THE INFLUENCE OF ESTRADIOL-17 β ON CORTISOL METABOLISM *IN VITRO*

AKIRA KUMAGAI, SABURO YANO, NOZOMU TAKEUCHI
KAZUHIKO NISHINO AND HYO UEDA

Third Department of Internal Medicine, Osaka University, Osaka

KEISHI MATSUMOTO

Department of Pathology, Osaka University, Osaka

KAZUYUKI TAKEUCHI

Central Clinical Laboratory, Osaka University, Osaka

Through demonstrating the inhibition of cortisol metabolism by other steroids in rat liver *in vitro* (Kumagai *et al.*, 1959), the authors have suggested a metabolic interaction among various steroids in the liver. The disappearance of infused steroids in dogs was also referred as an evidence of such action. While the metabolism of cortisol was found to be inhibited to some extent by an equal or larger amount of other steroids, natural estrogen showed very little inhibition, but rather activation of cortisol metabolism in smaller doses.

This paper deals with the stimulation of cortisol metabolism *in vitro* by the addition of a small amount of estradiol-17 β .

MATERIALS AND METHODS

Wistar strain female rats weighing approximately 150 g were used throughout the experiments. In a crude enzyme system, the rat liver homogenate was prepared as described in the previous reports (Kumagai *et al.*, 1959, 1958). In a purified enzyme system, the microsome-supernatant (24,000 $\times$ g, 30 mins.) and the microsome fractions (100,000 $\times$ g, 60 mins.) obtained by Schneider's differential centrifugation method (Schneider and Hogeboom, 1950) using 0.25 M sucrose were used as enzyme solutions for the studies of cortisol and estrogen metabolism respectively.

The crude system consisted of enzyme solution (0.5 g wet weight of liver), cofactor (boiled supernatant of liver homogenate), 0.1 M citrate, phosphate buffer pH 7.4, cortisol 250 μ g and estradiol-17 β in doses indicated below. The total volume was made to 4.5 ml.

The purified system consisted of enzyme solution (0.5 g wet weight of liver), 1.0 μ M TPN,*¹ 5 μ M glucose-6-phosphate*², 0.5 K U. glucose-6-phosphate dehydrogenase*³, 10 μ M MgCl₂, 1.0 μ M KCl, 10 μ M phosphate buffer pH 7.4, cortisol 250 μ g and estradiol-17 β in given doses. In some of the experiments, 1.0 μ M TPNH*⁴ was used instead of TPN, glucose-6-phosphate and glucose-

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*^{1,2} N.B.C. preparation, purchased.

*³ Prepared from brewer's yeast following Kornberg's method (Kornberg, 1950).

*⁴ Sigma preparation, purchased.

6-phosphate dehydrogenase, and *d,I*-aldosterone*⁵ was used in place of cortisol. The total volume was adjusted to 4.0 ml.

Cortisol was added in aqueous solution containing a trace of ethanol. Estradiol-17 β was added in propylene-glycol water solution. The concentration of KCN was 0.01 *M*. Anaerobic experiments were carried out in Warburg's apparatus filled with N₂ gas.

All experiments were performed in duplicate. Each vessel containing the above solution was incubated at 37°C for 30 mins. with a continuous shaking. After the incubation, the reaction was stopped by the addition of 10 ml of ice-cold water.

Extraction was performed immediately with 20 ml of chloroform with vigorous shaking for 2 mins., centrifugation and separation of the chloroform phase from the aqueous phase. The separated chloroform phase was washed with water and dried over anhydrous Na₂SO₄. After evaporation of 5 ml of the chloroform by warm air stream, the amount of cortisol was determined using isonicotinic acid hydrazide(INH)-reaction (Umberger, 1955; Weichselbaum and Margraf, 1957), ultraviolet absorption at 240 m μ , and Porter-Silber reaction (Porter and Silber, 1950).

Aldosterone was extracted with a method similar to the one used for cortisol, and assayed by ultraviolet absorption and Johnson's bioassay method (Johnson, 1954).

Estrogen was extracted twice with 10 ml of ether, and after dehydration, 5 ml of the ether was evaporated. This was dissolved in 1 ml of ethanol-water (300:190 v/v), and fractionated by ion exchange column chromatography according to Seki's method (Seki, 1958), using a column of 0.8 $\times$ 66 cm and partially esterified Duolite CS-101*⁶ as adsorbent.

Elution was carried out at room temperature (25 $\pm$ 2°C) for 30 hrs. with ethanol-water (300:190 v/v). The flow rate was 2 ml per hr. and each fractions contained about 40 drops (ca. 1 ml). Estrogen in these fractions was determined fluorophotometrically in H₂SO₄ by Bate and Cohen's method (1947).

RESULTS

A. Crude Enzyme System

The influence of estradiol-17 β on the activation rate of cortisol metabolism The metabolic rate of 250 μ g of cortisol was influenced by the concentration of added estradiol-17 β . Estradiol-17 β had a marked effect on the reduction of Δ^4 -3-ketone, but had no effect on the degradation of the side chain. The activation rate for the reduction of Δ^4 -3-ketone caused by the addition of estradiol-17 β was expressed in percentage, that is, an increase in the metabolic rate of cortisol due to estradiol-17 β against a control metabolic rate. As shown in Figure 1, the amount of estradiol-17 β equal to the amount of cortisol (250 μ g) showed no effect. The maximum rate (50%) of activation of cortisol metabolism was observed in the presence of 100 μ g of estradiol-17 β . Below 100 μ g, the activation rate was roughly proportional to the concentration of estradiol-17 β . A marked effect of estradiol-17 β on the reduction of Δ^4 -3-ketone was observed when the metabolic rate of the control was low because of a decreased activity of the added cofactor. The following experiments were therefore carried out.

*Effects of estradiol-17 β on cortisol metabolism in the presence of 0.01 *M* potassium cyanide* The metabolism of cortisol was inhibited by potassium cyanide as reported by Bacchus (1953). It was assumed that potassium cyanide blocked hydrogen transport

*⁵ Kindly supplied by Ciba Pharmaceutical Co.

*⁶ The same results could be obtained with Duolite CS-101 as with Amberlite IRC-50 (Seki, personal communication).

Fig. 1. The influence of estradiol-17 β in various concentrations on the activation rate of cortisol metabolism: 250 μ g of cortisol was incubated with various doses of estradiol-17 β at 37°C for 30 mins. in the crude enzyme system. Activation rate is an increase of cortisol metabolic rate due to estradiol-17 β against the control metabolic rate $\left(\frac{\text{MR studied} - \text{MR control}}{\text{Control metabolic rate (MRC)}} \times 100 \right)$.

Fig. 2. Effects of estradiol-17 β on cortisol metabolism in the presence of 0.01M KCN.

system as a cytochrome inhibitor, only secondarily inhibiting the metabolism of cortisol. In the present experiment, the metabolism of cortisol was reduced to about 1/3 the control following the addition of 0.01 M potassium cyanide (Fig. 2). It was found, however, that the metabolism of cortisol recovered nearly to the control level following the addition of estradiol-17 β even in the presence of potassium cyanide.

Effects of estradiol-17 β on cortisol metabolism under an anaerobic condition Under an anaerobic condition, the metabolism of cortisol was reduced to half the aerobic control level (Fig. 3). Estradiol-17 β restored, however, the cortisol metabolism to a normal level even under an anaerobic condition. This suggests that the addition of estradiol-17 β to the reaction system may result in hydrogen transport, even under an anaerobic condition, leading to the metabolism of cortisol.

B. Purified Enzyme System

The results in the crude enzyme system led the authors to a speculation that the action of estradiol-17 β to increase the reduction of Δ^4 -3-ketone of cortisol was not related with Δ^4 -steroid hydrogenases, but with the TPNH-generating system. In order to investigate further, experiments were performed in a purified enzyme system, *i.e.*, in microsome-supernatant fraction.

As shown in Table 1, the experiments comprise 5 groups. The complete system (group 5) showed 55% reduction of A-ring of cortisol, but no reduction was observed in the groups without the TPNH-generating system (groups 1, 3).

Fig. 3. Effects of estradiol-17 β on cortisol metabolism under an anaerobic condition: 250 μ g of cortisol was incubated in Warburg's apparatus filled with N₂ gas with or without 100 μ g of estradiol-17 β . White bars indicate aerobic (air), black bars anaerobic (N₂ gas).

Table 1. Metabolic rate of A-ring of cortisol in the purified enzyme system

Group	Reaction system	Metabolic rate of cortisol (%) (<i>Δ</i> ⁴ -3-ketone)
1	Enzyme + Cortisol	0
2	Enzyme + Cortisol + TPN	18
3	Enzyme + Cortisol + Estradiol-17 β	0
4	Enzyme + Cortisol + TPN + Estradiol-17 β	36
5	Enzyme + Cortisol + TPN + G-6-P + G-6-P dehydrogenase	55

Enzyme was microsome-supernatant of rat liver (24,000 $\times$ g) and incubation was performed at 37°C for 30 mins. in the final concentrations of cortisol 250 μ g, estradiol-17 β 100 μ g, TPN 1 μ M, glucose-6-phosphate 5 μ M, glucose-6-phosphate dehydrogenase 0.5 KU.

The reduction of *Δ*⁴-3-ketone of cortisol was determined by ultraviolet absorption at 240 m μ and INH reaction.

In group 2 containing only TPN but no glucose-6-phosphate system, the metabolic rate of A-ring of cortisol was 18%, suggesting an intact hydrogen transport system in the microsome-supernatant fraction. It was found, however, that the metabolic rate of cortisol was significantly increased when estradiol-17 β was added instead of glucose-6-phosphate system, the level being 36% in group 4, twice as great as that in group 2. This fact may be regarded as an evidence that estradiol-17 β can be replaced with glucose-6-phosphate system, acting as a hydrogen donor towards TPN to increase the reduction of A-ring of cortisol. It was also found that estradiol did not possess a similar effect on cortisol metabolism in this system as estradiol-17 β did.

The same effect of estradiol-17 β was observed on aldosterone metabolism in the same system (Table 2).

Table 3 shows the metabolic rate of cortisol in the presence of TPNH. The addition of estradiol-17 β caused a decrease of the metabolic rate of cortisol, the level being 61%, compared to 75% of the control.

Table 2. Aldosterone metabolism either in the presence of glucose-6-phosphate system or estradiol-17 β

Group	Reaction system	Metabolic rate of aldosterone (%) (4^4 -3-ketone)
1	Enzyme + Aldosterone + TPN + Estradiol-17 β	30
2	Enzyme + Aldosterone + TPN + G-6-P + G-6-P dehydrogenase	30

250 μ g of *d,l*-aldosterone was incubated under the the same condition as cortisol and the determination was made by ultraviolet absorption at 240 m μ .

Table 3. Effects of estradiol-17 β on cortisol metabolism in the presence of TPNH

Group	Reaction system	Metabolic rate of cortisol (%) (4^4 -3-ketone)
1	Enzyme + Cortisol + TPNH	75
2	Enzyme + Cortisol + TPNH + Estradiol-17 β	61

No activation of cortisol metabolism was observed by the addition of estradiol-17 β in the presence of 1 μ M TPNH.

C. Estrogen Metabolism

As suggested in the foregoing experiments, if estradiol-17 β coupled with cortisol metabolism acts as a hydrogen donor towards TPN, the production of estrone should eventually increase, though the metabolism of estradiol-17 β can take place independently of cortisol metabolism in the microsome fraction with the addition of DPN or TPN. For this purpose, estrone produced in the following system was quantitatively determined, using the column chromatography.

Percentage of produced estrone: added estradiol-17 β (EO/ED $\times$ 100) is also shown in the table as an index to express the estradiol-17 β served as a hydrogen donor towards TPN.

Elution patterns and recoveries of the standard samples are shown in Figure 4 and Table 4, with excellent recovery more than 90%.

Table 4. Recovery rate of standard samples by the column chromatography

Standard sample	ET	16-epi-ET	ED-17 β	EO
Recovery (%) (M $\pm$ S.D.) *1	93 $\pm$ 6.2	96*2	92 $\pm$ 1.5	91 $\pm$ 3.3

*1 Mean and standard deviation. ET; estriol, ED; estradiol, EO; estrone.

*2 Only one recovery test was made in the case of 16-epi-estriol.

No fluorescence of estrogen with sulfuric acid was detected in the microsome preparation (Fig. 4b). The production of estrone was very low in the absence of TPN, regardless of the addition of cortisol (groups 1 and 2 in Table 5). It was increased when TPN was added to the incubation medium (group 3). The addition of cortisol with TPN further increased the production of estrone (group 4). When estriol was added instead of estradiol-17 β , estriol was almost completely recovered.

Table 5. Production and production rate of estrone in the microsome of rat liver

Group	Reaction system	Production of estrone (μg)	EO/ED $\times$ 100
1	Enzyme + Estradiol-17 β	0.5	2.5
2	Enzyme + Estradiol-17 β + Cortisol	0.6	3.0
3	Enzyme + Estradiol-17 β + TPN	1.4	7.0
4	Enzyme + Estradiol-17 β + TPN + Cortisol	4.2	21.0

Enzyme was microsome of rat liver and incubation was made at 37°C for 30 mins. in the final concentrations of estradiol-17 β 80 μg , cortisol 250 μg , TPN 1 μM .

A quarter of each extract was separated by the column chromatography and determined fluorophotometrically. Production rate is EO/ED $\times$ 100, where EO is presented in the third column of the table and ED is the 1/4 amount (20 μg) of estradiol-17 β incubated.

DISCUSSION

Cortisol is known to be metabolized mostly in the liver, requiring TPNH as a cofactor, and enzymes reducing A-ring of cortisol are present in the microsome fraction of rat liver. Tomkins (1956) studied the metabolic pathway of the reduction of A-ring of cortisol and found that 2 sequential double bond saturation occurs initially and then the reduction of a 3-keto-group follows. Later, Forchielli *et al.* (1958) investigated the steroid Δ^4 -hydrogenases of rat liver in whole homogenate and microsome, and showed evidence that the female rat liver had 3 to 4

times greater activity in the rate of Δ^4 reduction than the male liver and contained only Δ^4 -5 α -hydrogenase in microsome whereas the male liver had soluble Δ^4 -5 β -hydrogenase as well.

Although detailed metabolic pathways of estrogen are not clearly understood at present, it is now known that enzymes of conversion between estradiol and estrone are present in the microsome, requiring either DPN or TPN as a cofactor. Recently Talalay and Williams-Ashman (1958) demonstrated that estradiol-17 β or estrone catalyzed transhydrogenation between DPN and TPN by 17 β -hydroxysteroid dehydrogenase in the soluble fraction of the placenta. This was later confirmed by Hollander *et al.* (1959).

The results of the present experiments would indicate a possible interaction between cortisol and estrogen metabolism through TPN. This is schematically represented in Figure 5. In cortisol metabolism, the conversion of estradiol-17 β to estrone results in the reduction of TPN to TPNH, leading to the metabolism of cortisol. Estrogen metabolism may act as a TPNH-generating system for cortisol metabolism like glucose-6-phosphate or isocitric acid dehydrogenation reaction.

Fig. 5. Mode of action of estradiol-17 β in the activation of cortisol metabolism *in vitro*. Conversion of estradiol-17 β to estrone results in the reduction of TPN to TPNH, thus leading to the metabolism of cortisol (see text).

The stimulatory effect of estradiol-17 β on cortisol metabolism, therefore, cannot be observed when such a natural TPNH-generating system is satisfactorily working or the amount of TPNH is sufficient in the reaction system. In other words, the effect of estradiol-17 β is not observed in the optimal condition, but becomes apparent in specifically induced conditions, such as by addition of potassium cyanide, in an anaerobic condition or microsome fraction without the TPNH-generating system. Moreover, the concentration of estradiol-17 β employed in the present experiments is so much greater than a physiological level that the physiological significance of the findings *in vitro* is still an open question.

Several points are also left to be solved for future investigations. In the present experiments, the degradation of the side chain of cortisol was not influenced by

estradiol-17 β in test doses. Troop (1959) recently reported that liver homogenate from male rats had greater activity in the degradation of the side chain than from female rats. Whether this sex difference was due to the enzyme activity or to cofactors should be decided.

Specificity of estradiol-17 β on the activation of cortisol metabolism should also be tested. Hurlock and Talalay (1958) showed that transhydrogenation between di- and triphosphopyridine nucleotide was catalyzed by other steroids such as androsterone in the presence of 3 α -steroid dehydrogenase like estradiol-17 β or estrone in the presence of 17 β -steroid dehydrogenase. It is also possible that these steroids also possess a stimulatory effect on cortisol metabolism.

The difference between natural and synthetic estrogens has been discussed by many investigators. It was already reported from the authors' laboratory that ethinyl-estradiol had a inhibitory effect on cortisol metabolism (Kumagai *et al.*, 1959), though estradiol-17 β in a small dose was now found to have a stimulatory effect.

Concerning the effect of estrogen on the adrenocortical function, it is generally agreed that synthetic estrogen inhibits corticoid production (McKerns, 1957; Berliner and Dougharty, 1958). Many reports on the effect of natural estrogen, however, have been contradictory; some reporting no effect (McKerns, 1957), other stimulatory effect (Holzbauer, 1957), and still others inhibitory effect (Clayton and Hammant, 1957). In this laboratory, the corticoid production has been studied *in vitro*, using the slice of rat adrenals according to Saffran and Schally's method (1955). The addition of estradiol-17 β to the incubation medium increased the production of corticosterone, similar to the addition of ACTH (Kumagai *et al.*, 1960). The mechanism of ACTH action in the biosynthesis of corticosteroids is explained on the basis of an activation of phosphorylation and resulting increase of TPNH in the adrenal (Haynes and Berthet, 1957). When there is a sufficient amount of 17 β -steroid dehydrogenase in the adrenal, the addition of estradiol-17 β should increase the amount of TPNH in the gland, leading to the biosynthesis of corticoids.

Further studies on these problems might provide an important clue for the elucidation of hormone activity.

SUMMARY

1. The influence of estradiol-17 β on cortisol metabolism was studied *in vitro* using the rat liver homogenate and microsome fraction.
2. It was found in the experiments using homogenates that 4^t-3-ketone reduction of cortisol was considerably increased following the addition of a given amount of estradiol-17 β to the medium and this effect of estradiol-17 β became more pronounced under an anaerobic condition or in the presence of potassium cyanide.
3. The stimulatory action of estradiol-17 β on cortisol metabolism and its relation to TPNH-generating system were investigated in the purified enzyme system (24,000 $\times$ g supernatant fraction). It was found that estradiol-17 β was able to replace glucose-6-phosphate system and no effect of estradiol-17 β was observed in the presence of TPNH. Similar stimulatory effect of estradiol-17 β was noted on aldosterone metabolism.

4. Experiments on estrogen metabolism in microsome fraction of the rat liver showed evidence that the addition of cortisol with TPN increased the production of estrone from estradiol-17 β .

5. The results obtained suggest that estradiol-17 β acts as a hydrogen donor to TPN to increase the reduction of A-ring of cortisol.

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