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Stimulation of Mammalian Erythropoiesis by 5β-H Steroid Metabolites*

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Abstract. The effects of a number of steroid compounds on erythropoiesis in normal and polycythemic mice were examined. Of the steroids that stimulated erythropoiesis, the hormone testosterone and certain 5β-H C_{19} and C_{21} nonhormonal metabolites were the most effective. Anti-erythropoietin abolished the erythropoiesis-stimulating effects of testosterone but not those exerted by the 5β-H steroid, 11-ketopregnanolone. Similarly, testosterone but not 11-ketopregnanolone evoked the production of erythropoiesis-stimulating factor in rats. It is concluded that two mechanisms underlie the stimulating actions of steroids on erythropoiesis; one through the production of erythropoietin and the second involving a more direct influence on the blood-forming tissues. The 5β-H steroid metabolites are postulated to act on erythropoiesis via the latter mechanism.

Three mechanisms have been proposed for the effects exerted by steroids on mammalian erythropoiesis.[1] One involves the capacity of certain steroids to influence production of the erythropoiesis-stimulating factor (ESF or erythropoietin),[2–4] in some instances through an action on the production and release of the renal erythropoietic factor (REF or erythrogenin).[1, 5] An ability to alter the effectiveness of the ESF in stimulating erythropoiesis has been described as another possible mode of action of steroids.[6–8] Evidence for a direct influence of steroids on the blood-forming tissues has also been presented.[9–11] Of special interest in this regard is the recent demonstration of a potent stimulatory action of some 5β-androstane and 5β-pregnane steroids on heme synthesis in chick embryo liver cells and in the erythroid cell precursors of the chick blastoderm.[12–15] The suggested mechanism for the steroid action described in the latter studies has involved the induction of δ-aminolevulinic acid synthetase, the rate-limiting enzyme in the heme biosynthetic pathway. An enhancing effect of a 5β-H steroid metabolite on heme synthesis *in vitro* has also been recently described for human bone marrow cells.[16]

The present study was undertaken to investigate the mechanisms underlying the influence of a series of natural steroids on erythropoiesis in mice and rats and to determine whether the ability of 5β-H steroids to enhance avian hemoglobin synthesis extended to these mammalian species as well.

Two types of effects were obtained: (1) a stimulatory action on erythropoiesis due to an increased production of the ESF, as shown with testosterone, and (2) increased erythropoiesis via a mechanism not associated with heightened levels of the ESF as observed with 5β-H C_{19} and C_{21} steroid metabolites. Evidence is presented that the latter mechanism probably involves a direct stimulatory action of the 5β-H steroid metabolite on erythroid cell precursors.

Materials and Methods. Studies in polycythemic mice: Adult female mice (CF-1 strain) rendered polycythemic by exposure to a lowered atmospheric pressure were used to determine the erythropoietic effects of six steroid compounds† (pregnanolone; 17α-OH, 11-ketopregnanolone; 11-ketopregnanolone; etiocholanolone; 5β-dihydrotestosterone; pregnandione). Each compound, administered as a single intraperitoneal injection, was tested at four dose levels (0.25, 0.75, 1.5, and 2.5 mg) dissolved in 0.1 ml of propylene glycol. The schedule of injections and estimation of erythropoietic activity were similar to those used in assessing ESF activity.[17]

Studies in normal mice: The effects of testosterone, etiocholanolone, 11-ketoprenanolone, cortisol, and estradiol were determined on erythropoiesis in normal mice at three dose levels (0.25, 0.75, and 2.5 mg). Mice were injected intraperitoneally with a single dose on day 1 of the experiment and each received 0.5 μCi ^{59}Fe Cl_3 intravenously 48 hr later. The per cent incorporation of radioiron into the circulating red blood cells was determined on day 5. As in polycythemic mice, 11-ketopregnanolone was found to be the most active in stimulating erythropoiesis. In subsequent experiments, therefore, this steroid and testosterone propionate were used exclusively. A similar study was performed in adult normal rats (Long-Evans strain) employing the latter two compounds.

Studies with anti-ESF: Six groups of normal mice (six mice/group) were established. The animals in group 1 were given intraperitoneal injections of 0.1 ml propylene glycol (the solvent vehicle for the steroids), while those in the second group received two daily intraperitoneal injections of 0.5 ml anti-ESF in addition to the 0.1 ml propylene glycol. Groups 3 and 4 were injected with a single 2.50 mg dose of testosterone propionate while those in groups 5 and 6 were given 2.50 mg of 11-ketopregnanolone. The mice in groups 4 and 6 also received two injections of 0.5 ml anti-ESF each. All mice were given intravenous injections of 0.5 μCi radioiron 48 hr after the administration of the steroid and the percentage of red blood cell radioiron incorporation values were determined 48 hr later. This method is now generally accepted as a reliable and sensitive index of the rate of erythropoiesis, paralleling the reticulocyte response to the same stimuli.[18]

Experiments in rats: These studies were performed to determine the effects of testosterone propionate and 11-ketopregnanolone on the production of the ESF in adult female rats of the Long-Evans strain. The animals were injected intraperitoneally with 5.0 mg testosterone or with 2.5, 5.0, and 10 mg of 11-ketopregnanolone daily for four days. On day 5 the rats were killed by exsanguination and their plasmas collected, pooled separately, and assayed for ESF activity in exhypoxic polycythemic mice.[17]

Results. Experiments in mice: Table 1*A* indicates that single injections of a number of steroids resulted in augmented erythropoiesis, as judged by percentage of red blood cell ^{59}Fe incorporation values, in normal adult mice.‡ The most significant effects here as well as in exhypoxic polycythemic mice (Table 1*B*) were noted with 11-ketopregnanolone at all dose levels examined. As may be seen from Table 1*B*, control per cent red blood cell radioiron incorporation values were considerably lower in the exhypoxic polycythemic mice than in normal mice (Table 1*A*). This marked depression in erythropoiesis occurs as a result of the negative feedback exerted by the excess numbers of red cells when the polycythemic mice are restored to ambient pressures. Such mice are known

TABLE 1A. *Effects of five steroid compounds on erythropoiesis in normal mice.*

Treatment	Mean percentage of red blood cell-^{59}Fe incorporation ± 1 SEM*		
Propylene glycol (control)	26.8 ± 3.2		
	0.25 mg	0.75 mg	2.50 mg
Testosterone propionate	...	28.6 ± 2.1	30.1 ± 3.2
Etiocholanolone	34.4 ± 3.7	21.8 ± 3.5	17.2 ± 1.9
11-Ketopregnanolone	43.7 ± 3.3	49.0 ± 3.7	53.7 ± 2.2
Cortisol	17.0 ± 3.0	12.4 ± 1.0	15.1 ± 5.0
Estradiol	19.4 ± 2.0	16.3 ± 2.5	8.2 ± 1.0

* Standard error of the mean.

TABLE 1B. *Effects of six steroid compounds on erythropoiesis in exhypoxic polycythemic mice.*

Treatment	Mean percentage of red blood cell-^{59}Fe incorporation ± 1 SEM*			
Propylene glycol (control)	0.9 ± 0.1			
	0.25 mg	0.75 mg	1.50 mg	2.50 mg
Pregnanolone	2.6 ± 0.2	2.6 ± 0.3	3.0 ± 0.6	3.6 ± 0.6
5-β 17α-OH, 11-Ketopregnanolone	2.4 ± 0.2	2.7 ± 0.4	3.4 ± 1.5	6.7 ± 1.8
11-Ketopregnanolone	6.3 ± 1.1	9.1 ± 0.5	11.6 ± 2.1	6.3 ± 1.3
Etiocholanolone	5.9 ± 1.3	6.9 ± 1.8	6.3 ± 1.5	2.9 ± 0.3
5-β Dihydrotestosterone	3.2 ± 0.4	3.0 ± 0.7	3.3 ± 0.7	...
Pregnane-3, 20-dione	2.9 ± 0.6	3.2 ± 0.5	2.9 ± 0.4	4.5 ± 0.5

* Standard error of the mean.

to respond more sensitively to erythropoietic stimuli, as for example the ESF.[5] Table 1*B* indicates that, as in normal mice, 11-ketopregnanolone increased the red blood cell radioiron incorporation values in polycythemic mice to a greater extent than did the other steroids tested. Etiocholanolone was stimulatory at the 0.25 mg level in normal mice and at the four levels tested in polycythemic mice. A reduction in the red blood cell radioiron incorporation values was noted with the 2.5 mg dose in normal mice. The reason for this is not clear but higher concentrations of certain 5β-steroids have been shown to have similar effects on porphyrinogenesis in liver cell culture, presumably due to toxic effects of the steroids on the cells. Testosterone administration led to a mild increase in erythropoiesis at the 2.5 mg dose level (Table 1*A*). As previously shown,[20] estradiol inhibited erythropoiesis in normal mice, an effect seen most significantly in this study at the 2.5 mg dose level. Somewhat similar inhibitory effects were noted with cortisol (Table 1*A*).

Table 2 shows the action of anti-ESF serum on the stimulatory effects evoked by steroids on erythropoiesis in normal mice. Two injections of anti-ESF markedly depressed erythropoiesis in these otherwise untreated animals indicating that normal erythropoiesis is largely under control of the ESF. Anti-ESF completely abolished the erythropoiesis-stimulatory influence of testosterone; however, it exerted only a slight, statistically insignificant ($P = 0.2$), depressive action on the erythropoietic effect of 11-ketopregnanolone. Similar results not indicated in the tables were obtained when transfusion-induced plethoric mice[2, 5]

were used instead of normal mice. Here, although the red blood cell radioiron incorporation values were as expected much lower than in normal mice), the effects were in the same direction and statistically significant. These results are consistent with the interpretation that the erythropoietic action of 5β-H steroids is mediated largely via a non-ESF mechanism as suggested earlier.[12]

TABLE 2. *Effects of anti-ESF on the erythropoietic response of normal mice to testosterone propionate and 11-ketopregnanolone.*

Treatment	Mean percentage of red blood cell-^{59}Fe incorporation ±1 SEM*
Propylene glycol† (control)	19.8 ± 2.4
Propylene glycol + 0.5 ml anti-ESF‡ (×2)	5.6 ± 1.1
Testosterone propionate (2.50 mg)†	26.2 ± 4.2
Testosterone propionate (2.50 mg) + 0.5 ml anti-ESF (×2)	4.0 ± 0.9
11-Ketopregnanolone (2.50 mg)†	31.9 ± 5.1
11-Ketopregnanolone (2.50 mg) + 0.5 ml anti-ESF (×2)	23.8 ± 3.1

* Standard error of the mean.

† Differences in RBC radioiron incorporation values in the mice of this series of experiments and those shown in Table 1A are attributable to interexperimental group variations in response of mice to the same agents. This necessitates the inclusion of a control group in each experimental run.

‡ We thank Dr. John C. Schooley (University of California) for the supply of anti-human urinary ESF. One ml of this anti-ESF preparation neutralized 20.25 International Units of ESF.

Experiments in rats: Table 3 demonstrates that stimulatory effects on both 24- and 48-hour red blood cell radioiron incorporation values in adult rats were exerted by four daily injections of three different doses (2.5, 5.0, and 10.0 mg)

TABLE 3. *Effects of testosterone propionate and 11-ketopregnanolone on erythropoiesis in normal rats.*

Treatment	Mean Percentage of Red Blood Cell-^{59}Fe Incorporation ± 1 SEM*	
	24-hr/incorporation	48-hr/incorporation
Propylene glycol × 4 (control)	21.2 ± 2.0	28.7 ± 3.7
Testosterone propionate (5.0 mg × 4)	31.8 ± 2.1	37.0 ± 4.1
11-Ketopregnanolone (2.5 mg × 4)	45.3 ± 3.7	41.2 ± 5.6
11-Ketopregnanolone (5.0 mg × 4)	50.6 ± 3.2	56.2 ± 2.7
11-Ketopregnanolone (10.0 mg × 4)	38.7 ± 3.9	42.3 ± 5.1

* Standard error of the mean.

of 11-ketopregnanolone. The most significant influence was seen in the rats receiving the 5.0 mg dose. Erythropoiesis was also significantly increased by four daily injections of testosterone, as expected.

Table 4 lists the results for the assay of ESF in plasma from the five groups of rats shown in Table 3. Hypoxia-induced polycythemic mice were used as the test animals.[2, 5] It will be noted that marked stimulatory effects on red blood cell radioiron incorporation were observed with plasma from testosterone-treated rats. That this plasma activity was due to ESF evoked by testosterone was seen from our unpublished findings that prior treatment of this plasma with anti-ESF resulted in complete loss of its erythropoietic activity. In contrast, the plasma from rats receiving the three different dose levels of 11-ketopregnanolone was devoid of erythropoiesis-stimulating activity.

TABLE 4. *Erythropoiesis-stimulating activity of plasmas obtained from testosterone propionate and 11-ketopregnanolone-treated rats as measured in hypoxia-induced polycythemic mice.*

Material assayed*	Mean percentage of red blood cell ^{59}Fe incorporation ± 1 SEM†
Saline (control)	1.1 ± 0.3
Plasma from propylene glycol-treated rats	2.1 ± 0.5
Plasma from testosterone-treated rats	11.7 ± 2.0
Plasma from 11-ketopregnanolone-treated rats	
Rats given 2.5 mg × 4	2.1 ± 0.6
Rats given 5.0 mg × 4	1.2 ± 0.1
Rats given 10.0 mg × 4	3.2 ± 0.8

* All samples were administered in a 1-ml dose.

† Standard error of the mean.

Discussion. The present experiments indicate that at least two different mechanisms underlie the erythropoietic actions of steroids and certain of their derivatives. It has been demonstrated previously that testosterone stimulates red cell formation in rodents[2, 3, 5–7] and in man[21] and that this is achieved, at least in part, by augmenting production of the ESF.[2, 3, 5, 21, 22] The experiments reported here confirm these findings in testosterone-treated rats. Additional evidence for this concept is derived from the ability of anti-ESF to completely abolish the erythropoiesis-stimulating action of testosterone (ref. 23 and the present paper).§ On the other hand, as shown in these experiments, the erythropoiesis-stimulating action of the 5β-pregnane compound 11-ketopregnanolone was not accompanied by an elevated level of circulating ESF, a finding that also receives support from our observation that its effects were not antagonized by anti-ESF. In view of the ability of 5β-H, C_{19}, and C_{21} steroids to directly stimulate hemoglobin synthesis in cultured chick blastoderms[12] and porphyrin-heme synthesis both *in vivo* and *in vitro* in chick embryo liver,[13–15] as well as to enhance heme production in cultured human bone marrow cells,[16] it seems highly probable that the effects of these 5β-H steroids on erythropoiesis in the mouse are exerted directly on the blood-forming tissues *in vivo*.

Testosterone has been noted to be weakly active, when compared to a series of 5β-H androstane and pregnane compounds, in stimulating heme synthesis in chick blastoderms and chick embryo liver cells *in vitro*.[14] Similarly, in the present study, testosterone proved to be less effective than 5β-H metabolites in augmenting erythropoiesis in both normal and plethoric mice. It is known that transfusion-induced plethora in the mouse induces a virtual disappearance of the nucleated erythroid cell compartment. Since the 5β-H steroid metabolites utilized in this study were shown not to stimulate ESF production, it would appear likely that their primary effect is exerted directly on the early erythroid cell precursors, inducing them to differentiate into the nucleated erythroid cell line. It is reasonable to postulate that, as for the hepatic and erythroid cell lines of the chick embryo, these steroids act by inducing the formation of δ-aminolevulinic acid synthetase in erythroid cell precursors of the mouse, thus leading to enhanced formation of heme and subsequently of hemoglobin in these elements. Of interest are recent findings[24] that ESF also enhances δ-aminolevulinic acid synthetase activity in rabbit bone marrow cells *in vitro*, suggesting the possibility

of a common mechanism of action of steroid metabolites and ESF on hemoglobin formation in early erythroid precursor cells.

It is also in order to suggest that the results obtained with steroids in the present experiments, by providing information on the basic underlying mechanisms of their action, will stimulate studies of their possible use—particularly certain of the 5β-H, C_{19}, and C_{21} metabolites not possessing classical sex hormone actions—in indicated refractory anemias in man. Considerable success has been reported recently with certain steroid derivatives in the treatment of patients with a variety of anemias[21, 25] and the availability of a group of erythropoietic steroids devoid of masculinizing side effects might prove to be of therapeutic value.

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† The following trivial names for steroid metabolites are used: Pregnanolone (5β-pregnane-3α-ol, 20 one; 17α-OH, 11-ketopregnanolone (5β-pregnane-3α,17α-diol, 11, 20, dione); 11-ketopregnanolone (5β-pregnane-3α-ol, 11, 20 dione); etiocholanolone (5β-androstane, 3α-ol, 17 one); 5β-dihydrotestosterone (5β-androstane, 17β-ol, 3 one); pregnandione (5β-pregnane-3, 20 dione).

‡ To ensure that the radioiron was incorporated specifically into heme, the erythrocytes were treated with methylethylketone according to the method of Teale.[19] Over 90% of the ^{59}Fe activity was found in the heme-containing methylethylketone extract. In addition, reticulocytosis was evident in these steroid-treated animals.

§ Since the erythropoietic effect of testosterone is fully inhibited by anti-ESF, it would appear that insufficient quantities of steroid metabolite are formed from the administered testosterone to stimulate red cell production directly.

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