

Diethylstilbestrol Selectively Modulates Plasma Coagulation Protein Synthesis by the Perfused Rat Liver

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The combined effects of orchietomy and estrogen administration on synthesis of selected hepatic secretory proteins—antithrombin (AT) III, plasminogen, fibrinogen, factor II (prothrombin), factor VII, fibronectin, and albumin—were studied using the isolated rat liver perfused *in vitro* for ten hours. Male rat liver donors underwent orchietomy under ether anesthesia and then received 5.0 mg of diethylstilbestrol (DES) by subcutaneous pellet implantation or a placebo pellet; 14 days later the livers were extracted and perfused *in vitro* for ten hours. In DES experiments, 1.0 mg of DES was also added directly to the liver perfusate at the outset. Pretreatment with DES resulted in significant increases in cumulative synthesis of factors II (65%) and VII (76%) and significant reductions in cumulative synthesis of both antithrombin III (20%; $P = .03$) and plasminogen (27%; $P < .01$) compared to control perfusions, but synthesis of fibrinogen, fibronectin, and albumin was not significantly affected by addition of the hormone. Plasma samples collected from rat liver

donors that had received DES showed similar effects on protein concentrations: significant decreases in concentration of plasminogen and antithrombin III were apparent with no significant changes in concentrations of fibrinogen, fibronectin, or albumin. In additional perfusions, "dose-response" experiments were conducted in which rat liver donors received a subcutaneous DES pellet of 0.5, 5.0, or 50 mg. Synthesis of plasminogen in this group of perfusions was progressively decreased as the concentration of DES administered to the rat liver donor increased. Synthesis of AT III was reduced to the same degree by 5.0 or 50 mg of DES, both being substantially lower than the 0.5-mg experiments. Concentrations of these two proteins in plasma samples from rat liver donors showed changes quite similar to those seen in perfusion experiments; however, plasma fibrinogen concentrations were not different among the three groups of rats.

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ESTROGEN is an established risk factor in the development of thromboembolic disease, and clinical evidence suggests a role for the hormone in the development of coronary artery thrombosis as well.¹⁻⁵ In the Coronary Drug Project of 1970, therapeutic use of diethylstilbestrol (DES) led to a twofold increase in coronary vessel thrombosis as well as other thromboembolic complications.⁶ Elevations of serum estradiol have been noted in males with coronary disease and may precede cardiac thrombosis.⁷⁻⁹ Recent evaluations of the Framingham cohort of patients (Framingham Study, Mass) also suggest that hyperestrogenemia may be a risk factor for development of coronary artery thrombosis.¹⁰

Estrogen therapy of neoplastic disease has also been complicated by thrombosis.¹¹⁻¹³ In the Veterans Administration Cooperative Urological Research Group Study of prostatic carcinoma, a daily dosage of 5 mg of DES, which gives a plasma concentration within the range found in pregnant females, was markedly thrombogenic in treated males.¹⁴ Use of estrogen-containing oral contraceptives also predisposes to thromboembolic disease. A dose-response relationship is suggested in some reports, with a strong correlation between the estrogen content of oral contraceptives and the degree of risk for thromboembolic disease.¹⁻⁵

Estrogen may produce changes in various elements of the

blood coagulation process, including vascular integrity, platelet function, and plasma coagulation proteins.¹⁵⁻²¹ Among the plasma coagulation proteins, the most consistently observed change is a decrease in plasma antithrombin III (AT III) concentration or activity.²²⁻²⁶ Also known as heparin-cofactor, AT III is the principal inhibitor of activated plasma coagulation factors.²⁷⁻³⁰ Family groups that are genetically deficient in AT III show marked susceptibility to thromboembolic events.³¹⁻³³ Surgical patients who have decreased plasma AT III are at considerable risk for postoperative thrombosis.³⁴ Decreased AT III levels have been frequently found in women receiving oral contraceptives, and this finding seems linked to thromboembolic complications.²²⁻²⁶ Other changes in plasma coagulation proteins described with estrogen treatment include increased plasma fibrinogen, decreased fibrinolytic activity, and increased vitamin K-dependent clotting factors.¹⁵⁻²¹ Whether these changes reflect altered protein synthesis, degradation, or other factors cannot be determined from the available clinical reports. Whether such changes are significant risk factors for the development of thrombotic disease is also unclear.

Many factors affect plasma protein concentrations in intact animals, thus making conclusions about rates of protein synthesis most difficult. Most of the plasma coagulation proteins are synthesized by the liver, and studies with the isolated perfused rat liver have provided much information about synthesis.³⁵⁻⁴² Although extensive work has been done with regulation of hepatic coagulation factor synthesis by nutritional factors such as vitamin K, the effects of sex hormones have not been extensively examined in the liver perfusion system. Estrogen effect on protein synthesis by the male rat liver has not been demonstrated *in vitro*, but *in vivo* studies indicate that this organ is estrogen-responsive.⁴³⁻⁴⁵ In the studies described here, the isolated perfused male rat liver was used to investigate the effects of DES on synthesis of selected hepatic secretory proteins, with emphasis on principal coagulation proteins.

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MATERIALS AND METHODS

Rat liver donors. Rat liver donors were male Sprague-Dawley rats, 78 to 90 days of age, all of which had undergone bilateral orchietomy while under diethyl ether anesthesia 14 days before killing. At the time of orchietomy, rat liver donors had subcutaneous implantation of an estrogen-containing pellet or a placebo pellet consisting of cholesterol, methylcellulose, and α -lactose, as described below. Blood samples were taken from all rat liver donors at the time of sacrifice.

Perfusion of the isolated rat liver. Hepatectomy was performed under ether anesthesia, and perfusion of the isolated rat liver was carried out *in vitro* as previously described.^{41,42} The perfused rat liver, mounted on a glass platform, was placed inside a closed lucite case for the duration of the perfusion experiment. Temperature of the liver was maintained constant at 38 °C by means of a Thermistor probe (Yellow Springs Instrument Co, Yellow Springs, Ohio) inserted between the lobes of the liver. All perfusion experiments were carried out for a period of ten hours, and samples of the liver perfusate were withdrawn for assay at intervals of two hours. At the start of each perfusion, the first 10 mL of perfusate to pass through the liver was collected and discarded to eliminate any washout of preformed rat liver proteins.

The isolated liver was perfused through the portal vein with a mixture consisting of 38 mL washed bovine RBCs (GIBCO, Grand Island, NY) suspended in 50 mL of Krebs-Ringer bicarbonate buffer to which was added 7 mL of 3.8% sodium citrate. Sufficient Ringer solution was added to bring the perfusate volume to 100 mL. The perfusate also contained 3 g/100 mL of bovine serum albumin, 100 mg of glucose, 3,000 units of penicillin, and 3 mg of streptomycin-HCl. A constant infusion consisting of 18 mL of 3.8% sodium citrate containing 500 mg of glucose, 320 mg of amino acids, 5 mg of cortisol, 6.8 units of insulin, 3,000 units of penicillin, and 1 mg of streptomycin-HCl was added to the perfusate at a rate of 1.5 mL/h. In all perfusion experiments, the pH of the perfusate was maintained constant at 7.40 by an infusion of 1.0 mol/L NaHCO₃ from a Radiometer Autoburette (ABU-12, MKS Co, Cheektowaga, NY) and Titrator (TTT-60) equipped with a combined glass calomel electrode (GK3201-C).

Perfusion experiments. Initial experiments were designed to compare the effects on protein synthesis of DES plus orchietomy (group 1) v orchietomy alone (group 2). All rat liver donors in these two groups underwent orchietomy 14 days before killing. In group 1 animals, continuous administration of hormone to the rat liver donors for 14 days was accomplished by subcutaneous implantation of a pellet containing 5.0 mg of DES (Innovative Research, Rockville, Md). The efficacy of this method of hormone delivery has been established.⁴⁶⁻⁴⁸ One milligram of DES was added directly to the liver perfusate at the outset. Group 2 animals received a subcutaneous placebo pellet (no DES) at the time of orchietomy, and no DES was added to the liver perfusate. To determine effects of the "stat" dose of DES (1.0 mg) added at the outset of the perfusion, another set of perfusion experiments was performed in which castrated rat liver donors received a placebo pellet, and 1.0 mg of DES was added directly to the liver perfusate at the outset (group 3).

Dose-response experiments. Additional experiments were designed to assess the effects of administration of different doses of DES to rat liver donors on coagulation protein synthesis. All rat liver donors in this group underwent orchietomy 14 days before death. At the time of orchietomy, the animals were placed in one of the three groups, in which each animal received a subcutaneous pellet containing a single dose of 0.5, 5.0, or 50 mg of DES. No DES was added to the liver perfusate in these studies.

Blood samples were taken from each rat liver donor at the time of

hepatectomy and protein measurements were performed on the plasma as described below.

Assays. In each perfusion experiment, aliquots (3.0 mL) of the liver perfusate were withdrawn every two hours, and measurement of coagulation protein concentrations made in each sample. Activity of two vitamin K-dependent proteins, factors II and VII, was measured in liver perfusate samples by methods described earlier.⁴⁹⁻⁵¹ Each of the other five proteins was measured in liver perfusate samples and rat liver donor plasma samples using a monospecific antiserum raised against the purified protein. Techniques of protein purification have been described elsewhere; in brief, AT III was purified by affinity chromatography of pooled rat plasma on heparin sepharose by the methods of Rosenberg and Damas,^{28,41} fibronectin was purified from pooled rat plasma by affinity chromatography on gelatin Sepharose by the methods of Engvall and Rouslahti,^{42,52} and plasminogen was isolated from pooled rat plasma by affinity chromatography on lysine Sepharose by the method of Deutsch and Mertz.⁵³ In each case, the monospecific antiserum raised in rabbits to the purified protein was used in a quantitative rocket immunoelectrophoresis assay for protein measurement. Development of antibodies of rat fibrinogen and albumin have also been described earlier; immunologic assay of these two proteins was performed using the single radial immunodiffusion technique of Mancini et al as modified by Fahey and McKelvey.^{41,54,55}

RESULTS

Initial experiments compared effects on protein synthesis of a 5-mg DES pellet implanted subcutaneously in the rat liver donor plus DES added to the perfusate (group 1), a subcutaneous placebo pellet with no DES added (group 2), or subcutaneous placebo pellet with 1 mg of DES added to the perfusate at the outset (group 3). Fig 1 shows the striking increase in cumulative synthesis of factors II and VII when rat liver donors were pretreated with DES (group 1) compared to that when animals received a placebo pellet (group 2). Enhanced synthesis and release of the two coagulation proteins was apparent by the second hour of perfusion, and by ten hours, a 76% increase in factor VII and 65% increase in factor II was reached. The addition of DES to the perfusate where rat liver donors received only a placebo pellet (group 3) was associated with a more modest increase in synthesis and release of both factors. Fig 2 shows mean cumulative synthesis of AT III and plasminogen in these three groups of perfusion experiments. Cumulative synthesis of AT III was significantly reduced in group 1, 2.29 ± 0.18 (SE) mg/300 cm² body surface area of the rat liver donor for the DES treated animals, compared to 2.89 ± 0.7 mg for group 2 animals and 3.15 ± 0.37 mg for group 3 animals, which only had DES added to the perfusate. AT III synthesis was not significantly different between groups 2 and 3, but the decrease in group 1, a 20% reduction compared to group 2, is significant ($P = .03$). A similar reduction was seen in the synthesis of plasminogen. In group 1 perfusions, 1.44 ± 0.13 mg of plasminogen was produced, compared to $1.98 \pm .07$ in group 2 and 1.95 ± 0.1 mg in group 3. In group 1, the implanted DES pellet was associated with a 27% reduction in plasminogen biosynthesis compared to group 2, which had no DES ($P = .01$). Other liver secretory proteins were not significantly affected by the presence of DES, either in pellet form or when added directly to the liver perfusate. Produc-

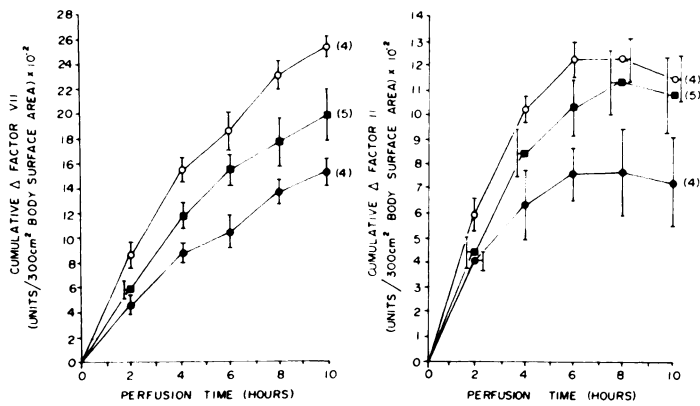


Fig 1. Cumulative synthesis of factors II and VII in group 1 perfusions (O), group 2 (●), and group 3 (■). Vertical bars indicate SEM, and the numbers in brackets indicate the number of livers perfused under each set of conditions.

tion of fibrinogen, fibronectin, and albumin was unchanged by the hormonal manipulations (Fig 3).

To investigate the effects of different doses of DES, perfusion experiments were performed in which rat liver donors underwent orchietomy, and at the same time, had a pellet containing 0.5, 5.0, or 50 mg of DES implanted subcutaneously. No additional DES was added to the liver perfusate in these experiments. Cumulative synthesis of plasminogen and AT III was measured in these perfusion studies (Fig 4). Synthesis of plasminogen showed a pro-

gressive decrease as the quantity of DES administered to the rat liver donor increased. Synthesis of AT III was diminished equally by 5.0 or 50 mg DES, compared to 0.5 mg.

Examination of plasma samples from rat liver donors used in these experiments revealed changes in protein concentrations similar to those observed in liver perfusion experiments (Table 1). Plasma from animals that had received a DES pellet contained significantly lower concentrations of AT III and plasminogen than did those from animals that had received only a placebo pellet containing no DES. Concentrations of fibrinogen, fibronectin, and albumin were not different between the two groups. In contrast to the effects on Factor VII synthesis observed in perfusion experiments, the concentration of this protein was decreased in animals that had received DES compared to those receiving placebo pellets.

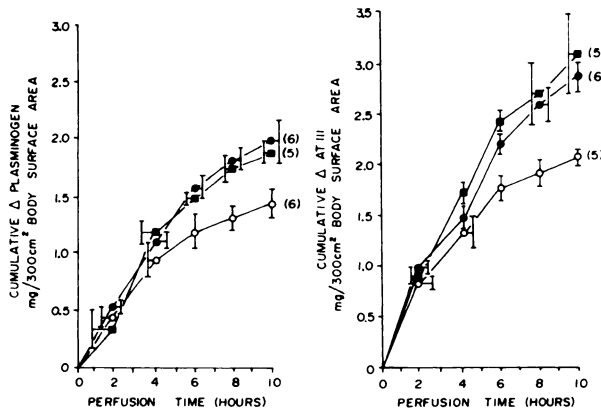
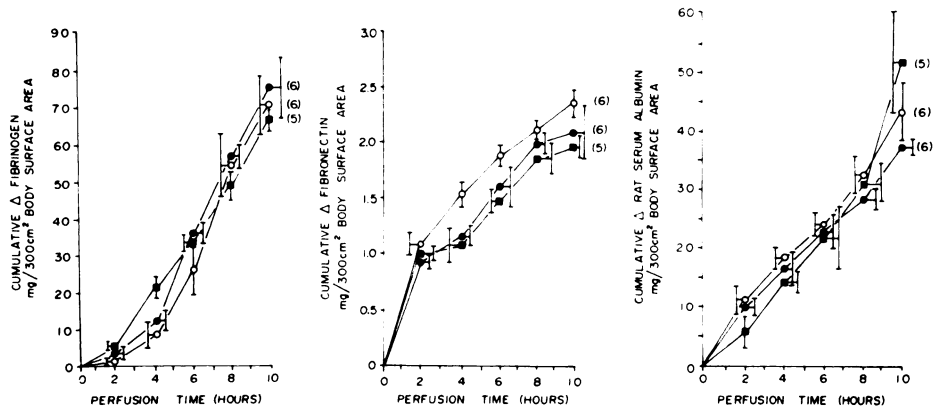


Fig 2. Cumulative synthesis of plasminogen and antithrombin III in group 1 perfusions (O), group 2 (●), and group 3 (■).

DISCUSSION

The liver perfusion experiments described here clearly demonstrate that in vitro synthesis of certain hepatic secretory proteins can be significantly affected by the combination of orchietomy and pretreatment of the rat liver donor with DES. Cumulative production of two principal procoagulant proteins, factors II and VII, was greatly increased by pretreatment with DES, whereas production of AT III and plasminogen was significantly decreased in these same experiments. Production of albumin, fibrinogen, and fibronectin was unchanged by such treatment. In the case of AT

Fig 3. Cumulative synthesis of fibrinogen, fibronectin, and albumin in group 1 perfusions (O), group 2 (●), and group 3 (■).



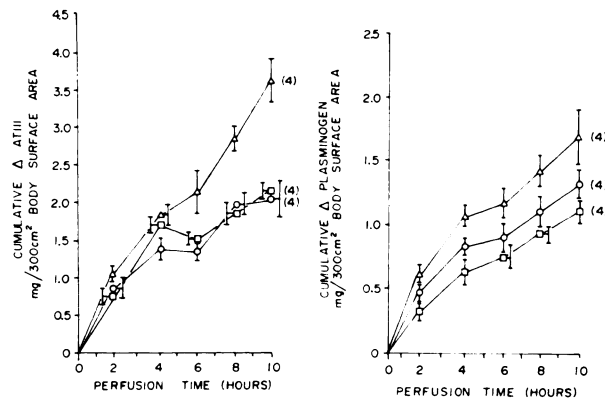


Fig 4. Cumulative synthesis of plasminogen and antithrombin III in dose-response perfusion experiments where rat liver donors were pretreated with DES—0.5 mg (Δ), 5.0 (O), or 50 mg (\square).

III and plasminogen, the observed effects on synthesis required prolonged exposure to DES; the addition of the hormone immediately after the onset of the perfusion did not cause changes in protein synthesis. In contrast, addition of the hormone was associated with increased production of factors II and VII whether the rat liver donor had been treated with DES or a placebo pellet, although the increase was more pronounced when the liver donors received DES. This may indicate that, in the case of the two vitamin K-dependent factors, DES may have effects on both the synthesis and release of the protein. In an earlier report of perfusion studies in which female rat liver donors were used, the addition of DES to liver perfusate greatly enhanced

Table 1. Plasma Protein Concentrations in Castrated Male Rats Pretreated With Diethylstilbestrol or Placebo 14 Days Before Death

Protein	DES (5.0 mg)	Placebo
Antithrombin III (mg/mL)	0.31 \pm 0.03	0.40 \pm 0.02 ($P = .014$)*
Plasminogen (mg/mL)	0.35 \pm 0.01	0.52 \pm 0.04 ($P < .01$)
Factor VII (U/mL)	67.3 \pm 4.2	128.2 \pm 4.4 ($P < .01$)
Fibrinogen (mg/mL)	13.4 \pm 1.3	14.6 \pm 0.9 (NS)
Albumin (mg/mL)	26.1 \pm 2.6	29.4 \pm 3.5 (NS)
Fibronectin (mg/mL)	0.38 \pm 0.1	0.38 \pm .02 (NS)

Data shown are means \pm SE. NS, no significance.

*Statistical comparisons were made using the Student's *t* test.

hepatic release of angiotensinogen.⁵⁶ This effect was apparent by the second hour of perfusion, in contrast to that observed with cortisol and insulin where induction of the "acute phase response" in the perfused rat liver requires at least four to six hours of hormone exposure.⁵⁷ The immediate effects of DES on angiotensinogen, as well as some of the results reported here with factors II and VII, occur too early to be mediated by synthesis of specific messenger RNA; however, the effects of DES on the synthesis of AT III and plasminogen clearly require prolonged exposure to the hormone. The minimum duration of exposure to DES that might alter protein synthesis was not defined in these studies since all estrogen-treated rat liver donors were exposed to DES for 14 days before hepatectomy.

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