The Renal Erythropoietic Factor (REF) VII. Relation to Sex Steroid Hormone Effects on Erythropoiesis* (33446)

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Increasing attention has been directed to the mechanisms by which steroid hormones influence erythropoiesis (1–5). Among those proposed have been: (1) a direct effect on the blood-forming organs (6, 7); (2) modification of erythropoietin (ESF) action (8–10); (3) changes in the production of the ESF (1, 2, 5). In the latter regard, androgens have been shown to stimulate the production of the ESF (1, 2, 5), an effect mediated most likely through the renal erythropoietic factor (REF), a principle which, although not erythropoietically active when administered alone, engenders the production of the ESF when incubated *in vitro* with normal serum (11, 12). That the factor formed in this *in vitro* incubation is actually the ESF is seen from the ability of anti-ESF serum globulin to abolish the generated activity (13). The REF appears to be an enzyme that converts a serum protein into the ESF (14).

The estrogenic hormone, administered in large doses, depresses production of the ESF (3). One possibility here is that estrogen inhibits production of the REF. It has been reported, however, that whereas the stimulatory action of androgen on erythropoiesis requires the presence of the kidneys (3, 15), the erythropoiesis-inhibiting effects of estrogen are evident in nephrectomized as well as in intact mice (3).

The present work deals with further studies of androgen and estrogen influences on the REF-serum-ESF axis.

Materials and Methods. In all experiments, adult male rats (250–270 g) of the Long-Evans strain served as plasma, serum, and kidney donors.

Experiments with androgen. Four groups, each consisting of 6 rats, were established. Group I received a single s.c. injection of 12.5 mg long-acting testosterone cyclopentylpropionate (TCP) (Depo-testosterone, Upjohn Co.) in 1 ml of cottonseed oil. The rats of Group II, comprising the controls, were injected s.c. with 1 ml vehicle, cottonseed oil. Group III was administered a single s.c. injection of 12.5 mg TCP and in addition was exposed to 19 hr of hypoxia (0.42 atm. of air) 5 days after the androgen injection. The rats of Group IV received a single s.c. injection of vehicle and 5 days later were exposed to a hypoxic stimulus similar to that employed in Group III. All rats were killed by exsanguination on day 6 post-injection.

Experiments with estrogen. Experiments on two groups of donors, each consisting of 5 rats, were conducted. The rats of Group V were given a single s.c. injection of 5 mg estradiol benzoate (EB) made up in 1 ml peanut oil and 24 hr later were exposed to 8 hr of hypoxia (0.42 atm. of air). Group VI was administered a s.c. injection of 1 ml vehicle (peanut oil) and 24 hr later was subjected to 8 hr of hypoxia. The animals of these two groups were killed by exsanguination immediately after the termination of the hypoxic period.

The bloods of Groups I–VI were heparinized, the plasmas collected, pooled, and stored at −20° until used. The kidneys of the six groups were likewise separately pooled and subjected to the following procedure for the extraction of the REF. After washing and mincing the organs, 10 ml of cold 0.25 *M* sucrose were added for each gram of kidney used. The material was then homogenized in a Potter-Elvehjem homogenizer. After centri-

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TABLE I. Influence of Androgen (TCP) and Hypoxia on Plasma ESF and on REF in Rats.

Group	Donors and materials tested[a]	% RBC ^{59}Fe inc. (mean ± SEM)	Mean ESF units/ml plasma or /2 ml of REF-serum incubate[b]
I	TCP-treated, nonhypoxic		
	A. Plasma	9.53 ± 0.98	0.085
	B. REF + EDTA-dial. NRS	7.59 ± 0.45	0.065
II	Oil-treated, nonhypoxic		
	A. Plasma	2.11 ± 0.63	NS
	B. REF + EDTA-dial. NRS	4.19 ± 0.61	NS
III	TCP-treated, hypoxic		
	A. Plasma	32.73 ± 3.01	1.5
	B. REF + EDTA-dial. NRS	13.85 ± 0.97	0.18
IV	Oil-treated, hypoxic		
	A. Plasma	23.81 ± 2.62	1.0
	B. REF + EDTA-dial. NRS	9.18 ± 1.02	0.08

[a] NRS = normal rat serum.
[b] NS = not significant.

fuging at 6300 *g* for 15 min at 5°, the sediment was discarded. Recentrifugation was now carried out for the sucrose supernatant at 21,000 *g* for 20 min at 5°. The sediment obtained ("light mitochondrial" fraction) was suspended in 0.02 *M* phosphate buffer, pH 6.8, and the mixture immediately frozen. After thawing, the material was centrifuged at 37,000 *g* for 30 min. The supernatant fluid containing the REF was kept frozen until used.

The incubation procedure for each of the groups involved adding 6 ml REF-containing fluid (extracted from 3 g hypoxic rat kidneys) to 6 ml ethylenediamine tetraacetate (EDTA)–dialyzed normal rat serum (12). The incubations were conducted for 60 min in a water bath shaken at 37°. In the experiments involving estrogen, two additional incubations were carried out to determine whether the estrogen had affected the production and/or activity of the serum substrate. These involved addition of: (1) 6 ml of REF from peanut oil–treated hypoxic rats to 6 ml EDTA-dialyzed serum from nonhypoxic rats that had received a single s.c. injection of 12.5 mg EB 32 hr previously (Group VII); and (2) 6 ml REF from peanut oil–injected hypoxic rats to 6 ml EDTA-dialyzed serum from nonhypoxic rats that had been administered a single s.c. injection of 1 ml peanut oil 32 hr earlier (Group VIII).

Assay methods. All plasmas and REF-serum incubation mixtures were assayed for ESF activity in hypoxia-induced polycythemic mice (16). Five to 6 mice were used to test each sample. Each mouse received either 1.0 ml plasma or 2.0 ml of incubation mixture as a single i.p. injection on day 3 posthypoxia. On day 5, they were given an i.v. injection of 0.5 μCi ^{59}Fe in 0.2 ml saline. They were killed on day 7 and the percentage of RBC-radioiron incorporation determined (16). These values were also converted into Erythropoietin Units by reference to the standard curve for the International Reference Preparation (17).

Results. Androgen groups. Table I indicates that plasma obtained from TCP-injected rats (Group IA) showed significantly greater erythropoiesis-stimulating activity than did plasma from the vehicle-treated rats (Group IIA). The ESF concentration in the plasma rose impressively in rats exposed to 19 hr of hypoxia (Group IVA). An even greater increase was noted in the plasma of rats that received the combined TCP-hypoxia treatment (Group IIIA). TCP and hypoxia (Groups IB and IVB) caused significant increases in the REF activity over that noted

TABLE II. Influence of Estrogen (EB) and Hypoxia on Plasma ESF and on REF in Rats.

Group	Donors and materials tested[a]	% RBC ^{59}Fe inc. (mean ± SEM)	Mean ESF units/ml plasma or /2 ml of REF-serum incubate[b]
V	EB-treated, hypoxic		
	A. Plasma	8.66 ± 0.73	0.075
	B. REF + EDTA-dial. NRS	10.57 ± 2.10	0.09
VI	Oil-treated, hypoxic		
	A. Plasma	28.61 ± 3.92	1.2
	B. REF + EDTA-dial. NRS	9.31 ± 1.30	0.085
VII	REF (oil-treated, hypoxic) + EDTA-dial. serum (EB-treated, nonhypoxic)	4.28 ± 0.93	NS
VIII	REF (oil-treated, hypoxic) + EDTA-dial. serum (oil-treated, nonhypoxic)	11.46 ± 1.19	0.11

[a] NRS = normal rat serum.
[b] NS = not significant.

for the vehicle-injected controls (Group IIB). A greater rise in REF activity was evoked by the combination of TCP and hypoxia than that observed for either stimulus alone (Group IIIB).

Estrogen groups. A single injection of EB markedly inhibited the rise in erythropoiesis-stimulating activity of plasma induced by hypoxia (Table II, *cf.* Groups VA and VIA). This inhibitory effect of estradiol on plasma ESF in rats subjected to hypoxia was not accompanied by any alteration in REF activity (Groups VB and VIB). Incubation of REF from hypoxia-exposed rats with EDTA-dialyzed serum from oil-injected controls resulted in the development of significant quantities of ESF (Group VIII). Considerably less ESF was generated when EDTA-dialyzed serum from estradiol-injected rats was used in the incubation system (Group VII).

Discussion. The present experiments confirm and extend previous findings that the stimulatory effects of androgen in rats are mediated, at least in part, through an increased production of the ESF (1, 2, 5). The rise in REF activity evoked by the androgen is most likely the basis for the augmented ESF production. The data also indicate that the combination of androgen and hypoxia induces greater increases in REF and ESF than those resulting from either stimulus administered separately. Reference of the radioiron incorporation values to the log-dose response curve for the International Reference Preparation permits determination of the actual ESF unitage. Such analysis indicates that the increased effects of the combination of androgen and hypoxia on both the REF and ESF are more likely synergistic than additive in nature.

It seems apparent that the depressive influence exerted by estradiol on ESF production in rats exposed to hypoxia cannot be attributed to alterations in REF activity. Thus the REF values in rats receiving estradiol prior to exposure to hypoxia are not significantly different from those of vehicle-injected controls subjected to the same hypoxic stimulus. On the other hand, incubation of the REF from hypoxic rats with serum from estradiol-treated rats results in the generation of considerably less ESF than when serum from oil-injected controls is employed in the incubation system. Thus it seems probable that the inhibitory effect exerted by estradiol on ESF production stems from a lowering in the amount and/or the activity of the serum factor serving as the substrate for the REF. In this regard, the reduced erythropoiesis-stimulating activity of this incubation mixture cannot be attributed to the presence of erythropoiesis-inhibiting agents that would act to decrease RBC radioiron incorporation values in

the assay mice. Thus plasma obtained from animals administered comparable large doses of estrogen does not contain residual biologically active estrogen (3). Nor does such plasma depress erythropoiesis in normal mice or antagonize the erythropoietic action of exogenous ESF (3).

Studies are in progress to determine more specifically by chemical methods, whether the serum proteins (particularly beta globulin) demonstrated by us (Wong *et al.*, unpublished) to serve as substrates for the REF are reduced in concentration by the dosages of estradiol employed in this study. In this regard, estrogen has been reported to depress some phases of protein metabolism in liver, e.g., the glutamic-oxaloacetic and glutamic-pyruvic transaminase systems (18).

Summary. Previous observations that testosterone incrases production of the ESF and the REF are confirmed and extended. The combination of testosterone and hypoxia act synergistically in augmenting plasma ESF and REF activities. The depressive effect of estradiol on ESF production is not accompanied by a decrease in REF activity. However, the serum from estradiol-treated rats exhibits a reduced capacity to serve as a substrate for the REF in the generation of the ESF.

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Protection by Orotic Acid Against the Renal Necrosis and Fatty Liver of Choline Deficiency* (33447)

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Choline deficiency produces fatty infiltration of the liver in rats (1). Recently, Porta, Manning, and Hartroft reported that feeding orotic acid hinders hepatic fat accumulation in choline deficiency (2). This observation is unexpected, because orotic acid feeding itself causes fatty infiltration of the liver (3, 4), an effect that can be prevented by simultaneous administration of adenine sulfate (4, 5).

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