Increase in Calcium Binding Protein-D28K Contents in the Shell Gland by an Injection of 1,25-Dihydroxyvitamin D3 into the Shell Gland Lumen in Laying Hens

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The objective of this study was to determine whether vitamin D directly stimulates the shell gland to induce calcium binding protein-D28K (CaBP-D28K) in vivo, which plays an essential role in calcium transport for shell formation. White Leghorn laying hens were injected with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or saline (control) into the shell gland lumen at 3 h (precalcification phase) or 8 h (early calcification phase) after oviposition. The CaBP-D28K levels in the shell gland and intestine were examined by immunocytochemistry and Western blot analysis. Also, the changes in the serum calcium levels were examined. The immunoreaction products for CaBP-D28K were localized in the tubular glands of the shell gland at precalcification and calcification phases in both the 1,25(OH)₂D₃ treated and control hens. At the calcification phase the density of immunoreaction band for CaBP-D28K was greater in the 1,25(OH)₂D₃ treated hens than in the control hens, whereas difference in the densities of CaBP-D28K was not found between 1,25(OH)₂D₃ treated and control hens at the precalcification phase. No significant differences in the densities of immunoreaction products for the intestine CaBP-D28K and in the serum calcium concentrations were observed between the 1,25(OH)₂D₃ treated and control hens. These results suggest that 1,25(OH)₂D₃ directly stimulates the shell gland to induce CaBP-D28K.


Key words : shell calcification, shell gland, calcium binding protein, vitamin D

Introduction

It has been suggested that calcium binding protein-D28K (CaBP-D28K) plays an essential role in calcium absorption in the intestine (Nys, 1993; Wu et al., 1994) and calcium transport for shell calcification in the shell gland (Bar et al., 1992 b; Nys, 1993). Sequential hydroxylations of vitamin D₃, first in the liver and then in the kidney, give rise to the hormonally active form 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (Taylor and Dacke, 1984). Laying hens maintained under a vitamin D deficient condition produce soft shelled eggs, whereas they lay normal shelled eggs by addition of vitamin D in feed (Narbaizt et al., 1987; Bar et al., 1988). In the intestine 1,25(OH)₂D₃ induces CaBP-D28
K by the interaction with nuclear vitamin D receptor (VDR) (Christakos et al., 1979; Zhou and Norman, 1995) and via nongenomic pathway (Kim et al., 1996). Such an increase in the intestine CaBP-D28K content is responsible for the increase in the plasma calcium level. CaBP-D28K contents in the shell gland are increased by the oral or intramuscular administration of vitamin D (Corradino et al., 1968; Navickis et al., 1979; Striem and Bar, 1991) and estrogen (Navickis et al., 1979; Corradino et al., 1993; Bar et al., 1996). However, it is also possible that the plasma calcium, the level of which is increased by the action of vitamin D, may affect the CaBP-D28K level in the shell gland, because CaBP-D28K mRNA in the shell gland significantly increases with calcium influx (Bar et al., 1992a; Nys et al., 1992). Although Corradino (1993) found that 1,25(OH)2D3 induced the shell gland CaBP-D28K in vitro, it still remains to be determined whether 1,25(OH)2D3 directly stimulates the shell gland to induce CaBP-D28K in vivo.

Coty (1980) reported the presence of specific and high affinity binding protein for 1,25(OH)2D3 in the mucosal tissue of the shell gland. Also, we localized VDR in the tubular glands and mucosal epithelium in the shell gland (Yoshimura et al., accepted). Ieda et al. (1995) showed that the expression of CaBP-D28K mRNA increased in a correlation with the increase of VDR mRNA in the shell gland, and their expression was greater during shell calcification than the other phases. Therefore, there may be a close relationship between the CaBP-D28K induction and the interaction of 1,25(OH)2D3 with VDR in the shell gland. The objective of this study was to determine whether 1,25(OH)2D3 directly stimulates the shell gland to induce CaBP-D28K in vivo. We injected 1,25(OH)2D3 directly into the shell gland lumen at precalcification and calcification phases, and examined the changes in the immunoreactive CaBP-D28K level in the shell gland. Also we examined the changes in the intestinal CaBP-D28K level and plasma calcium level to confirm that the injection of 1,25(OH)2D3 into the shell gland lumen does not affect the levels of them.

Materials and Methods

Treatment of Birds and Sample Collections

White Leghorn hens (approximately 400 days old) regularly laying four or more eggs in a sequence were kept in individual cages under a light condition of 14 L : 10 D, and provided with feed and water ad libitum. In the experimental group, birds were treated with 100 nM 1,25(OH)2D3 (Nakarai, Kyoto, Japan) in 1 ml saline by an injection into the shell gland lumen through the vagina, using a glass tube and syringe. Injection was performed at 3 h (precalcification phase, n=4 birds) or 8 h, which is the early calcification phase, (calcification phase, n=4 birds) after oviposition of the first or second egg in a sequence. It has been accepted that ovulation occurs approximately 30 min after oviposition in hens (Warren and Scott, 1934). Control hens were subjected to same treatment except that the 1,25(OH)2D3 solution was replaced with 1 ml saline (n =4 birds in each precalcification and calcification phase group). Blood samples (2 ml) were collected before and 2 h after the treatment with 1,25(OH)2D3 or saline for the measurement of calcium contents in the serum. Two hours after the treatment, hens
were killed by decapitation, and the mucosal tissues of the shell gland and duodenum were collected.

**Tissue Preparation for Western Blot of CaBP-D28 K**

Mucosal tissues of the shell gland and duodenum were homogenized in 5 volumes of buffer (13.7 mM Tris-HCl, pH 7.4, 0.12 M NaCl, 4.74 mM KCl, 1 mM phenylmethylsulfonyl fluoride) with a Polytron homogenizer. The homogenates were centrifuged at 10,000 g for 10 min and 38,000 g for 20 min. Proteins in the supernatant were precipitated with sodium sulfate at a final concentration of 48%, and were centrifuged at 10,000 g for 20 min. The precipitates were dissolved in water before use. These processes were carried out at 4°C.

The isolated proteins (1 to 10 μg/lane) were run on SDS-PAGE (4% stacking gel and 15% separation gel) as described by Laemmli (1970). Then the separated proteins were electrophoretically transferred onto the nitrocellulose membrane (Amersham Int., Amersham, UK). The membrane was washed with Western buffer consisting of 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% (v/v) Tween 20, and 0.05% (w/v) BSA for 15 min. The membrane was then incubated with 5% (w/v) milk casein for 30 min in Western buffer, and with anti-CaBP-D28 K antibody (Sigma Chem, St. Louis, MO, USA) diluted to 1:1,000 in Western buffer for 1 h. After being washed with Western buffer for 30 min (10 min × 3 times), the membrane was incubated with anti-mouse IgG conjugated with alkaline phosphatase (Vector Lab., Burlingame, CA, USA) diluted to 1:5,000 in Western buffer. The membrane was then washed with Western buffer for 30 min (10 min × 3 times) and incubated with a reaction mixture (3 mg 5-bromo-4-chloro-3-indolyolphosphate and 3 mg nitro blue tetrazolium and 1 mM MgCl₂ in 10 ml 0.1 M Tris-HCl, pH 9.6) to develop the color showing the immunoprecipitates. The density of each band was examined with an image analyzer (NIH Image 1.60) after scanning the image with an image scanner (Epson GT 5000 ART, Epson Co., Tokyo, Japan).

**Tissue Preparation for Immunocytochemistry of CaBP-D28 K**

Shell gland tissues were fixed with Bouin’s solution and embedded in paraffin. Paraffin sections (6 μm thick) of them were air dried on slides. After deparaffinized, the sections were incubated with 5% milk casein in PBS for 30 min, and then incubated with anti-CaBP-D28 K antibody diluted to 1:200 in PBS. The immunoreaction products of CaBP-D28 K were detected by Vectastain ABC kit for mouse IgG (Vector Lab., Burlingame, CA, USA). Control slides were also prepared as described above, except that the primary antibody (anti-CaBP-D28 K antibody) was replaced with normal mouse IgG (Vector Lab., Burlingame, CA, USA).

**Measurement of Calcium Concentrations in Serum**

Serum samples were diluted to 1:4 with water, and the calcium concentrations in them were examined by Fuji Dry-Chem 5,000 (Fuji Film Co., Kanagawa, Japan). Then the serum calcium concentration (mg/dl) was obtained.
**Statistical Analysis**

The differences in the serum calcium concentrations between pre- and post-1,25(OH)\(_2\)D\(_3\) injections were examined by Student’s t test, and significance of difference was taken when \(P<0.05\).

**Results**

The hens of precalcification phase (4.5 h after the expected time of ovulation) and those of calcification phase (9.5 h after expected time of ovulation) contained an egg in the isthmus and shell gland, respectively, when examined 2 h after the treatment. The immunoreaction products for CaBP-D28 K were localized in the tubular glands of the shell gland in all hens examined (Fig. 1 a, b). These immunocytochemical results on the localization of CaBP-D28 K were equally observed in four repeated observations. No staining was observed in the control sections which were stained without the anti-CaBP-D28 K antibody (Data not shown).

Western blot analysis showed the presence of CaBP-D28 K, whose molecular weight was 28 KDa, in the shell gland and intestine of all hens examined (Fig. 2 a, b and 3). In the calcification phase, the density of immunoreaction band for CaBP-D28 K was greater in the 1,25(OH)\(_2\)D\(_3\) treated hens than in control hens which received saline (Fig. 2 a). In contrast, differences in the density of CaBP-D28 K were not observed between 1,25(OH)\(_2\)D\(_3\) treated and control hens at precalcification phase (Fig. 2 b). Similar results were observed in four replicate experiments.

In the calcification phase, no significant difference in the density of immunoreactive bands for intestine CaBP-D28 K was observed between the 1,25(OH)\(_2\)D\(_3\) treated and control hens (Fig. 3). The same results were observed in the precalcification phase. In both precalcification and calcification phases, there was no significant difference in the serum calcium concentrations between the 1,25(OH)\(_2\)D\(_3\) treated and control hens (Fig. 4).

![Fig. 1a, b. Sections of the shell gland 10 h after oviposition (calcification phase), treated with or without 1,25(OH)\(_2\)D\(_3\), and immunostained for CaBP-D28 K. Immunoreactions for CaBP-D28 K are localized in the tubular glands (arrow) in both 1,25(OH)\(_2\)D\(_3\) stimulated (A) and non-stimulated (B) shell gland. E = mucosal epithelium. Scale bar = 30 μm.](image-url)
Fig. 2a, b. Western blot for CaBP-D28K in the shell gland treated with (treatment) or without (control) 1,25(OH)2D3 2h before tissue collection. Ten, 5, 2.5 or 1 µg of sample proteins (values are shown in the bottom of the figure) obtained from one hen in each treatment (lane T) and control group (lane C) were applied in each lane. (A) Shell gland 10h after oviposition (calcification phase). A band is observed (arrow head) in the treated hen but not in the control hen within 1 µg sample lanes. (B) Shell gland 5h after oviposition (precalcification phase). No difference is observed in the density of bands between the treated and control hen.

Discussion

The significant finding of this study was that injection of 1,25(OH)2D3 into the shell gland lumen increased CaBP-D28K contents in the shell gland during calcification phase. CaBP-D28K plays an essential role in calcium transport for shell calcification in the shell gland (Bar et al., 1992b; Nys, 1993). In this study, CaBP-D28K was localized in the tubular glands of the shell gland in control hens, supporting the reports of Lippiello and Wasserman (1975). Furthermore, the results of the present study indicated that treatment with 1,25(OH)2D3 did not affect the localization of CaBP-D28K in the shell gland as compared with the control.
Fig. 3. Western blot for CaBP-D28K in the intestine obtained from the hens 10 h after oviposition. 1,25(OH)2D3 or saline (control) were injected into their shell gland 2 h before tissue collection. Ten, 5, 2.5 or 1 µg of sample proteins (values are shown in the bottom of the figure) obtained from one bird in each treatment (lane T) and control group (lane C) were applied in each lane. The density of bands is not different between the treated and control hens.

Fig. 4. Changes in the serum total calcium concentrations before and 2 h after the injection with 1, 25(OH)2D3 or saline into the shell gland lumen. 1, 25(OH)2D3 (treatment) or saline (control) was injected 3 h (precalcification phase) or 8 h (early calcification phase) after oviposition. There is no significant difference in the calcium concentrations 2 h after the treatment between the 1, 25(OH)2D3 treated and control hens within each precalcification and calcification phase. PC0 = precalcification phase hens before treatment, PC2 = precalcification phase hens 2 h after treatment, C0 = calcification phase hens before treatment, C2 = calcification phase hens 2 h after treatment.
Oral or intramuscular administration of vitamin D increases CaBP-D28K contents in the shell gland (Corradino et al., 1968; Navickis et al., 1979; Striem and Bar, 1991). However, it is unclear whether vitamin D which is provided by such manners directly stimulates the shell gland tissue to increase CaBP-D28K contents, because there is a possibility that circulating vitamin D may increase calcium absorption in the intestine (Christakos et al., 1979; Zhou and Norman, 1995; Kim et al., 1996), and then the increased plasma calcium level may lead to the increase in CaBP-D28K in the shell gland (Bar et al., 1992a; Nys et al., 1992). In this study, we injected 1,25(OH)₂D₃ directly into the shell gland lumen, and confirmed that treatment of birds with 1,25(OH)₂D₃ during calcification phase increased shell gland CaBP-D28K contents without significant changes in the intestine CaBP-D28K level and also plasma calcium level. These results suggest that 1,25(OH)₂D₃ directly stimulates the shell gland tissue to induce CaBP-D28K.

Interaction of 1,25(OH)₂D₃ with VDR in the shell gland (Coty, 1980; Yoshimura et al., accepted) may be responsible for this induction of CaBP-D28K.

It was observed in this study that the shell gland CaBP-D28K content was increased by an injection with 1,25(OH)₂D₃ during calcification phase, but not during precalcification phase. Therefore, it is likely that the induction of CaBP-D28K in response to 1,25(OH)₂D₃ is greater during shell calcification phase than during precalcification phase. This result also supports the previous report suggesting that the expression of CaBP-D28K mRNA in the shell gland is greater during calcification phase than the other phases (Ieda et al., 1995). Nys et al. (1989) found daily increase of CaBP-D28K mRNA associated with shell formation and the absence of concomitant changes in CaBP-D28K concentration in the shell gland. Based on these results, they suggested that post-transcriptional process might exist and stimuli other than the sex steroids or 1,25(OH)₂D₃ may be involved in the regulation of CaBP-D28K synthesis in the shell gland. However, the present results clearly suggest that 1,25(OH)₂D₃ significantly increases CaBP-D28K content in the shell gland during calcification phase.

There are reports suggesting that estrogen plays a significant role in inducing shell gland CaBP-D28K (Navickis et al., 1979; Corradino et al., 1993) or as a co-regulator for shell gland CaBP-D28K induction by 1,25(OH)₂D₃ (Corradino, 1993; Bar et al., 1996). Although the relationship between the sex steroids and 1,25(OH)₂D₃ in the regulation of CaBP-D28K induction was not examined in this study, we suggest that 1,25(OH)₂D₃ can induce shell gland CaBP-D28K which plays an essential role in calcium transport for shell formation.

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References


鶏卵殻腺部管腔内への 1,25-ジヒドロキシビタミン D3 直接投与による卵殻腺部カルシウム結合蛋白質-D28K の誘導

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産卵鶏卵殻腺部のカルシウム結合蛋白質 (CaBP-D28 K) 誘導におけるビタミン D の役割を検討した。1,25-ジヒドロキシビタミン D3 [1,25(OH)2D3 投与区] または生理的食塩水（対照区）を卵殻 3 時間後（卵殻形成期）または卵殻形成初期である 8 時間後（卵殻形成期）に卵殻腺部管腔内へ直接投与し、その 2 時間後に卵殻腺部の CaBP-D28 K の局在と量的変化を免疫組織化学的検索及びウェスタンプロット法で解析した。また、この時、十二指腸 CaBP-D28 K 量及び血清中総カルシウム濃度の変化も測定し、これらに及ぼす卵殻腺部管腔内への 1,25(OH)2D3 投与の影響も調べた。全ての供試鶏で CaBP-D28 K は卵殻腺部の管状腺に局在することが認められた。卵殻形成期では、1,25(OH)2D3 投与区で対照区より濃い CaBP-D28 K のバンドが認められたが、卵殻非形成期では CaBP-D28 K のバンドの濃さは両区の間で同程度であった。十二指腸 CaBP-D28 K のバンドの濃さ及び血清中総カルシウム濃度は、卵殻形成期と非形成期の両時期とも、1,25(OH)2D3 投与区と対照区との間で差を示さなかった。以上の結果から、1,25(OH)2D3 は卵殻腺部を直接刺激して CaBP-D28 K を誘導するものと考えられた。

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キーワード：卵殻形成、卵殻腺部、カルシウム結合蛋白、ビタミン D