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ACUTE EFFECT OF ESTRADIOL ON THE RENAL VITAMIN D HYDROXYLASES IN JAPANESE QUAIL

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Abstract—It has been reported previously by us and others that *in vivo* estradiol administration stimulates *in vitro* 1,25-$(OH)_2D_3$ production in the Japanese quail and chicken. The present investigation was done to study the dose- and time-response relationships of estradiol-induced stimulation of *in vitro* 1,25-$(OH)_2D_3$ production. In addition, the reported permissive role of androgen in this response was studied. Four-week-old immature female Japanese quail, either primed with testosterone propionate (5 mg/kg) or without androgen priming, were injected i.m. with three different doses (0.01, 0.1 and 1.0 mg/kg) of estradiol benzoate dissolved in 95% ethanol. One, 4, 12 and 24 hr after *in vivo* estradiol injection, kidney homogenates were incubated with tritiated 25-$(OH)D_3$. The results were similar whether or not the birds were primed with androgen. Under both conditions all three doses of estradiol stimulated 1,25-$(OH)_2D_3$, and inhibited 24,25-$(OH)_2D_3$, production at 12 and 24 hr with one exception (0.01 mg/kg dose at 12 hr in androgen-primed birds). At 4 hr these responses were seen only at the higher doses. The most rapid response was suppression of 24,25-$(OH)_2D_3$ synthesis by the 0.1 mg/kg dose of estradiol at 1 hr in the androgen-primed birds. These responses in 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ production after a single dose of estradiol may be seen prior to a detectable rise in plasma calcium.

Vitamin D_3 becomes metabolically active only after it undergoes two hydroxylations, initially in the liver to 25-hydroxyvitamin D_3 [25-$(OH)D_3$] [1] and subsequently in the kidney to 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] [2]. The kidney also hydroxylates 25-$(OH)D_3$ to 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$], which has some biological activity [3, 4]. In stimulating both intestinal calcium transport and bone calcium mobilization, 1,25-$(OH)_2D_3$ is the most active and fastest acting metabolite of vitamin D_3 [5, 6] and has been designated as a hormone [7].

The synthesis of 1,25-$(OH)_2D_3$ is influenced by the calcium and vitamin D [8, 9], phosphate [10, 11], and parathyroid [12, 13] status of the animal, although the direct regulatory roles of parathyroid hormone [14–16] and phosphate are disputed [17, 18]. Recently, we and others have reported that estradiol administration *in vivo* stimulates *in vitro* renal production of 1,25-$(OH)_2D_3$ in Japanese quail and chickens [19–22]. Our previous studies were concerned with chronic (4 weeks) and subacute (3 days) effects of estradiol injections in Japanese quail. Tanaka *et al.* [21] and Castillo *et al.* [22] have reported that estradiol injections in Japanese quail and chickens can stimulate *in vitro* 1,25-$(OH)_2D_3$ production as early as 24 hr; these authors also concluded that estradiol can influence renal 1-hydroxylase activity only in the presence of androgen.

The present investigation was designed to study the immediate (1–24 hr) effects of estradiol injection on the renal vitamin D hydroxylases, both in androgen-primed and non-androgen-primed immature, 4-week-old female Japanese quail. Vitamin D metabolism was monitored *in vitro* using kidney homogenates and tritiated 25-$(OH)D_3$ as a substrate.

MATERIALS AND METHODS

Birds

Four-week-old female Japanese quail (*Coturnix coturnix japonica*) with an average body weight of 62 ± 3 g (mean ± S.E.) were used. They were maintained and fed a normal diet (2.3 to 3.3% Ca and 0.8% P) as described elsewhere [23].

Hormones, substrates, incubation of kidney homogenates and plasma calcium determination

17β-Estradiol-3-benzoate (General Biochemicals, Chagrin Falls, OH) was dissolved in the required volume of 95% ethanol and injected intramuscularly (0.5 ml/kg). The androgen-primed groups were injected (5 mg/kg i.m.) with Testosterone Propionate Injection, U.S.P. (Eli Lilly Co., Indianapolis, IN) in oil 24 hr before receiving estradiol benzoate. Control birds received an equal volume of 95% ethanol or oil (1 mg/kg). Three dose levels of estradiol benzoate (0.01, 0.1 and 1.0 mg/kg) were used. A group of three birds at each dose level was sacrificed at 1, 4, 12 and 24 hr after receiving estradiol.

25-[26,27^{3}H]-$(OH)D_3$ (Amersham Searle, Chicago, IL; sp. act. 11.3 Ci/m-mole) was used as the substrate. The preparation of kidney homogenates, the incubation with tritiated 25-$(OH)D_3$, and subsequent extraction and separation of the metabolites were performed according to the method of Kenny [24] as modified by Baksi and Kenny [23]. The substrate concentration in the incubation mixture was controlled at 1.0×10^{-7} M by the addition of unlabeled 25-$(OH)D_3$ (kindly donated by Hoffmann La Roche, Nutley, NJ) to give a final specific activity of 1.13 Ci/m-mole. The metabolite production was expressed as pmoles min^{-1} (g kidney)$^{-1}$.

Heparinized plasma was analyzed for calcium by the automated method of Kessler and Wolfman as modified by Gitelman [25] and subsequently by Technicon for the AutoAnalyzer II system (Technicon Method No. SE4-0003FJ4, Technicon Instruments Corporation, Tarrytown, NY).

Periodate oxidation of metabolites

In addition to their chromatographic behavior on Sephadex LH-20 columns, the metabolites were subjected to periodate oxidation for further identification [26, 27]. The fractions containing the suspected peaks of 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ were combined, evaporated under nitrogen, and dissolved in methanol. To 2 ml methanol containing either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ was added 400 μl of 5% aqueous $NaIO_4$ solution or 400 μl water; all solutions were incubated at room temperature in the dark for 3 hr. The metabolites were extracted from each tube and subjected to Sephadex LH-20 chomatography using chloroform–hexanes (65 : 35) as described by Kenny [24].

Statistics

Where appropriate, the data are presented as mean values accompanied by the standard errors of the mean. Comparisons were made using Student's *t*-test.

RESULTS

In vivo *estradiol benzoate injection in non-androgen-primed birds*

1,25-$(OH)_2D_3$ *production.* The non-androgen-primed birds were treated with three different dose levels (0.01, 0.1 and 1.0 mg/kg) of estradiol benzoate (E_2), and 1, 4, 12 and 24 hr later their *in vitro* kidney production of 1,25-$(OH)_2D_3$ was measured. The data, expressed as a rise in 1,25-$(OH)_2D_3$ production over the control levels, are summarized in Fig. 1 (top panel). The results are similar to those seen in androgen-primed birds (Fig. 2), except that the lowest dose (0.01 mg/kg) gave a significant response at 12 hr. Control birds (95% ethanol) produced between 24 and 52 pmoles min^{-1} (g kidney)$^{-1}$. At 12 and 24 hr, all three doses increased 1,25-$(OH)_2D_3$ production. At 4 hr, the response was significant only at the highest dose. None of the doses was effective at 1 hr.

24,25-$(OH)_2D_3$ *production.* The data, expressed as a fall in 24,25-$(OH)_2D_3$ production, are summarized in Fig. 1 (middle panel). The results are similar to those seen in androgen-primed birds. However, there was no suppression of 24,25-$(OH)_2D_3$ production at 1 hr at any dose levels of E_2. Control levels of 24,25-$(OH)_2D_3$ production ranged from 27 to 68 pmoles min^{-1} (g kidney)$^{-1}$. All three doses suppressed production to non-detectable levels at 12 and 24 hr.

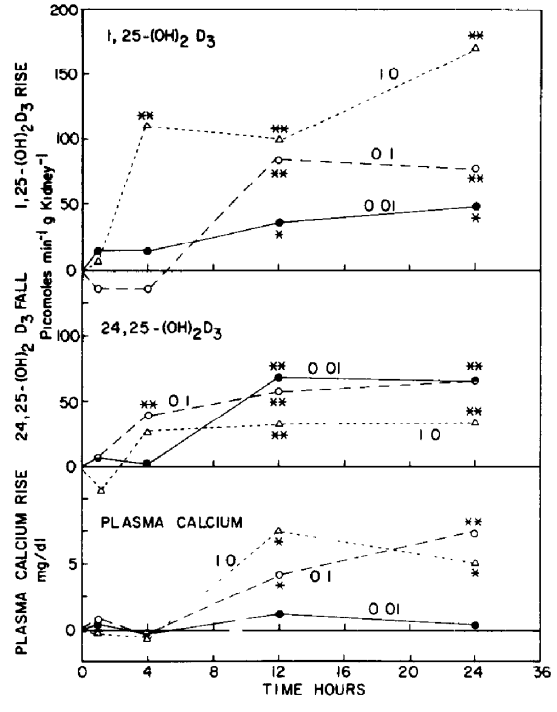

Fig. 1. Effect of estradiol benzoate injection on *in vitro* renal metabolism of tritiated 25-$(OH)D_3$ and on plasma calcium in normal immature (4-week-old) female Japanese quail. Estradiol benzoate was injected at 0.01 (●——●), 0.1 (○- - -○), or 1.0 (△- - -△) mg/kg i.m. The kidneys were removed 1, 4, 12 or 24 hr later, homogenized, and incubated with 10^{-7} M [^{3}H]-25-$(OH)D_3$ for 20 min. The rise in 1,25-$(OH)_2D_3$ or fall in 24,25-$(OH)_2D_3$ production is expressed in pmoles min^{-1} (g kidney)$^{-1}$ over control birds receiving vehicle alone. Asterisks indicate significant responses (single, $P<0.05$; double, $P<0.01$).

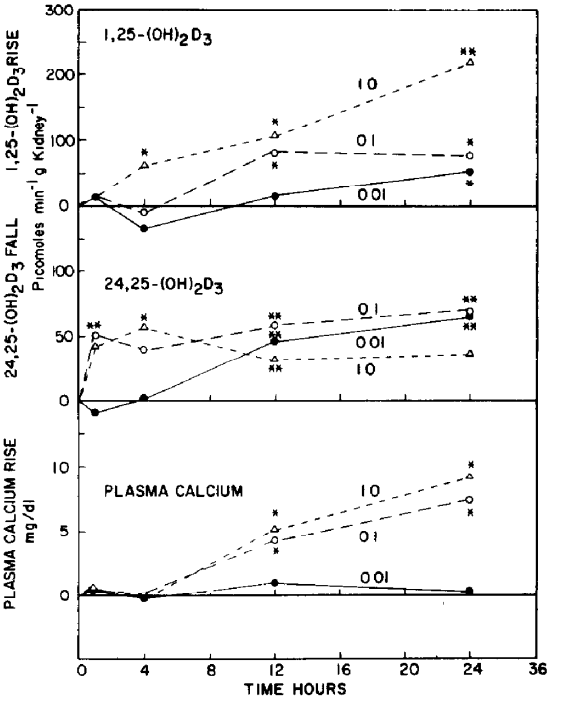

Fig. 2. Effect of estradiol benzoate injection on *in vitro* renal metabolism of tritiated 25-$(OH)D_3$ and on plasma calcium in androgen-primed immature (4-week-old) female Japanese quail. The conditions were as described for Fig. 1 except the birds were pre-treated with Testosterone Propionate Injection, U.S.P. 5 mg/kg i.m. 24 hr prior to estradiol treatment.

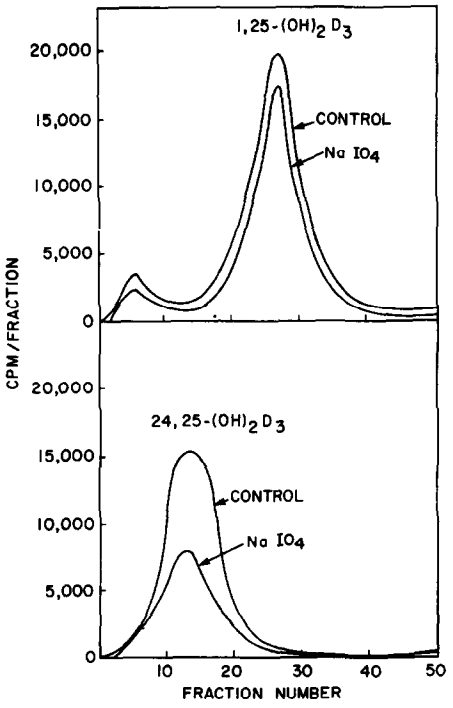

Fig. 3. Periodate treatment of suspected peaks of 1,25-$(OH)_2D_3$ (upper panel) and 24,25-$(OH)_2D_3$ (lower panel) followed by Sephadex LH-20 (chloroform–hexanes, 65 : 35) chromatography. Only a small amount (13 per cent) of the 1,25-$(OH)_2D_3$, but most (60 per cent) of the 24,25-$(OH)_2D_3$, disappeared after periodate treatment for 3 hr, indicating that 25,26-$(OH)_2D_3$ is not a major contaminant of the 1,25-$(OH)_2D_3$ peak.

At 4 hr only the 0.1 mg/kg dose gave a significant response.

Plasma calcium. The plasma calcium responses were similar to those seen in androgen-primed birds. Plasma calcium rose only at 12 and 24 hr and only in birds receiving the 0.1 and 1.0 mg/kg doses of E_2 (Fig. 1, bottom panel).

In vivo *estradiol benzoate injection in androgen-primed birds*

1,25-$(OH)_2D_3$ *production.* The androgen-primed birds were treated with three dose levels (0.01, 0.1 and 1.0 mg/kg) of estradiol benzoate (E_2), and 1, 4, 12 and 24 hr later their *in vitro* kidney production of 1,25-$(OH)_2D_3$ was measured. The data, expressed as a rise in 1,25-$(OH)_2D_3$ production over control levels, are summarized in Fig. 2 (top panel). Control birds (95% ethanol) produced between 50 and 39 pmoles min^{-1} (g kidney)$^{-1}$. At 24 hr, all three doses increased 1,25-$(OH)_2D_3$ production whereas, at 12 hr, only the two higher doses were effective. At 4 hr, the response was significant only at the highest dose. None of the doses was effective at 1 hr.

24,25-$(OH)_2D_3$ *production.* The data, expressed as a fall in 24,25-$(OH)_2D_3$ production, are presented in Fig. 2. Control levels of 24,25-$(OH)_2D_3$ production ranged from 32 to 68 pmoles min^{-1} (g kidney)$^{-1}$. All three doses suppressed production to non-detectable levels at 12 and 24 hr although the response at 1.0 mg/kg at 25 hr was not significant. At 1 and 4 hr, only the 0.1 and 1.0 mg/kg doses, respectively, gave significant responses.

A comparison between the control levels of the activities of the enzymes in the normal birds with those of the androgen-primed birds revealed that acute administration of testosterone did not affect 1,25-$(OH)_2D_3$ or 24, 25-$(OH)_2D_3$ production.

Plasma calcium. Plasma calcium showed a significant increase at 12 and 24 hr but only in the groups treated with 0.1 and 1.0 mg/kg of E_2. There was no significant difference in plasma calcium levels at 1 and 4 hr after E_2 injection (Fig. 2, bottom panel).

Periodate oxidation of vitamin D_3 metabolites

The results of periodate oxidation of the suspected 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ for 3 hr and subsequent Sephadex LH-20 chromatography of the end-products are presented in Fig. 3. Incubation with $NaIO_4$ destroyed 40 per cent of the 24,25-$(OH)_2D_3$ whereas 87 per cent of the 1,25-$(OH)_2D_3$ remained after similar treatment. These observations indicate that 25,26-$(OH)_2D_3$ is not a major contaminant of the 1,25-$(OH)_2D_3$ peak.

DISCUSSION

The present study indicates that the estradiol-induced stimulation of 1,25-$(OH)_2D_3$ production is much more rapid in onset than has been reported before. A response can be seen within 4 hr of estradiol benzoate (1.0 mg/kg) injection. A significant stimulation of 1,25-$(OH)_2D_3$ was noticed at lower doses (0.01 and 0.1 mg/kg) but only at 12 and 24 hr after estradiol injection. The suppression of 24-hydroxylase activity in the androgen-primed birds was even more rapid, occurring within 1 hr of administering estradiol benzoate at the 0.1 mg/kg dose.

The requirement for androgen in the stimulation of estradiol-induced 1,25-$(OH)_2D_3$ was insignificant in our study. This is in contrast to the findings of Tanaka *et al.* [21] and Castillo *et al.* [22], who reported an essential role of androgen for the response in chickens. Although the immature female birds in our study were not gonadectomized, the ovarian tissues were extremely rudimentary and were unlikely to be secreting any appreciable amounts of androgenic steroid. Nevertheless, in view of the potential for adrenal tissue to synthesize and secrete androgens, we cannot be certain that the birds were androgen deficient. More definitive experiments, involving removal of all steroidogenic endocrine tissues, must be performed before the role of androgens in the response of the renal–vitamin D_3 endocrine system to estrogen in Japanese quail is clarified. Our findings do confirm those of others [21, 22] that acute doses of testosterone do not affect the renal hydroxylases in avian species. On the other hand, we have reported that chronic administration of testosterone to mature female quail suppresses 1,25-$(OH)_2D_3$, and stimulates 24,25-$(OH)_2D_3$, production [28].

The rise in plasma calcium after estradiol treatment is much slower than the changes in activities of the renal hydroxylases; the hypercalcemic response

was noticeable only at 12 and 24 hr and only at the higher doses (0.1 and 1.0 mg/kg) of estradiol. Castillo *et al.* [22] also reported a similar observation, but in their study they did not claim any significant stimulation of 1,25-$(OH)_2D_3$ production until 24 hr after estradiol injection. The 24-hydroxylase activity, on the other hand, was totally suppressed as early as 12 hr after injection. The slower onset of the responses may be due to the fact that, in their study, estradiol valerate was dissolved in oil and injected subcutaneously whereas in our study estradiol benzoate was dissolved in 95% ethanol and injected into the breast muscle. It may be anticipated that the pharmacokinetic behavior of the estradiol esters under these two sets of conditions would differ; absorption would be more rapid under our conditions.

Our study does not answer the question of whether the estradiol-induced stimulation of 1,25-$(OH)_2D_3$ is direct, or indirectly mediated through another messenger. Spanos *et al.* [29] have recently reported that ovine prolactin injection *in vivo* in immature chickens can stimulate *in vitro* 1,25-$(OH)_2D_3$ production within 1 hr. These authors suggested that estradiol probably provokes prolactin secretion which in turn stimulates 25-$(OH)_2D_3$-1-hydroxylase activity.

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