EVIDENCE FOR CIRCADIAN RHYTHMS IN THE SERUM LEVELS OF THE VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN AND IN THE ACTIVITY OF THE 25-HYDROXYVITAMIN D_3-1-α-HYDROXYLASE IN THE CHICK

Studies on the mode of action of calciferol

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1. Introduction

The hormonally active metabolite of vitamin D_3, $1,25(OH)_2D_3$, is responsible for increasing intestinal absorption and bone mobilization of both calcium and phosphate [1]. Studies on the molecular action of vitamin D have shown that in the intestine $1,25(OH)_2D_3$ induces the synthesis of a vitamin D-dependent calcium binding protein (CaBP) [2]. While the exact function of this protein is unknown, CaBP appears to have a role in calcium absorption. Evidence for this comes from studies in which the steady state level of CaBP in the intestine has been shown to be highly correlated with the magnitude of vitamin D-stimulated intestinal calcium absorption [3]. This CaBP has also been detected in serum and a positive correlation has been shown between the serum levels and the intestinal levels of this protein [4,5].

In the non-pregnant animal [6], $1,25(OH)_2D_3$ is produced exclusively through the action of the renal, mitochondrially localized, cytochrome P450 containing enzyme, the 25-hydroxyvitamin D_3-1α-hydroxylase (1-hydroxylase) [7,8]. It is recognized that control of the vitamin D endocrine system occurs through the stringent regulation of this enzyme. Therefore studies on the regulation of $1,25(OH)_2D_3$ production have focused on factors which affect the activity of this enzyme. Among the known regulatory factors are dietary and serum calcium levels [9]. When dietary calcium is restricted, the activity of the 1-hydroxylase increases; when adequate calcium is available, the activity of the enzyme decreases [10].

In [11] we reported an apparent rhythm in the activity of the renal 1-hydroxylase from measurements at regular intervals following a sudden dietary calcium deprivation. Circadian rhythms have been reported in both 1-hydroxylase activity and in plasma levels of $1,25(OH)_2D_3$ in egg-laying hens, and correlated with the hen's ovulatory cycle [12]. This agreed with observations that hens with eggs in their oviducts exhibit an increase in 1-hydroxylase activity [13,14]. However, neither of these studies attempted to determine if other aspects of the vitamin D endocrine system, such as serum CaBP levels, also exhibit a diurnal rhythm. It is not clear whether the apparent rhythm that was observed in the activity of the 1-hydroxylase is endogenous or whether it resulted from the stress of the sudden dietary calcium deprivation in the cockerels, or from the ovulatory cycle in the egg-laying hens.

This report describes experiments in which both serum CaBP levels and the activity of the 1-hydroxylase were measured at 1 h and 0.5 h intervals over 26 h in sexually immature male chicks which were maintained on constant dietary calcium and phosphorus conditions.

2. Materials and methods

White Leghorn cockerels were obtained on the day of hatch from Pace/Setter Hatchery (Alta Loma CA). They received ad libitum a moderately low calcium (0.7%), normal phosphate (0.7%), vitamin D_3-supplemented diet [15] and were maintained in a room providing constant temperature and a 12:12 h light:dark cycle. When 3–4 weeks old, the birds were sacrified

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by decapitation in groups of 4 at 0.5 h and 1 h intervals over 26 h. At the time of sacrifice care was taken not to interrupt the established light:dark cycle by exposing the birds to light during their normal dark periods.

2.1. *25-Hydroxyvitamin D_3-1α-hydroxylase activity measurements*

Upon sacrifice, kidneys were removed from each bird, washed in ice-cold 0.15 M NaCl and immediately homogenized in 0.25 M sucrose to yield a 10% whole kidney homogenate. 25-Hydroxyvitamin D_3-1α-hydroxylase activity was determined as in [8]. This method involves the conversion of 25-[26,27-^{3}H]-$(OH)D_3$ into 1,25-[^{3}H]$(OH)_2D_3$ or 24,25-[^{3}H]$(OH)_2D_3$. Aliquots were removed from each incubation at 0, 4, 8 and 12 min following initiation of the reaction, and the lipids were extracted from each aliquot as in [16]. The chloroform layer was dried under N_2 and, following filtration through a 0.5 μm Millipore Teflon filter, the sample was transferred in 200 μl 5% isopropanol in hexane to a WISP 710 (Waters Assoc.) automatic injector for high-pressure liquid chromatography. A solvent gradient from 5–40% isopropanol in hexane was run for 20 min at a flow rate of 2 ml/min through a 50 cm μ-Porasil column (Waters). This system results in the complete separation of the substrate 25-[^{3}H]-$(OH)D_3$ and the products of the reaction, 1,25-[^{3}H]-$(OH)_2D_3$ and 24,25-[^{3}H]$(OH)_2D_3$. Fractions from the HPLC were collected in liquid scintillation vials and the solvent was evaporated under air. Liquid scintillation mixture (5 g phenylbiphenyloxadiazole-1,3,4/l toluene) (8 ml) was added to each vial and the radioactivity measured in a Beckman LS-233 liquid scintillation counter. The % radioactivity recovered as $1,25(OH)_2D_3$ was plotted as a function of incubation time and the rate of $1,25(OH)_2D_3$ production was determined from the slope of the line thus formed. Products produced (pmol/min) were calculated and the protein concentration of each kidney homogenate was determined by the biuret method [17].

2.2. *Serum CaBP determination*

At the time of sacrifice blood was immediately collected and allowed to coagulate at 4°C. Serum was then removed and frozen at −20°C until the time of assay. Levels of CaBP were measured by a radioimmunoassay as in [5]; 400 μl serum were required for assay.

2.3. *Chemicals*

25-[26,27-^{3}H]Hydroxyvitamin D_3 (obtained from Amersham-Searle, Chicago IL) was added to unlabeled 25-hydroxyvitamin D_3 (kindly supplied by M. R. Uskoković, Hoffmann-LaRoche, Nutley NJ) to yield spec. act. 75 Ci/mol.

3. Results and discussion

In the chick serum [CaBP] fluctuates with a diurnal rhythm (fig.1). Levels of CaBP during the peak periods of this rhythm are ~2.5-fold higher than the levels during the nadir periods. For birds maintained under a 12:12 h light:dark cycle with lights on at 06:00 h, the peak periods in this rhythm occur around 24:00 h and the nadir levels occur between 10:00–12:00 h. Fig.2 indicates that the activity of the 1-hydroxylase also exhibits a diurnal rhythm and there is ~2.5-fold change between the peaks and nadirs of this rhythm. Peak periods in the activity of the 1-hydroxylase for these birds were observed to occur between 16:00–18:00 h and nadir periods were observed between 04:00–06:00 h.

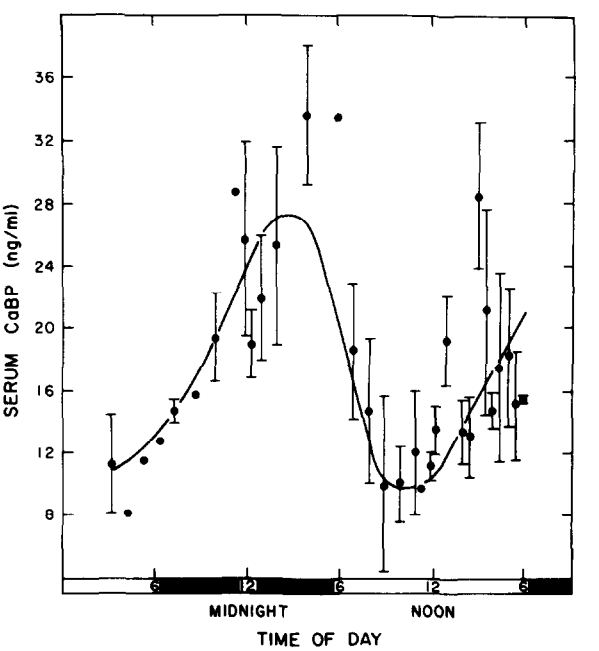

Fig.1. Serum calcium-binding protein levels in chicks over 26 h measured by radioimmunoassay. Each point represents the mean of 4 birds ± SE. Darkened bars on the abscissa correspond to periods of darkness in the bird's light:dark cycle.

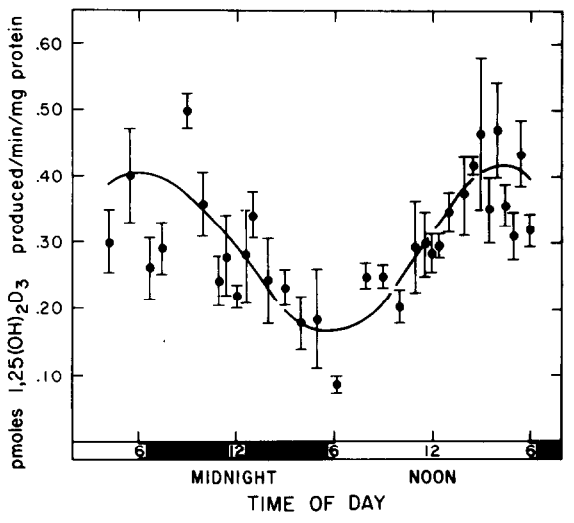

Fig.2. Specific activity of the renal 25-hydroxyvitamin D_3-1α-hydroxylase over 26 h. Each point represents the mean of 4 birds ± SE. Darkened bars on the abscissa correspond to periods of darkness in the bird's light:dark cycle.

The appearance of circadian rhythms in serum CaBP levels and in 1-hydroxylase activity in sexually immature male chicks, maintained under constant environmental and dietary conditions, strongly indicates that these rhythms are endogenous. Other aspects of the vitamin D endocrine system may fluctuate diurnally; studies are underway to detect such rhythms. The effect on these rhythms of the light:dark cycle, which affects other circadian rhythms [18,19], is also under investigation.

Circadian rhythms exist for the levels of many hormones [19]. Diurnal rhythms have also been reported for plasma calcium levels in both the rat [20,21] and in humans [22], and for active calcium transport in rat intestine [23]. Similar diurnal rhythms are also observed in the levels of serum CaBP and in the activity of the 1-hydroxylase.

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