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CIRCADIAN RHYTHM OF 1α,25-DIHYDROXYVITAMIN D_3 PRODUCTION IN EGG-LAYING HENS

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Summary: The activity of renal 25-hydroxyvitamin D_3(25-OH-D_3)-1α- and 24-hydroxylase and the plasma concentrations of vitamin D metabolites were investigated in relation to the ovulatory cycle in egg-laying hens. The time after ovulation was estimated from the position of the egg in the oviduct and the dry weight of the egg-shell. The in vitro renal 25-OH-D_3-1α-hydroxylase activity was significantly enhanced 14-16 hr after ovulation, whereas 25-OH-D_3-24-hydroxylase activity remained unchanged. The plasma level of 1α,25-dihydroxyvitamin D [1α,25-$(OH)_2$-D] was also increased 14-16 hr after ovulation in accord with the enhancement of the renal 1α-hydroxylase activity. The plasma level of 24,25-dihydroxyvitamin D did not change during the ovulatory cycle. These results strongly suggest that 1α,25-$(OH)_2$-D_3 production in the kidney varies in a circadian rhythm during the ovulatory cycle in egg-laying hens.

INTRODUCTION

It is well known that vitamin D_3 is first metabolized to 25-hydroxyvitamin D_3 (25-OH-D_3) in the liver and subsequently to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3] in the kidney (1). Studies of the metabolism and action of vitamin D have been shown that 1α,25-$(OH)_2$-D_3 is the major, metabolically active form of the vitamin, since this metabolite induces a remarkable change in the levels of plasma calcium and phosphate (1).

Recently it has become apparent that 1α,25-$(OH)_2$-D_3 is predominantly synthesized in the kidney during calcium-requiring states such as growth, mammalian pregnancy, lactation and avian egg-laying (2-5). Kenny (5) reported

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Abbreviations used : 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2$-D_3, 24,25-dihydroxyvitamin D_3; M, magnum; I, isthmus; U, uterus.

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for the first time that ovulation in the Japanese quail is accompanied by enhanced renal biosynthesis of $1\alpha,25\text{-}(OH)_2\text{-}D_3$. The present report describes the production of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ in the kidney in a circadian rhythm which is synchronized with the ovulatory cycle in egg-laying hens.

MATERIALS AND METHODS

Renal $25\text{-}OH\text{-}D_3$-hydroxylases activity and plasma levels of vitamin D metabolites were measured at specific time intervals during the ovulation process as indicated by the egg stages along the reproductive tract. These measurements were analyzed in time series related to the circadian rhythm of the ovulation process.

Animals: One hundred egg-laying hens, 500-day-old, Shaver strain, were placed in individual cages for 4 weeks prior to sacrifice. The birds were maintained on a 15 hr-light and 9 hr-dark cycle and were fed a diet for laying hens containing 3.2% Ca and 1.5 IU of vitamin D_3 per gram diet. The number of eggs laid and the average weight of their egg-shells were examined for 3 weeks prior to sacrifice. The birds were killed by decapitation either at 9-11 o'clock in the morning, 14-17 o'clock in the afternoon, or 22-24 o'clock at night. They were then classified into 4 groups according to the presence or absence of an egg and its position in the oviduct: no egg in oviduct, or an egg in the magnum (M), isthmus (I), or uterus (U). Egg-shells found in the uterus (shell gland) were washed thoroughly with water, dried for 3 hr at 100°C and weighed. They were subdivided into 3 groups (U_1, U_2 and U_3) according to the egg-shell weight and the results of Talbot et al.(6), who reported that there is a linear relation between the amount of egg-shell calcification and the time from 8 to 22 hr after ovulation (6). Five birds randomly selected as representatives of each group were used in this experiment. For comparison, two 90-day-old immature hens, maintained on a diet containing 1.5% Ca and 1 IU of vitamin D_3 per gram diet, were used.

Measurements of renal $25\text{-}OH\text{-}D_3$-hydroxylases activity: Kidney homogenates were prepared and incubated with 1.96 nmol (0.1 μCi) of $[26,27\text{-}^3H]\text{-}25\text{-}OH\text{-}D_3$, as described previously (7). Extraction and chromatography of the extracts (Sephadex LH-20 and high pressure liquid chromatography, HPLC, Waters Model 204 equipped with a Zorbax-Sil column) were also performed, as previously described (7). Renal $25\text{-}OH\text{-}D_3$-1α- and 24-hydroxylase activity was calculated from radioactivities of the respective metabolites and expressed in terms of pmol/300 mg tissue/20 min.

Determination of plasma levels of vitamin D metabolites: Four ml of blood plasma was extracted 3 times with dichloromethane. The extracts were applied to a microcolumn (0.7 x 14 cm) of Sephadex LH-20 (2 g) using a solvent of 60% chloroform - 40% hexane. The 25-OH-D fraction in the Sephadex column was repurified on a microcolumn (1 x 8.5 cm) of Celite according to the method of Hughes et al.(8). The $24,25\text{-}(OH)_2\text{-}D$ and the $1\alpha,25\text{-}(OH)_2\text{-}D$ fractions in the Sephadex column were individually applied to HPLC columns. To compensate for recovery during extraction and chromatography, 3000 cpm of $[^3H]\text{-}25\text{-}OH\text{-}D_3$, $[^3H]\text{-}24,25\text{-}(OH)_2\text{-}D_3$ and $[^3H]\text{-}1\alpha,25\text{-}(OH)_2\text{-}D_3$ were added to each plasma sample prior to extraction.

The assay for 25-OH-D and $24,25\text{-}(OH)_2\text{-}D$ was performed by the competitive protein binding method using plasma from rats fed a low Ca, vitamin D deficient diet as a binding protein (9).

The assay for $1\alpha,25\text{-}(OH)_2\text{-}D$ was carried out by Eisman's radioreceptor assay utilizing the chick intestinal receptor (10). The sensitivity of this assay was increased to 4 pg/tube by using $[23,24\text{-}^3H]\text{-}1\alpha,25\text{-}(OH)_2\text{-}D_3$ of high specific activity (100 Ci/mmol).

Chemicals: Crystalline 25-OH-D_3 was purchased from Philips-Duphar Co. of the Netherlands, and crystalline 1α,25-$(OH)_2$-D_3 and 24R,25-$(OH)_2$-D_3 were kindly donated from Dr. M. R. Uskokovic, Hoffmann-LaRoche Inc., Nutley, New Jersey. [23,24-^{3}H]-1α,25-$(OH)_2$-D_3 and [26,27-^{3}H]-24,25-$(OH)_2$-D_3 were synthesized biologically from respective radioactive 25-OH-D_3, as previously reported (7).

RESULTS

The average time after ovulation was calculated from the data of Warren et al.(11) and Talbot et al.(6). They reported that the ovum spends its first 5 hr in the magnum and isthmus, and the last 19 hr of the 24 hr ovulatory cycle in the uterus. Therefore, eggs found in the uterus were sub-

Table 1. Subgrouping of the birds with an egg in uterus.

Hen No.	Egg-shell weight*	Estimated time after ovulation**		Groups
		hrs	hrs	
9	4.5	6.3		
10	3.0	5.8		
11	6.2	7.2	6.3 ± 0.4	U_1
13	2.3	5.3		
14	5.7	7.0		
28	65.7	15.8		
30	59.3	15.0		
44	51.9	14.1	14.8 ± 0.4	U_2
46	49.7	13.8		
48	59.7	15.3		
54	100.5	21.6		
55	110.4	22.8		
67	101.9	21.8	21.4 ± 0.5	U_3
84	88.2	19.5		
87	98.0	21.4		

* The egg-shell weight is expressed as percentage of the individual average weight of egg-shell laid for 3 weeks prior to sacrifice.

** The time after ovulation was estimated from the egg-shell weight according to the report of Talbot et al.(6).

divided into 3 groups (U_1, U_2 and U_3) according to the dry weights of their egg-shells. The egg-shells produced by each hen during the 3 weeks prior to sacrifice were weighed individually. Each of the shell weights presented in Table 1 is expressed as a percentage of the average weight of the egg-shells laid by each hen during the 3 weeks prior to sacrifice. The time after ovulation was calculated from these values according to the report of Talbot et al.(6)(Talbe 1).

The radioactive peak suspected to be [^{3}H]-1α,25-$(OH)_2$-D_3 in the Sephadex LH-20 column migrated to exactly the same position as synthetic 1α,25-$(OH)_2$-D_3 on HPLC columns. No other radioactive peaks were detected. When the 24, 25-$(OH)_2$-D_3 fraction in the Sephadex LH-20 column was applied to HPLC columns, at least 85% of the radioactivity coincided with authentic 24R,25-$(OH)_2$-D_3. The radioactive peak suspected to be [^{3}H]-24,25-$(OH)_2$-D_3 was found to be periodate sensitive after treatment with 5% sodium periodate at room temperature overnight.

The in vitro renal 25-OH-D_3-1α-hydroxylase activity was significantly greater in group U_2 than that in groups M, I and U_1, and it fell to the latter level in group U_3. The 25-OH-D_3-24-hydroxylase activity, on the other hand, did not change at all during the ovulatory cycle (Fig 1).

Plasma levels of vitamin D metabolites coincided with the results of the in vitro renal hydroxylases activity. The plasma concentration of 1α,25-$(OH)_2$-D was 3 times higher in group U_2 than that in the other groups, whereas plasma 24,25-$(OH)_2$-D levels remained constant during the reproductive cycle. The plasma levels of 25-OH-D in groups I and U_1 appeared to be somewhat lower than those in groups M, U_2 and U_3 (Fig 2).

Laying hens without eggs in their oviduct were assumed to be in a clutch. Their renal 1α-hydroxylase activity was only 78.7 $\pm$ 20.2 pmol/300 mg tissue/20 min. The 24-hydroxylase activity was, on the other hand, significantly enhanced in these birds. In some of the birds 24-hydroxylase activity was even higher than 1α-hydroxylase activity. Plasma 1α,25-$(OH)_2$-D levels also remain-

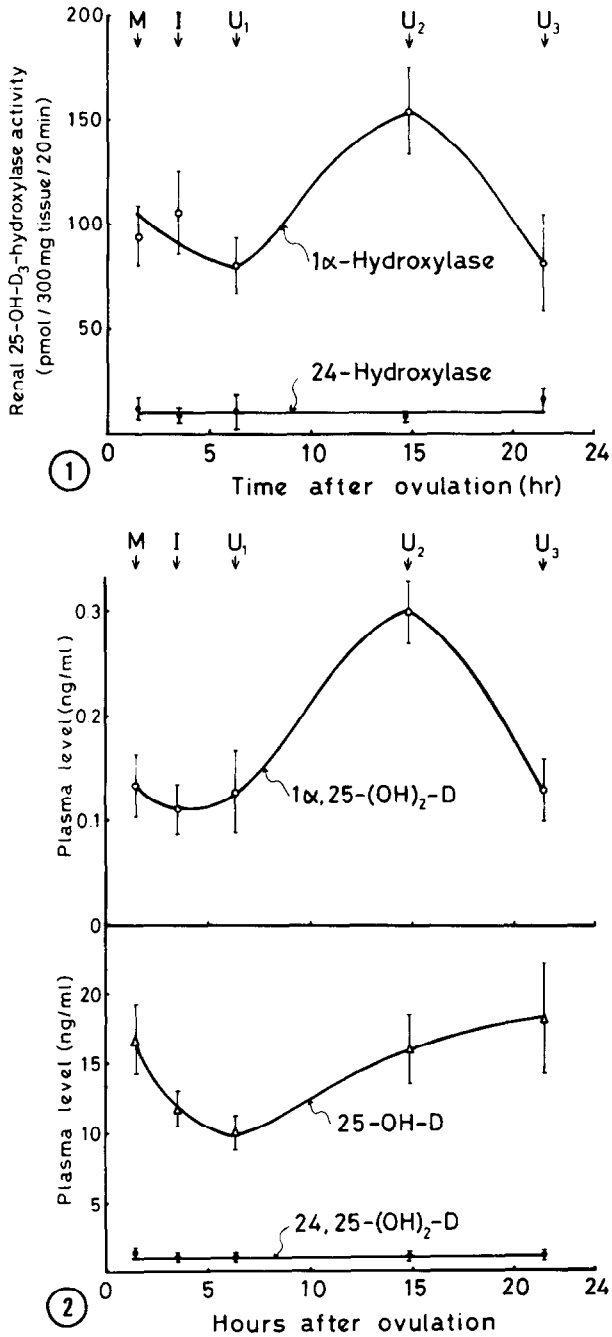

Fig 1. The time course of change in renal 25-OH-D_3-1α- and 24-hydroxylase activity after ovulation. The time period after ovulation was estimated from the position of the egg in the oviduct according to the results of Talbot et al.(6). The points and vertical bars represent the means and standard errors of 5 birds.

Fig 2. The time course of change in plasma levels of vitamin D metabolites after ovulation. The points and vertical bars represent the means and standard errors of 5 birds.

Table 2. Renal 25-OH-D_3-1α- and 24-hydroxylase activity and plasma concentrations of vitamin D metabolites in laying hens without egg in oviduct and in immature hens.

	Laying hens without egg in oviduct	Immature hens (90-day-old)
Renal hydroxylase activity	pmol/300 mg tissue/20 min	
1α-hydroxylase	78.7 ± 20.2	2.9, 6.3
24-hydroxylase	38.6 ± 12.6	1.3, 1.9
Plasma concentrations	ng/ml	ng/ml
25-OH-D	14.0 ± 2.0	14.5, 20.5
24,25-$(OH)_2$-D	0.63 ± 0.18	0.76, 1.74
1α,25-$(OH)_2$-D	0.14 ± 0.02	0.03, 0.05

ed within the levels of groups M - U_1. Ninety-day-old immature hens showed very low 1α- and 24-hydroxylases activity. Their plasma levels of 1α,25-$(OH)_2$-D were also very low (0.03-0.05 ng/ml)(Table 2).

DISCUSSION

Kenny (5), Montecuccoli et al.(12) have reported independently that renal 1α-hydroxylase activity is considerably enhanced in egg-laying hens with eggs in their oviducts, as compared to that in hens without eggs in their oviducts and in immature hens. Spanos et al.(13) has also reported that the circulating 1α,25-$(OH)_2$-D level is markedly increased during egg-laying.

The present report confirms their observations. In addition, our results demonstrate for the first time that 1α,25-$(OH)_2$-D is produced in a circadian rhythm during the ovulatory cycle in egg-laying hens. Both renal 1α-hydroxylase activity and the plasma 1α,25-$(OH)_2$-D level were significantly higher in stage U_2 (14-16 hr after ovulation) than those in the other stages. 1α,25-$(OH)_2$-D_3 has been thought to be essential for intestinal Ca absorption, bone mobilization and calcification, and possibly for Ca transport in the uterus. Since most of the birds laid eggs at 9-12 o'clock in the morning, the stage

U_2 appeares to be mainly during the dark period. The stage U_2 coincides with the most active period in egg-shell calcification. Thus the enhancement of the *in vitro* renal 1α-hydroxylase activity and that of the plasma 1α,25-$(OH)_2$-D level might suggest the important role of 1α,25-$(OH)_2$-D_3 in the remodeling of medullary bone and in Ca transport in the uterus.

Factors influencing 1α,25-$(OH)_2$-D production during the ovulatory cycle have not been established. Tanaka *et al.*(14) demonstrated a strong regulation by sex hormones of the renal 25-OH-D_3-hydroxylases in birds. Since sex hormones are secreted in a circadian rhythm during the ovulatory cycle (15), some of them (probably estrogen) may be closely related to the circadian rhythm of 1α,25-$(OH)_2$-D_3 production in egg-laying hens.

The mechanism and biological significance of the circadian rhythm of 1α,25-$(OH)_2$-D production are of considerable interest, and are currently under investigation in our laboratories.

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