ESTROGEN AND PROTHROMBIN SYNTHESIS. THE PROTHROMBINOGENIC ACTION OF ESTROGEN

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Summary: Vitamin K deficient castrate male rats exhibit more rapid prothrombin depletion after injection of 100 µg of methyltestosterone, decreasing from 25% to 8% of normal plasma prothrombin levels. During the same 96 hour period, control castrate male rats showed a decline from 25% to 16%. Injection of 100 µg ethynylestradiol to similar vitamin K deficient, castrate male rats increased plasma prothrombin levels from 23% to approximately 45% within 96 hours. The effect of estradiol on biosynthesis of prothrombin was investigated by measuring incorporation of [^{3H}]L-amino acids into the electrophoretically separable prothrombin. We conclude that the observed estrogen effect is due to a prothrombinogenic, rather than prothrombin-sparing mechanism.

The presence of a low concentration of vitamin K in the liver is both sufficient and necessary for continued synthesis of several blood coagulation factors (factors II, VII, IX and X) in higher animals. Several lines of evidence indicate that vitamin K is required to complete a post-ribosomal step in the synthesis of prothrombin (11, 12, 13, 17, 19, 21, 22, 23, 24), and this step has been shown recently to be the carboxylation of the glutamic acid residues near the NH_2-terminal of the prothrombin polypeptide. The altered amino acid residue was found to be γ-carboxy-glutamic acid (5, 9, 14, 25) but the actual mechanism of the carboxylation has not yet been delineated. Recent reports have indicated that it can take place in vitro (1, 2, 4, 20).

If rats are fed a vitamin K-free diet for 7-12 days, they develop hypoprothrombinemia and become quite susceptible to hemorrhagic death (3, 6). It has also been shown that intact (non-castrate) male rats and castrate male, or castrate female rats are more susceptible to vitamin K deficiency or coumarin drugs than are intact female rats (3, 7, 8, 16). In this paper we

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report the effect of single doses of an androgen (methyltestosterone) and an estrogen (ethynylestradiol) on the continued prothrombin depletion in vitamin K deficient castrate male rats. We conclude from prothrombin generation experiments using radioactive amino acids that estrogen reduces the severity of hypoprothrombinemia by increasing synthesis of prothrombin during vitamin K deficiency.

MATERIALS AND METHODS

Animals and Diet. Male Simonsen Albino rats (Simonsen Laboratories Inc.) weighing between 160 and 260 grams were used throughout. The animals were maintained on Purina Rat Chow (Ralston Purina Co.) for at least two weeks prior to the beginning of each experiment. They were castrated 7 to 12 days before placing them on a vitamin K-free diet prepared according to Mameesh and Johnson (6). During the course of each experiment, the animals were housed in suspended wire bottom cages with food and water supplied ad libitum.

Prothrombin Assays. One ml blood samples, withdrawn via cardiac puncture, were diluted 9:1 (blood:anticoagulant) with either 3.2% sodium citrate or Ware and Stragnell modified Owren anticoagulant obtained from Hyland Div., Travenol Labs. The blood was centrifuged (2000 x g for 20 minutes) and the plasma was frozen for subsequent analysis. The plasma prothrombin content was estimated by the modified Owren one-stage method of Ware and Stragnell (27), which is sensitive to proconvertin deficiency as well as hypoprothrombinemia, or by the two-stage assay of Ware and Seegers (26) which specifically measures prothrombin activity. An automated coagulation timer (Fibrometer, BBL-Bioquest) was used for all clotting assays. The blood sample, withdrawn before placing the animal on the vitamin K-free diet, served as a control for each animal hence, even though the prothrombin values varied between rats, the subsequent development of, or recovery from, hypoprothrombinemia could be recorded as a percentage of the control value for each individual rat rather than using pooled samples.

Prothrombin Isolation and Radioactive Assay. After assaying the prothrombin content of the plasma the prothrombin was adsorbed on barium sulfate, eluted, then purified by disk gel electrophoresis as described by Shah and Suttie (17, 18). The disk gel slice containing only the prothrombin band was placed in buffer to elute the protein for assay by the modified Lowry method (15) using a Cary Model 17 Spectrophotometer, or placed directly in a glass scintillation vial and liquified by incubation at 90° C for 12 hours with 1 ml of 30% H_2O_2. The vials were then cooled and, following the addition of 10 ml of a Triton X-100 based scintillation fluid mixture #3 (10), the radioactivity was determined in a Packard Tri Carb Scintillation Spectrophotometer Model 3380.

Chemicals and Reagents. Dietary components, with the exception of sucrose (C & H granulated sugar), were obtained from Nutritional Biochemicals Corp. and the ethynylestradiol and estradiol-17β were purchased from Sigma Chemical Corp. Methyltestosterone was obtained from Matheson Co., and the [^{3}H]L-amino acid mixture (specific activity = 80 mCi/m mole) was purchased from New England Nuclear Corp. Dade plasma test reagents and scintillation chemicals were purchased from Fisher Scientific Co. Chemicals and materials needed for electrophoresis were obtained from Bio-Rad Inc. All other chemicals and reagents used were of reagent grade.

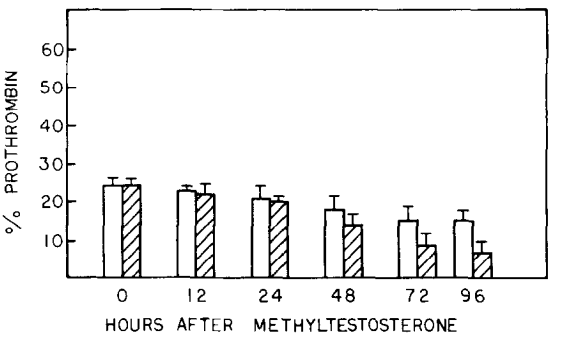

FIGURE 1. Effect of Androgen on Prothrombin Depletion in the Vitamin K Deficient Castrate Male Rat.
Castrate male rats, fed a vitamin K-free diet for 2 weeks, were injected (i.p.) with 100 µg methyltestosterone in 0.2 ml sesame oil or with 0.2 ml sesame oil vehicle alone and continued on the vitamin K-free diet. Cardiac blood samples (1 ml each) withdrawn at 0, 12, 24, 48, 72 and 96 hours following injection were assayed for prothrombin content by the two-stage assay of Ware and Seegers (24). Each value is the mean ± s.e.m. for 3 to 5 animals. Open bars represent control animals while crossed bars indicate androgen treated animals.

RESULTS

Effect of Androgen on Plasma Prothrombin Depletion

Following withdrawal of the control, 1 ml cardiac blood sample, castrate male rats were separated into two groups and placed on a vitamin K-free diet for 2 weeks. The animals then received intraperitoneal (i.p.) injections containing 100 µg methyltestosterone in 0.2 ml sesame oil (androgen treated) or 0.2 ml sesame oil vehicle alone (control) and continued on the vitamin K-free diet. Subsequent cardiac blood samples were withdrawn at 12, 24, 48, 72 and 96 hours following treatment. Figure 1 illustrates the effect of the androgen injection on the plasma prothrombin content assayed by the two-stage assay of Ware and Seegers (26).

Prothrombin depletion continues at a gradual rate in control castrate male rats (3). In this experiment control animals exhibit a decline from approximately 25% of normal plasma prothrombin to values of approximately 15% of normal in 4 days. The androgen treated, castrate male rats also exhibit decreasing plasma prothrombin levels during the ensuing 96 hours following

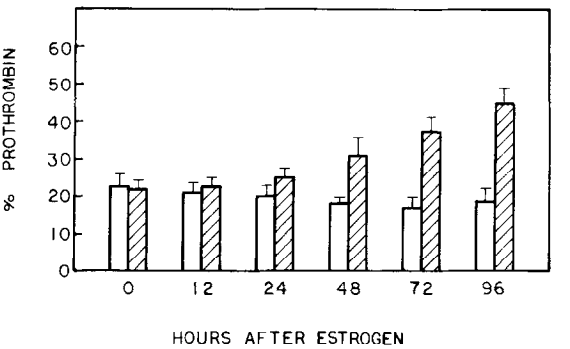

FIGURE 2. Effect of Estrogen on Prothrombin Depletion in the Vitamin K Deficient Castrate Male Rat.
Castrate male rats were treated similarly to those in experiments presented in Figure 1 with the exception that they were injected with 100 µg of ethynylestradiol in 0.2 ml sesame oil or sesame oil vehicle alone. Cardiac blood samples were withdrawn and assayed as in Figure 1. Each value represents the mean ± s.e.m. for 3 to 5 animals. Open bars represent control animals while crossed bars represent estrogen treated animals.

injection. Up to 24 hours both androgen treated and control animals maintained similar plasma prothrombin values, however by 48 hours the androgen treated animals had shown a more rapid decline. By 72 hours androgen treated animals had shown a decline to 10% of their normal plasma prothrombin in comparison to 16% for the control group. Ninety-six hours after injection there was a 7% difference between the two groups suggesting that the injection of methyltestosterone can actually increase the susceptibility of the castrate male rat to the vitamin K deficiency.

Effect of Estrogen on Plasma Prothrombin Depletion

Castrate male rats were treated as in the previous experiment (Figure 1) but with the following modification. Two groups of animals were injected i.p. with 100 µg of the long-acting estrogen ethynylestradiol in 0.2 ml sesame oil (estrogen treated) or 0.2 ml sesame oil vehicle alone (control). Figure 2 presents the results obtained from two-stage prothrombin assay of the blood drawn during the 96 hour experimental period.

Control castrate male rats again exhibited a gradual decrease in plasma prothrombin during the 96 hour experiment. The plasma prothrombin of

control animals decreased from 23% to 18% of normal levels during the time constraints of the experiment. In contrast to the decreasing plasma prothrombin levels seen in control or androgen treated castrate male rats, estrogen treated castrate male rats demonstrated increased plasma prothrombin values following injection. By 24 hours, the control animals had decreased to approximately 18% of normal plasma prothrombin levels whereas the estrogen treated rats had attained only 26% of normal plasma prothrombin levels. In this experiment the control animals continued at 16% to 18% of their normal plasma prothrombin over the remaining 72 hours of the experiment while the estrogen treated animals increased to 45% of normal by 96 hours after estrogen administration. It would appear that estrogen administration reduces the susceptibility of castrate male rats to sustained vitamin K deficiency but the mechanism by which estrogen causes this effect cannot be determined from these experiments. Both estrogen-mediated prothrombin-sparing or estrogen-stimulated prothrombin synthesis could conceivably increase the plasma prothrombin values in estrogen treated castrate male rats.

Effect of Estrogen on Prothrombin Synthesis

Castrate male rats were placed on a vitamin K-free diet and given i.p. injections of either 50 µg of the short acting estrogen, estradiol-17β, in 0.2 ml sesame oil or 0.2 ml sesame oil vehicle alone on even numbered days beginning on day zero. An i.p. injection of a mixture of [^{3}H]L-amino acids (50 µCi per injection) was administered on days 4, 5 and 6 of the experiment and cardiac blood samples (1 ml each) were withdrawn on days 0, 2, 4, 5, 6, 7, 8 and 10.

It may be seen from Figure 3A that estrogen treated castrate male rats maintained higher plasma prothrombin levels than the control castrate male rats throughout the experiment. It is noteworthy that the levels of hypoprothrombinemia reached in this experiment are lower than in previous

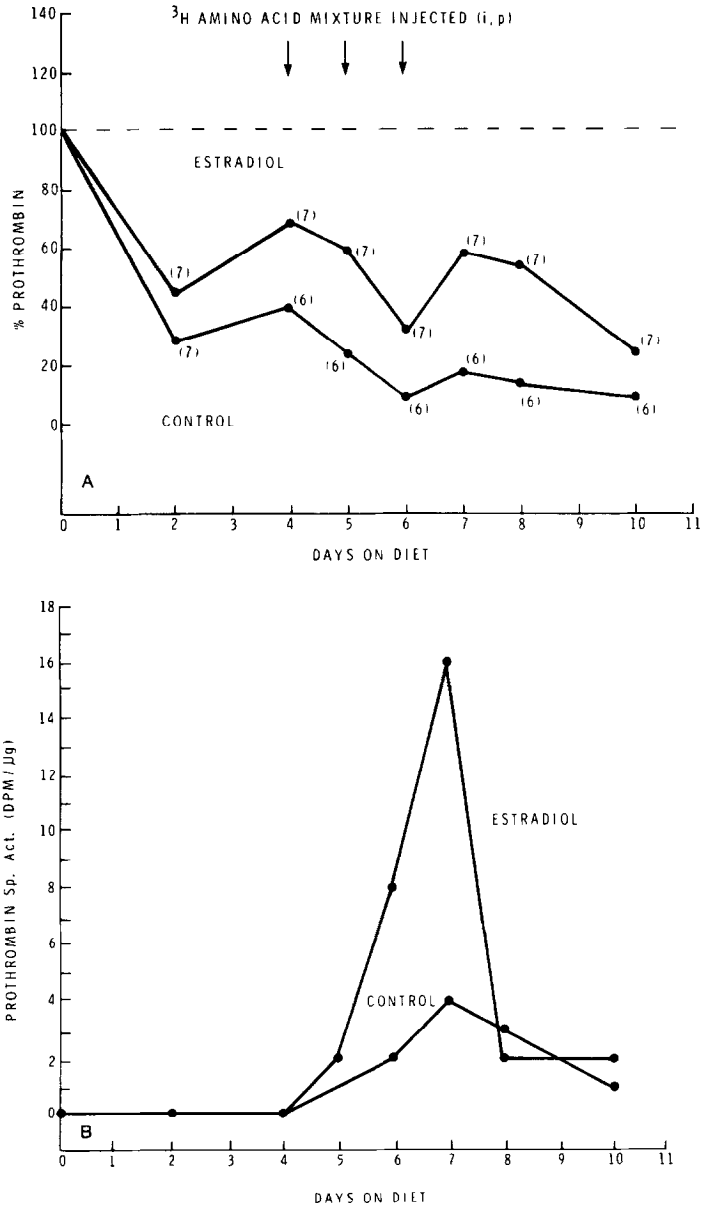

FIGURE 3A and B. Prothrombinogenic Effect of Estrogen.

A. Castrate male rats were placed on vitamin K-free diet and injected (i.p.) on even numbered days with 50 µg estradiol-17β in 0.2 ml sesame oil or with 0.2 ml sesame oil vehicle alone. On days 4, 5 and 6,50 µCi of a [^{3}H]L-amino acid mixture was injected i.p. and cardiac blood samples (1 ml each) were withdrawn on days 0, 2, 4, 5, 6, 7, 8 and 10. The plotted points represent the values obtained, using the modified Owren one-stage method of Ware and Stragnell (27), from the pooled plasma contributed by the number of rats indicated in parentheses.

B. Specific activity of the radioactive prothrombin isolated (see Methods) from the plasma obtained from the animals represented in A.

experiments in both the control and estrogen treated animals. This, of course, may only reflect the more frequent daily blood sampling in this experiment.

Radioactive analysis of the purified plasma prothrombin of both groups of animals is represented in Figure 3B. An increase (0.0 DPM/μg to 3.8 DPM/μg) in the specific activity of the prothrombin from the control rats indicates that these animals continue to synthesize prothrombin during dietary induced vitamin K deficiency. The prothrombin from the estrogen treated castrate male rats reached a much higher specific activity (16 DPM/μg) indicating that not only are the estrogen treated animals synthesizing prothrombin during dietary vitamin K deficiency but they are synthesizing the prothrombin at a much higher rate than the control castrate male rats.

Following termination of the [^{3}H]L-amino acid mixture injections, the specific activity of the prothrombin from both control and estrogen treated castrate male rats quickly returns to similar levels. This observation would seem to indicate that estrogen does not act by preventing the degradation of prothrombin in the castrate male rat during vitamin K deficiency.

DISCUSSION

Although it has been shown that intact male, castrate male and castrate female rats are more susceptible to hemorrhagic death from either vitamin K deficiency or coumarin drugs, neither the location nor the mechanism of this apparent protective action of estrogen has been defined (7, 8, 15). We have recently reported that estrogen seems to facilitate the intestinal absorption of vitamin K_1 in estrogen treated castrate male and female rats (3). In this report we show that the administration of an androgen (methyltestosterone) to vitamin K deficient castrate male rats enhances prothrombin depletion in these animals when compared to control castrate male rats (Figure 1). In contrast, the administration of an estrogen (ethynylestradiol) results in an increase in the plasma prothrombin levels of vitamin K deficient castrate male rats from 22% of normal levels to 45% of normal levels in 4 days

(Figure 2). This increase in plasma prothrombin apparently is due to a prothrombinogenic action of estrogen rather than a prothrombin sparing action. When tritium labelled amino acids are administered to estrogen treated and non-treated castrate male rats on vitamin K-free diets both groups of animals appear to exhibit a continued prothrombin synthesis in spite of their vitamin K deficient condition. However, the estrogen treated animals are seen to produce a radioactive prothrombin with approximately 4 times the specific activity of that shown by the control animals (16 DPM/µg versus 3.8 DPM/µg, Figure 3B)*.

These data confirm and extend our own and other previously published reports that estrogen and androgen by some still unknown mechanism, modify the synthesis of the vitamin K-dependent blood protein, prothrombin.

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*Even though the specific activity values shown in Figure 3B are small, we believe that the difference between them is significant. Low DPM values were obtained because only a small amount of radioactivity was injected per animal plus the fact that the relative prothrombin concentration in blood of vitamin K deficient animals is low.

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