermediates involved in the conversion of precursor pass through the catalytic process require enzymes. The conversion of radioactive pregnenolone (P5) to 17α-hydroxyprogesterone (17α-OHP) depends upon the activity of 3β-hydroxy-△^{5}-steroid dehydrogenase (3β-HSD) enzyme. One must take care, here, to recognize the dilution of exogenous radioactive precursor by endogenously produced precursor which is stimulated by regulatory factor. This phenomenon resulting in a decrease of specific activity of radiolabeled precursor (isotope dilution) can cause the apparent inhibition of enzymatic activity. Thus, it interrupts the real quantitative value.

In order to eliminate the difficulty described above, it is necessory for total inhibition of endogenous precursor production by using a specific inhibitor. In the present experiments, therefore, we have tested the effects of two metabolic inhibitors, cyanoketone (Lipner and Greep, 1971; Young et al., 1982a, b) and trilostane (Potts et al., 1978) which are known to inhibit the activity of 3β-HSD in mammals, amphibians and few fishes, by incubating ^{3}H-pregnenolone with ovarian thecal layers.

Materials and methods

Female rainbow trouts (weight 1.5~2.5 kg) were obtained from the experimental fish farm (France).

After anesthetizing with phenoxy-2-ethanol(0.3 ml/l), the ovaries (subperipheral germinal vesicle or peripheral germinal vesicle stages) were removed and placed in TBSS (trout balanced salt solution)(Jalabert and Fostier, 1984), and ovarian follicles were isolated with the aid of fine forceps. Thecal layers (Fig. 1),

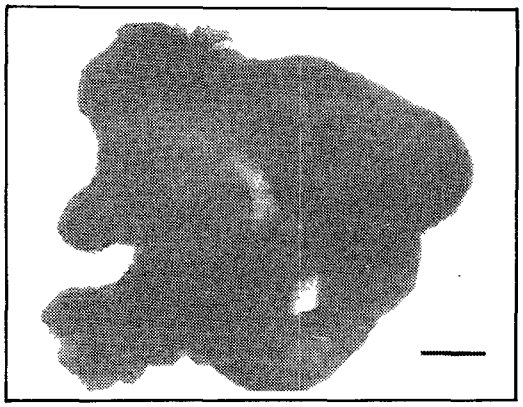

Fig. 1. Manually isolated thecal layer following removal of extrafollicular tissue(Scale bar =75μm).

apparently contaminated with granulosa cells, were manually peeled away after exerting a moderate pressure with a small glass cuplet on the follicle wall. Usually, 5 thecal layers were preincubated for 24 hr in 1 ml of Leibovitz L-15 medium with trilostane or cyanoketone before the addition of radiolabeled pregnenolone (Amersham, 4, 7-^{3}H-pregnenolone).

Incubations (duplicate per treatment) were maintained for 4 hr at 12℃. At the end of incubations, the steroid carriers were added to each incubation. Medium and tissues from each incubation were homogenized and extracted twice in 80% ethanol and dichloromethane. The organic phase (free steroids) was evaporated to dry, dissolved in 200μl ethanol and stored at −30℃ until analysis. The aqueous phase (conjugated steroids) was also evaporated and dissolved in 100μl ethanol plus 100μl distilled water to estimate the part of conjugated steroid and the total extractable radioactivity.

The extracts (free steroids) were separated by thin layer chromatography (TLC) on silica gel plates 60F^{254} (Merck) in benzene : acetone (80 : 20) system. Subsequently, the chromatograms were analyzed with a radiochromatograph scanner (Packard 7222). The radioactive metabolites were identified by checking the isopolarity of the peak of radioactivity with that of the steroid standard in different systems (Table 1). The peaks were eluted (dichloromethane : methanol =9 : 1), and then radioactivities were determined using an Intertechnique SL 4000 liquid scintillation counter. The relative percentage of radioactivity associated with each isolated steroid (Δ^{5}-steroids: P5, 17α-OHP5; Δ^{4}-steroids: 17α-OHP, 17α20βOHP, andros-

Table 1. Thin layer chromatographic solvent systems used in TLC

Solvent system	Ratio	System number	Separated steroid
Benzene : Acetone	80 : 20	I	17α20βOHP/ T+17αOHP5/ 17αOHP+P5/A4
Cyclohexane : Ethylacetate	50 : 50	II	17αOHP/P5
Benzene : Ethanol	90 : 10	III	T/17αOHP/A4

Steroid abbreviations: pregnenolone(P5); 17α-hydroxypregnenolone(17αOHP5); 17α-hydroxyprogesterone (17αOHP); 17α-hydroxy, 20β-dihydroprogesterone(17α20βOHP); androstenedione(A4); testosterone(T).

tenedione and testosterone) was calculated to total radioactivity eluted from initial TLC.

Results

Initial experiments were designed to confirm the effects of cyanoketone (10^{-6} and 10^{-5} M) and trilostane (10^{-5} and 10^{-4} M) on 3β-HSD activity in isolated thecal layers using exogenous radiolabeled pregnenolone. Both cyanoketone and trilostane at all doses used appeared to be efficient (Fig. 2). At a dose of 10^{-5} M trilostane was more efficient than cyanoketone; following experiment, we employed the trilostane as a specific inhibitor of 3βHSD activity. In this experimental procedure granulosa cells were not completely removed, showing that a low activity of 20 β-hydroxysteroid dehydrogenase (20β-HSD) was present in the thecal layer.

The effect of varying doses of trilostane(10^{-8}~10^{-5} M) was presented in Fig. 3. The production of Δ^4-steroids was inhibited in a dose-dependent manner by trilostane treatment, but the proportion of 17α-OHP product was noted at doses of 10^{-7} and 10^{-8} M (32% and 38%, respectively, compared to 22% for the control value).

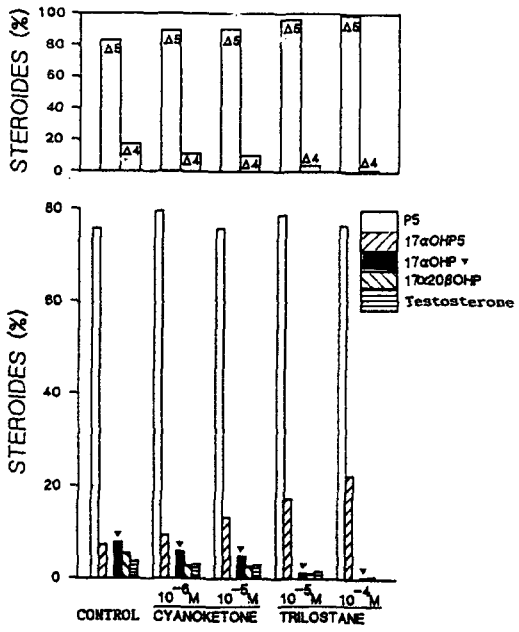

Fig. 2. Effects of cyanoketone and trilostane (3β-HSD inhibitors) on ^{3}H-pregnenolone (172 pmol ml^{-1}) metabolism by isolated thecal layers. Theca layers were incubated with cyanoketone or trilostane for 24 hr and reincubated in the presence of ^{3}H-pregnenolone for 4 hr. The percentage of radioactivity associated with each isolated steroid was calculated to the percentage of total steroid recovered from initial TLC.

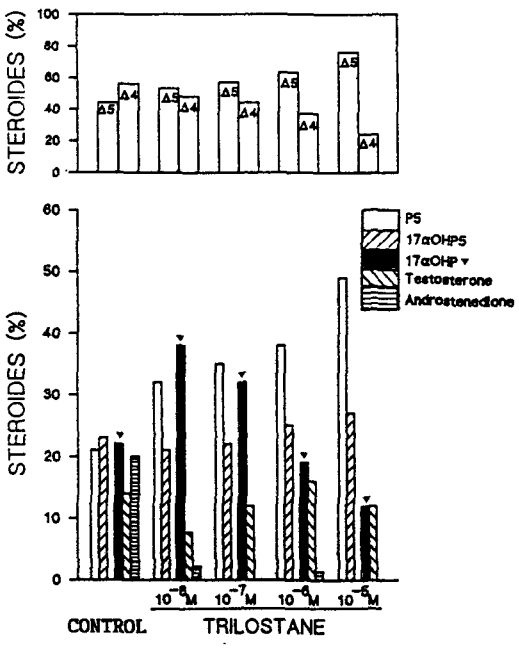

Fig. 3. Effects of trilostane on ^{3}H-pregnenolone (157 pmol ml^{-1}) metabolism by isolated thecal layers. Thecal layers were incubated with trilostane for 24 hr and reincubated in the presence of ^{3}H-pregnenolone for 4 hr. The percentage of radioactivity associated with each isolated steroid was calculated to the percentage of total steroid recovered from initial TLC.

Discussion

Cyanoketone and trilostane are related specifically to 3β-HSD, preventing conversion of $\triangle^5$ steroids to $\triangle^4$ steroids (Petrino et al., 1989; Potts et al., 1979; Young et al., 1982). The doses of inhibitors used in the present experiments were followed a similar levels as described in other experimental models; considered to be effective in inhibiting 3β-HSD activity in fish ovarian follicles [see Young et al., 1982a, b (cyanoketone $3 \cdot 10^{-6}$ M); Petrino et al., 1989 (trilostane $3 \cdot 10^{-5}$ M and $3 \cdot 10^{-6}$ M)].

Compared to the dose of 10^{-5} M (Fig. 2), cyanoketone is a less effective inhibitor of 3β-HSD enzyme than trilostane in the thecal layers when P5 was the precursor. The specificity of cyanoketone in mammals was a less strict (Wolfson et al., 1983). It has been previously used in fishes with different *in vitro* experimental conditions (Young et al., 1982).

Trilostane exerted a dose related inhibitory effect on $\triangle^4$ steroids production from ^{3}H-pregnenolone while elevated 17α-hydroxypregnenolone (17α-OHP5) accumulation. At doses of 10^{-8} and 10^{-7}M, although the total production of $\triangle^4$ steroids is depressed, 17α-hydroxyprogesterone(17α-OHP) production appeared to be increased as compared to control; it may be explained by an inhibition of the metabolism of 17α-OHP. Petrino et al. (1989) reported that trilostane blocked 3β-HSD activity in ovarian follicles of *Fundulus heteroclitus*. It appears that only 10μg ml^{-1} (3×10^{-5} M) or more completely blocked the conversion of pregnenolone to 17α20βOHP in this species, indicating an inactivation of 3β-HSD enzyme.

On the other hand, our results (unpublished data) showed that 20β-HSD was also inhibited at 10^{-5} M and below trilostane dose. It is probably risky to use trilostane as a specific inhibitor of 3β-HSD and to lead the conclusions on this sole enzyme step. Enzyme systems responsible for the metabolism of labeled P5 are susceptible to interference by an inhibitor of 3β-HSD.

Further studies on the enzymatic mechanism may provide useful information relevant to the regulation of steroid biosynthesis.

References

Baek, H. J. 1990. Biosynthèse du stéroîde inducteur de la maturation ovocytaire par les cellules de granulosa du follicule ovarien de truite arc-en-ciel, *Oncorhynchus mykiss*. Thèse de docteur de l'Université Pierre et Marie Curie, Paris 6.

Baek, H. J. and T. Y. Lee. 1993. Analysis of steroid metabolism in the ovarian theca layers of rainbow trout, *Oncorhynchus mykiss*. Korean J. Zool. 36, 77~83.

Jalabert, B. and A. Fostier. 1984. The modulatory effect *in vitro* of oestradiol-17β, testosterone or cortisol on the output of 17α-hydroxy, 20β-dihydroprogesterone by rainbow trout (*Salmo gairdneri*) ovarian follicles stimulated by the maturational gonadotropin s-GtH. Reprod. Nutr. Develop. 24, 127~136.

Lipner, H. and Greep, R. O. 1971. Inhibition of steroidogenesis at various sites in the biosynthetic pathway in relation to induced ovulation. Endocrinology 88, 602~607.

Petrino, T. R., Y. W. P. Lin and R. A. Wallace. 1989. Steroidogenesis in *Fundulus heteroclitus*. I. Production of 17α-hydroxy, 20β-dihydroprogesterone, testosterone and 17β-estradiol by prematurational follicles *in vitro*. Gen. Comp. Endocrinol. 73, 147~156.

Potts, G. O., J. E. Creange, H. R. Harding and H. P. Schane. 1978. Trilostane, an orally activity inhibitor of steroid biosynthesis. Steroids 32, 257~267.

Wolfson, A. J., J. Richards and D. Rotenstein. 1983. Cyanoketone competition with estradiol for binding to the cytosolic estrogen receptor. J. Steroid Biochem. 19, 1817~1818.

Young, G., H. Kagawa and Y. Nagahama. 1982. Inhibitory effect of cyanoketone on salmon gonadotropin-induced estradiol-17β production by ovarian follicles of the amago salmon (*Oncorhynchus rhodurus*) *in vitro*. Gen. Comp. Endocrinol. 47, 357~360.

Young, G., H. Kagawa and Y. Nagahama. 1982a. Secretion of aromatizable $\triangle^4$ androgens by thecal layers during estradiol-17β production by ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) *in vitro*. Biomedical Res. 3, 659~667.

Young, G., H. Kagawa and Y. Nagahama. 1982b. Oocyte maturation in the amago salmon (*Oncorhynchus rhodurus*): *in vitro* effects of salmo gonadotropin, steroids, and cyanoketone (an inhibitor of 3β-hydroxy-$\triangle^5$-steroid dehydrogenase). J. Exp. Zool. 224, 265~275.

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무지개송어 (*Oncorhynchus mykiss*)의 난소내 협막층(theca layers)에서의 pregnenolone 대사: cyanoketone과 trilostane의 저해 효과

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3β-히드록시-△^{5}-스테로이드 탈수소효소 (3β-HSD, 3β-hydroxy-△^{5}-steroid dehydrogenase: △^{5}-스테로이드→△^{4}-스테로이드로의 대사경로에 관여하는 효소)에 대하여 특이적 저해제인 cyanoketone과 trilostane의 저해효과가 ^{3}H-pregnenolone 전구체를 이용하여 무지개송어 난소에서 분리한 여포층, 협막층(theca layers)을 대상으로 비교 관찰되었다. Pregnenolone으로부터 17α-hydroxyprogesterone으로의 대사과정에서 주요 효소인 3β-HSD 활성은cyanoketone 10^{-6}과 10^{-5} M, 그리고 trilostane 10^{-5}과 10^{-4} M의 농도에서 억제되었으며, trilostane이 cyanoketone보다 더 효과적인 억제반응을 보이는것으로 나타났다. Pregnenolone으로부터 △^{4}-스테로이드 대사물 축적에 대한 trilostane의 저해작용은 사용한 농도 즉, 10^{-8}, 10^{-7}, 10^{-6} 그리고 10^{-5} M에 비례하여 나타났으나 완전한 저해효과는 보이지 않았다.