Estrogen and prothrombin synthesis: effect of estrogen on absorption of vitamin K$_1$

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In this paper, we report experiments that undertake to a) demonstrate the effects of estrogen on the development of hypoprothrombinemia in intact and castrate animals, and b) determine whether estrogen affects the ability of the intestine to absorb available vitamin K into the thoracic duct lymph.

MATERIALS AND METHODS

Animals and Diet

Simonsen albino rats (Simonsen Laboratories, Inc., Gilroy, Calif.) weighing between 160 and 200 g were used in the hypoprothrombinemia experiments. The animals were maintained on Purina rat chow (Ralston Purina Co., St. Louis) for approximately 1 wk prior to the beginning of each experiment. In experiments involving castrate rats the animals were castrated approximately 1 wk before being placed on the vitamin K-free diet. During the course of each experiment, the animals were housed separately in suspended, wire-bottom cages with individual food and water supplies.

Food and water were supplied ad libitum, and food consumption and weight gain were recorded to assess acceptance of the diet. These data indicated that growth rate and dietary intake, although reduced, were acceptable after an initial adjustment to the synthetic diet, even when the diet contained ethynylestradiol (Fig. 1, B and D).

The vitamin K-free diet for all experiments was prepared in our laboratory according to Mameesh and Johnson (2). The estrogen-supplemented diet was prepared by first dissolving the ethynylestradiol in 5 ml of 100% ethyl alcohol and this solution was then dispersed in, and added with, the wheat germ-oil component. The diet was then completely mixed and reground 2 or 3 times to ensure a homogeneous preparation.

In the cannulation experiments larger Simonsen albino rats of both sexes were used, weighing between 325 and 450 g. These animals were maintained on Purina rat chow during the period between castration and lymph duct cannulation. The day of castration is referred to as day 0. Injections of 17β-estradiol, 100 µg in sesame oil, or sesame oil alone were given intraperitoneally on day 0 and once more on day 5. The animals were then fasted for 24 h, at which time they were force-fed 3 ml of whipping cream by stomach tube to visualize the ducts for cannulation. One hour later the thoracic

In the absence of dietary vitamin K the plasma levels of prothrombin (II), proconvertin (VII), Christmas factor (IX), and Stuart factor (X) in the rat drop, and male rats respond much more abruptly to vitamin K deficiency than female rats (4, 6). This phenomenon suggests that the sex hormones have a role in maintaining plasma prothrombin levels despite dwindling vitamin K intake. Both castrate female and castrate male rats are more susceptible to hemorrhagic death from either coumarin anticoagulants or vitamin K deficiency than are the intact female rats (5, 8), implying a possible protective role for estrogen. This effect might be mediated by a direct mechanism, such as the actual participation of estrogen in prothrombin synthesis, or by an indirect mechanism, wherein estrogen may modify the metabolism of vitamin K that, in turn, would then regulate prothrombin synthesis.
lymph ducts were cannulated at the level of the cisterna chyli. Pentobarbital sodium (Nembutal, 50 mg/kg) was used to anesthetize the animals prior to surgery, and fluid loss was replaced by intragastric injection of water (1–1.5 ml) hourly. Lymph samples were taken at 30-min intervals for the colorimetric assay, while 15-min intervals were found useful for the radiolabeled vitamin K experiments. Total lymph flow was measured gravimetrically and the samples were assayed for vitamin K content as described below.

**Prothrombin Factor VII Determination**

Blood samples (1 ml each) were drawn on alternate days by cardiac puncture. Plasma was separated by centrifugation (2,000 × g for 20 min) and frozen for subsequent analysis. The plasma prothrombin-proconvertin activity was estimated by the modified one-stage prothrombin assay of Ware and Stragnell (9) with an automatic coagulation timer (Fibro System, BioQuest). Although this assay does not discriminate well between prothrombin and proconvertin deficiency, actual prothrombin deficiency was confirmed by specific two-stage assay on several occasions. The blood sample obtained on day 0 served as the control value for each animal and the subsequent development of hypoprothrombinemia could be recorded as a percentage of the control value for each individual rat, rather than by the use of pooled control samples.

**Isolation of Vitamin K and Determination in Lymph**

**Colorimetric assay.** Each lymph sample was extracted twice with two volumes of diethyl ether and the ether was decanted and evaporated to dryness under nitrogen. The ether residue was then dissolved in 4 ml ethyl alcohol and the undissolved residue removed by centrifugation (2,000 × g for 20 min). The resulting clear alcohol solution was analyzed for vitamin K concentration by a colorimetric procedure (1). **Radioactivity assay.** Each lymph sample was added to 10 ml of Triton X-100 scintillation fluid, mixture no. 3 (7) and mixed vigorously for 30 s. The radioactivity of the sample was then determined in a Packard Tri-Carb scintillation spectrophotometer, model 3380. In order to demonstrate that the radioactivity was actually related to intact vitamin K\(_1\), two lymph samples from each experiment were randomly selected and the vitamin K was extracted as described under the procedure for colorimetric assay. In this determination, however, the volume of the alcohol solution was only 500 μl. The alcohol solutions, along with a vitamin K\(_1\) standard, were spotted on thin-layer Silica Gel-G-coated plastic chromatographic plates impregnated with liquid paraffin (3) and developed in acetone:water (92:8). After the plates were sectioned, the sections were transferred directly to vials containing scintillation fluid (0.5 g 1,4-bis-(5-phenyloxazoyl)-benzene and 5 g 2,5-diphenyloxazole per liter toluene) and the radioactivity was determined in the Packard Tri-Carb scintillation spectrophotometer. The vitamin K spots were found to contain 92–98% of the radioactivity of the lymph sample.

**Chemicals and Reagents**

Dietary components, with the exception of sucrose (C&H granulated sugar), were obtained from Nutritional Biochemical Corp., Cleveland, and ethynylestradiol and 17β-estradiol were purchased from Sigma Chemical Co., St. Louis. The radioactive \(^{14}C\)-labeled vitamin K, was a gift from Dr. John T. Matschiner, University of Nebraska School of Medicine, and the nonradioactive vitamin K\(_1\) used in our experiments was AquaMEPHYTON, kindly given to us by Merck Sharp & Dohme, Rahway, N.J. Ware and Stragnell anticoagulant, obtained from Hyland Division, Travenol Laboratories, Costa Mesa, Calif., was used with all blood samples for prothrombin assay. Dade plasma test reagents, scintillation chemicals, and solvents for thin layer chromatography were obtained from Fisher Scientific Co., Maryland Heights, Mo. The thin-layer Silica Gel-G-coated plastic chromatographic plates were obtained from Brinkmann Instruments, Inc., Westbury, N.Y.

**RESULTS**

**Development of Prothrombin-Proconvertin Deficiency in the Intact Male and Female Rat**

The effect of substitution of a vitamin K-free diet on intact male and female rats for a period of 19 days is shown in Fig. 1, A and B. In this experiment males exhibit a precipitous decline in plasma prothrombin-proconvertin levels whereas females maintained a much higher plasma prothrombin-proconvertin activity throughout the experiments. Only 30% of the intact females died during the 19-day experiment (Fig. 1, A), whereas 80% of the intact males had expired before day 16. In the second experiment (Fig. 1, C and D), under nearly identical conditions as possible with the exception that the vitamin K-free diet was fortified with ethynylestradiol to deliver an approximate dose of 5–10 μg/day, male rats did not succumb as rapidly as before. In the first experiment (Fig. 1, A and B), intact females showed a gradual increase in plasma prothrombin-proconvertin to as much as 140% of normal levels after initiation of the vitamin K-free diet, in contrast to the precipitous drop seen in the males. When the vitamin K-free diet was fortified with ethynylestradiol, both males and females responded differently. Comparison of the growth patterns shown in the two experiments suggests that the estradiol reduced the food intake while it acted to protect the animals from the hemorrhagic effects of vitamin K deficiency. All deaths in both experiments were due to hemorrhage, either spontaneous or associated with cardiac puncture during blood sampling.

**Effect of Castration on Development of Prothrombin-Proconvertin Deficiency in Female and Male Rats**

The development of prothrombin deficiency in the castrated male rat (Fig. 2) is less severe than in the intact male rat (Fig. 1, A). The castrate males show a more
FIG. 1. A: effect of vitamin K-free diet on plasma prothrombin-proconvertin levels in intact male and female rats. Animals were fed a vitamin K-free diet starting on day 0 and continuing for duration of experiment. Blood samples (1 ml) were drawn by cardiac puncture from each animal on days indicated and plasma prothrombin-proconvertin activity (% prothrombin) was determined and expressed as a percentage of individual animal's control level (day 0). Each point represents mean ± SE of number of surviving animals, indicated in parentheses. B: mean weight gain ± SE of animals represented in A, indicating continued growth and positive nutritional balance on this diet. Deviation in growth curve indicates temporary withdrawal of food for 24 h. C: experiment similar to A, except that vitamin K-free diet contained ethynylestradiol (5-10 μg/day). D: mean weight change ± SE for animals represented in C.

If estrogen serves a protecting function, then one would expect the development of prothrombin deficiency to be very similar, if not identical, in castrate male and female rats given a vitamin K-free diet supplemented with ethynylestradiol (5-10 μg/day). The responses of castrate male and female rats are indeed remarkably similar, as shown in Fig. 3, A. Both groups showed very little development of prothrombin deficiency and very few deaths due to hemorrhage. Again, this would suggest a protective function of estrogen in the maintenance of plasma prothrombin levels during dietary vitamin K deficiency.

We established the acceptance of the estrogen-fortified diet by the animals by monitoring their weight (Fig. 3, B). The castrate animals accepted the diet (illustrated by weight gain) after a short lag period lasting up to 4 days.

**Effect of Ethynylestradiol on the Development of Prothrombin-Proconvertin Deficiency in the Castrate Male and Female Rat**

Although estrogen has not been shown to substitute for vitamin K1, it does demonstrate, as we have seen, some type of prothrombinogenic action. Estrogen might act by modifying the metabolism of vitamin K or by facilitating its absorption from the gastrointestinal tract so that more vitamin K1 would be present at the site of synthesis.
The possibility that estrogen might facilitate vitamin K$_1$ absorption was tested by cannulating the thoracic lymph duct of castrate female and male rats, both with and without estrogen treatment. Thirty minutes after establishment of a control lymph flow, 10 mg of unlabeled vitamin K$_1$ (AquaMEPHYTON) was injected intragastrically. The total lymph collected was analyzed colorimetrically for vitamin K content. The untreated, castrate males had no detectable vitamin K$_1$ in their lymph during the 210 min of lymph collection, as shown in Fig. 4, whereas the control castrate females absorbed a cumulative 1-2 µg of vitamin K$_1$ during the last 60 min of lymph collection. Both estrogen-treated, castrate female and male rats showed significant levels of vitamin K$_1$ in their lymph in contrast to the very low values in control animals. The estrogen-treated, castrate females absorbed a cumulative 10-13 µg during the experiment, but the estrogen-treated, castrate males showed the greatest total absorption of vitamin K$_1$, 24-27 µg in 210 min. From the summary of the data (Table 1) it can be seen that all of the animals demonstrated similar body weight and lymph flows. The data obtained from the colorimetric lymph assays indicate that estrogen treatment increased the rate of vitamin K$_1$ absorption.

A similar set of experiments was performed with $^{14}$C-labeled vitamin K$_1$, added to unlabeled carrier vitamin K$_1$ to enable us to determine the radioactivity of the vitamin K$_1$ content of the lymph. The total injection of vitamin K$_1$ was reduced to 1 mg in order to simulate more accurately the physiologic concentrations. Unlike the nonradioactive experiments, the vitamin K$_1$ was injected intraduodenally to minimize differences in stomach-emptying time. Castrate males, rather than both sexes, were used in the four isotope experiments because males had responded with the highest rate of absorption in the colorimetric experiments. Again, the estrogen-treated, castrate male rats absorbed much more vitamin K$_1$ during the experiment than the control animals, as shown in Fig. 5. The control rats absorbed only 8-10 µg, cumulatively, in 175 min, whereas the
TABLE 1. Effect of estrogen treatment on in vivo intestinal absorption of vitamin K₁ in castrate male and female rats

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Body Wt, g</th>
<th>Treatment</th>
<th>Mean Lymph Flow, g/min</th>
<th>Duration of Expt, min</th>
<th>Total Lymph Collected, g</th>
<th>Total Vitamin K Absorbed, μg</th>
<th>Mean Vitamin K Absorbed, μg/g lymph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>360</td>
<td>Control</td>
<td>0.013</td>
<td>240</td>
<td>2,900 ± 0.13</td>
<td>0.0 ± 0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Female</td>
<td>376</td>
<td>Control</td>
<td>0.014</td>
<td>240</td>
<td>3,293 ± 0.09</td>
<td>1.2 ± 0.1</td>
<td>0.36</td>
</tr>
<tr>
<td>Male</td>
<td>393</td>
<td>Estradiol</td>
<td>0.015</td>
<td>240</td>
<td>3,603 ± 0.44</td>
<td>25.8 ± 3.0</td>
<td>7.16</td>
</tr>
<tr>
<td>Female</td>
<td>355</td>
<td>Estradiol</td>
<td>0.011</td>
<td>240</td>
<td>2,603 ± 0.21</td>
<td>11.8 ± 3.5</td>
<td>4.53</td>
</tr>
</tbody>
</table>

All values represent the mean of three animals ± SE (where given). Physiologic data of the animals represented in Fig. 4 is tabulated for each experimental group.

TABLE 2. Effect of estrogen treatment on in vivo intestinal absorption of ¹⁴C-labeled vitamin K₁ in castrate male rat

<table>
<thead>
<tr>
<th>Animal</th>
<th>Body Wt, g</th>
<th>Treatment</th>
<th>Mean Lymph Flow g/min</th>
<th>Duration of Expt, min</th>
<th>Total Lymph Collected, g</th>
<th>Total Vitamin K Absorbed, μg</th>
<th>Mean Vitamin K Absorbed, μg/μc A lymph</th>
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<tbody>
<tr>
<td>A</td>
<td>497</td>
<td>Control</td>
<td>0.012</td>
<td>165</td>
<td>1.986</td>
<td>11.60</td>
<td>5.84</td>
</tr>
<tr>
<td>B</td>
<td>502</td>
<td>Control</td>
<td>0.016</td>
<td>225</td>
<td>3.586</td>
<td>22.65</td>
<td>6.32</td>
</tr>
<tr>
<td>C</td>
<td>487</td>
<td>Estradiol</td>
<td>0.015</td>
<td>195</td>
<td>2.850</td>
<td>113.02</td>
<td>39.66</td>
</tr>
<tr>
<td>D</td>
<td>501</td>
<td>Estradiol</td>
<td>0.019</td>
<td>165</td>
<td>3.131</td>
<td>102.89</td>
<td>32.86</td>
</tr>
</tbody>
</table>

Physiologic data of the animals represented in Fig. 5 is tabulated for each individual animal.

castrate male and female rats absorbed 60-70 μg in the same period. The continuation of the experiment for an additional 60 min did not significantly alter the results, as shown by control animal B (Table 2). Although the animals were quite similar in body weight and lymph flow (Table 2), the total vitamin K₁ absorbed within the time constraints of our experiment differed by as much as 500%. These results would seem to indicate some type of facilitatory action of estrogen with respect to vitamin K₁ absorption in the castrate rat.

DISCUSSION

It has been reported that intact male rats and castrate male and female rats are much more susceptible to uncontrolled hemorrhage due to coumarin anticoagulants and vitamin K deficiency than are intact female rats (4-6, 8). These results seem to indicate a protective function of estrogen in the development of hypoprothrombinemia in the rat.

We have further investigated this protective phenomenon in the rat and shown that ethynylestradiol (5-10 μg/day) with the diet does indeed affect the prothrombin-proconvertin levels during dietary vitamin K deficiency. Intact male and female rats placed on a vitamin K-free diet respond quite differently. The female rat maintains a plasma prothrombin-proconvertin level compatible with life whereas the male rat responds with a dramatic, precipitous drop in plasma prothrombin-proconvertin, predisposing the animals to hemorrhage. Castration of the animals 1 wk prior to administration of the vitamin K-free diet greatly reduces the hypoprothrombinemic response of the male rat, and the castrate males do not exhibit the rapid life-threatening plasma prothrombin depletion seen in the intact rat. However, the castrate females respond in much the same manner as the intact females.

Castrate male and female rats, when supplemented with ethynylestradiol (5-10 μg/day orally) appear to respond identically (within experimental error).

Because vitamin K is an essential vitamin to the rat for prothrombin synthesis and is normally acquired by enteral absorption, it was of great interest to study the effects of estrogen on the intestinal absorption of vitamin K₁. Analysis of thoracic duct lymph collected from control and estrogen-treated castrate male and female rats after intragastric injection of unlabeled vitamin K₁ or intraduodenal injection of ¹⁴C-labeled vitamin K₁ was rewarding. There is little doubt that estrogen treatment increases the absorption of vitamin K₁, as determined by either colorimetric or radioactivity spectrophotometric assay and values were converted to cumulative total micrograms of vitamin K₁ absorbed. Each curve represents a single animal.

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treated, castrate male rats absorbed 24-27 \( \mu g \) of vitamin K, whereas nontreated controls absorbed essentially no detectable vitamin K.

In support of the colorimetric observations, the experiments using radioactive vitamin K\(_1\) (\(^{14}\)C uniform label in the phytol side chain) in castrate male rats probably show a more accurate physiologic response in that significant absorption is also detected in the nontreated controls. The estrogen-treated castrate rats absorbed 102-113 \( \mu g \), whereas the nontreated controls absorbed only 22 \( \mu g \) during a longer period than that recorded for the estrogen-treated rats.

These data suggest that estrogen can assist in maintaining plasma prothrombin-proconvertin levels during vitamin K deficiency. Furthermore, one possible mechanism by which this may be accomplished is through a facilitatory action of estrogen on the intestinal absorption of vitamin K, permitting larger quantities of the vitamin to be absorbed in the presence of similar intraluminal concentrations.

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