Effects of Several Androgens and Steroid Metabolites on Erythropoietin Production in the Isolated Perfused Dog Kidney

By Luiz G. Paulo, Gregory D. Fink, Byung L. Roh, and James W. Fisher

In an attempt to clarify the role of the kidney in the action of several androgens and steroid metabolites on erythropoietin (ESF) production, ESF titers were measured in perfusates of isolated kidneys from dogs previously exposed to 4-hr hypoxia and perfused with blood containing testosterone (Test), 5α-17β-hydroxy-androstane-3-one (5αDHT), 5β-17β-hydroxy-androstane-3-one (5βDHT), 19-nortestosterone (19-nor), oxymetholone (Oxy), fluoxymesterone (Fluoxy), 3α-hydroxy-5β-pregnane, 11,20-dione (3αOH5βpreg), or 3β-hydroxy-5β-pregnane-20-one (3βOH-5 preg). ESF levels in the perfusates were assayed in exhypoxic polycythemic mice. Test, 5αDHT, oxy, fluoxy, and 3βOH5β-preg were found to produce a significant increase in ESF levels in the kidney perfusates. 5βDHT, 19-nor, and 3αOH5βpreg failed to produce a significant elevation in erythropoietin titers in the perfusates of the isolated perfused kidneys. These data suggest that the 4-5 double bond and the spatial configuration of the hydrogen at the 5 position as well as the lack of a methyl group in the 19 position of the basic androstan nucleus are important in the ability of these steroids to stimulate kidney production of ESF.

Testosterone has been reported to induce remissions in some patients with refractory anemias.1,2,3,4,5 It has been postulated that the erythropoietic effects of androgens in normal and polycythemic mice is erythropoietin (ESF) dependent since their stimulatory effects were abolished by nephrectomy.6,7

Several 5β steroid derivatives have been reported by Gorshein and Gardner8 to stimulate radioactive iron (59Fe) incorporation into red blood cells of polycythemic mice. On the other hand, Samuels and Fisher9 found that 5β steroids were devoid of erythropoietic activity in either mildly plethoric or polycythemic mice, whereas 19-nortestosterone produced a marked increase in 59Fe incorporation in red blood cells of both mildly plethoric and polycythemic mice. Oxymetholone10,21 and fluoxymesterone11 have both been reported to induce remissions in hypoplastic anemias in humans. In order to clarify the mechanism of action of androgen derivatives and of several steroid metabolites which have been reported to stimulate erythropoiesis in other systems, the effects of these...
steroids on erythropoietin production in the isolated perfused dog kidney were studied.

MATERIALS AND METHODS

Forty-eight male or female mongrel dogs weighing 10-20 kg were exposed to hypoxia for 4 hr (0.42 atm) in a hypobaric chamber after which they were anesthetized with pentobarbital sodium (30 mg/kg). Both kidneys were then exposed through a midline incision and the left renal artery and vein dissected free of surrounding tissues. The dogs were heparinized with 5 mg/kg of sodium heparin and the left kidney carefully removed for perfusion. The renal artery was cannulated and the kidney placed in an organ-warming chamber connected to an isolated kidney perfusion unit. The perfusion apparatus consisted of a Davol air-actuated pump and a Waters organ perfusion apparatus including an organ-warming chamber and a membrane oxygenator. The kidney was initially perfused with 1000 ml of saline at 37°C to remove intrarenal blood containing detectable levels of ESF. This saline perfusate was discarded after passage through the kidney. After this washout period, perfusion was begun at 37°C with 600 ml of blood obtained from a donor dog not subjected to hypoxia. The kidneys were perfused for 5 hr. All steroids (kindly supplied by Dr. John C. Babcock from Upjohn Laboratories, Kalamazoo, Mich. and Dr. Judith Nadell, Syntex Research, Palo Alto, Calif.) were dissolved in 95% alcohol and added to the perfusion system at a concentration of 0.2 mg/ml of blood. An equivalent amount of 95% alcohol was added to the control perfusions.

The following steroids were used in the perfusion studies: testosterone (Test), 17β-hydroxy-androstan-3-one (5α-DHT), 17β-hydroxy-5β-androstan-3-one (5β-DHT), 19-nortestosterone (19-nor), 3α-hydroxy-5β-pregnane, 11, 21 dione (3αOH-5βpreg), 3β-hydroxy-5β-pregnane-20 one (3βOH-5βpreg), fluoxymesterone (Fluoxymest), and oxymetholone (Oxymeth). Perfusion pressure was monitored continuously with a Statham pressure transducer connected via a “T” connector to the perfusion system and recorded on a Grass Polygraph. Microhematocrits, blood PO2 pCO2, and pH, and renal blood flow were determined initially and at hourly intervals throughout the experiment with an Instrumentations Lab Model 113 Blood Gas Analyzer. Blood samples were removed from the oxygenated (arterial) side of the perfusion system initially at hourly intervals and assayed for erythropoietic activity in exhypoxic polycythemic mice, prepared according to a modification of the method of Cotes and Bangham.12

HAM/ICR strain female mice (22-25 g) were made polycythemic for the polycythemic mouse assay by exposure to 0.42 atm for 2 wk. The mice were injected subcutaneously with one-half the total dose of either saline, human urinary erythropoietin standardized against the International Reference Preparation Standard B, or 0.5 ml of the assay plasma on the fourth and fifth days following their removal from the hypobaric chamber. Each mouse received 0.5 μCi of radioactive iron citrate (59Fe), intravenously, on the sixth posthypoxic day. Two days later (eighth posthypoxic day) each animal was exsanguinated via cardiac puncture and the per cent 59Fe incorporation into red blood cells determined. All mice with hematocrit values below 50%, were discarded. The method of Dunnett13 for comparing several treatments with a single control was used for the statistical analyses.

RESULTS

As indicated in Fig. 1 testosterone produced a significant (p < 0.05) elevation in erythropoietin titers in the perfusates of isolated perfused kidneys as early as 2 hr perfusion. On the other hand, 19-nortestosterone, which has previously been demonstrated to be highly active in stimulating radioactive iron incorporation in polycythemic mice,9,16,17 did not produce a significant increase in ESF levels in the kidney perfusates. Perfusion of the isolated kidney with blood alone did not result in a detectable increase in the levels of ESF in the perfusates at any time interval studied.

Table 1 compares the effects of Test, 5α-DHT, 5β-DHT, 19-nor, oxymeth, fluoxymest, 3αOH-5β preg, and 3βOH-5β preg with that of the control perfu-
tion on ESF production in the isolated perfused kidney. It can be seen that 5α-DHT was the most active steroid studied on ESF production in the isolated perfused kidneys. On the other hand, 5β-DHT only showed a moderate increase in ESF titers after 2 hr of perfusion, which returned to preinfusion levels during the remaining time of the perfusion. Table 1 also shows that both fluoxymesterone and oxymetholone, androgens which have been reported to be effective in refractory anemias,10,11,23 produced significant increases in ESF levels in the perfusates of the isolated perfused kidneys. Note that the 1-hr ESF titers in the perfusates of both fluoxymesterone- and oxymetholone-treated kidneys were higher than that of testosterone but were generally not as high at the 2-, 3-, and 4-hr time. It can be observed that of the 5β-pregnanes, only the 3β-OH form was active in increasing ESF titers in perfusates of the isolated kidney. This compound was slightly less active than testosterone. The 5β-pregnanes have been reported to have variable erythropoietic activities in poly- 

cythemic mice.8,9

Table 2 presents the pO₂, pCO₂, pH, renal blood flow, perfusion pressure, and hematocrit during the course of the control perfusion as well as during perfusion of the kidneys with the steroid metabolites. The perfusion pressure showed either a moderate increase or decrease in some experiments, but the renal blood flow was maintained constant throughout the perfusion. Thus, it is not likely that vascular tone was changed sufficient to modify renal blood flow. Hematocrit values showed a slight decrease in all perfusions. Blood pO₂ levels were not changed significantly during the control perfusion or during perfusion with Test, 5α-DHT, and 19-nor. However, a moderate reduction in blood pO₂
Table 1. Erythropoietin Titers in Plasma Perfusates from the Isolated Dog Kidney Perfused With Several Steroids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of Perfusions</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>2.17 ± 0.44</td>
<td>2.61 ± 0.80</td>
<td>1.56 ± 0.33</td>
<td>1.66 ± 0.67</td>
<td>1.30 ± 0.23</td>
<td>1.41 ± 0.42</td>
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<tr>
<td>Testosterone</td>
<td>6</td>
<td>2.52 ± 0.35</td>
<td>3.80 ± 0.64</td>
<td>7.22* ± 0.86†</td>
<td>4.85 ± 1.05</td>
<td>6.92 ± 2.66</td>
<td>11.37* ± 3.21†</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>5</td>
<td>2.76 ± 0.48</td>
<td>7.66 ± 2.02†</td>
<td>9.72* ± 0.74†</td>
<td>7.76* ± 0.69†</td>
<td>12.5* ± 2.14†</td>
<td>10.99* ± 2.51†</td>
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<tr>
<td>5β-DHT</td>
<td>5</td>
<td>3.63 ± 0.62</td>
<td>6.42 ± 1.66</td>
<td>7.63 ± 2.38</td>
<td>4.22 ± 1.56</td>
<td>3.50 ± 2.09</td>
<td>6.47 ± 3.97</td>
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<tr>
<td>19-Nor</td>
<td>5</td>
<td>2.83 ± 0.66</td>
<td>3.21 ± 0.37</td>
<td>3.35 ± 0.69</td>
<td>4.07 ± 1.65</td>
<td>4.95 ± 1.53</td>
<td>3.24 ± 0.67</td>
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<td>Oxyhexth</td>
<td>5</td>
<td>3.05 ± 0.48</td>
<td>6.61* ± 0.41†</td>
<td>7.59* ± 1.73†</td>
<td>6.30* ± 1.51</td>
<td>5.81* ± 1.19</td>
<td>6.66* ± 1.75</td>
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<tr>
<td>Fluoxymest</td>
<td>5</td>
<td>4.03 ± 0.90</td>
<td>8.20 ± 1.93</td>
<td>7.25* ± 1.42</td>
<td>7.88* ± 1.77</td>
<td>6.94* ± 1.42</td>
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<td>3α-OH-5β-preg</td>
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<td>1.98 ± 0.70</td>
<td>3.12 ± 0.78</td>
<td>4.60 ± 1.40</td>
<td>4.05 ± 1.09</td>
<td>3.03 ± 0.78</td>
<td>3.59 ± 1.90</td>
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<td>3β-OH-5β-preg</td>
<td>5</td>
<td>2.81 ± 0.49</td>
<td>6.85 ± 1.39</td>
<td>5.13 ± 0.75</td>
<td>5.90* ± 0.83†</td>
<td>7.61 ± 2.35</td>
<td>5.65 ± 2.27</td>
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± SEM.
* Indicates significantly (p < 0.05) different from the control perfusion value at the same time interval.
† Indicates significantly (p < 0.05) different from 0-hr value.
<table>
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<tr>
<th>Treatment</th>
<th>No of Perfusions</th>
<th>No of Venous Blood</th>
<th>Arterial Blood pO₂ (mm Hg)</th>
<th>Blood pCO₂ (mm Hg)</th>
<th>Blood pH</th>
<th>Hours of Perfusion</th>
<th>Renal Blood Flow (ml/g/min)</th>
<th>Mean Perfusion Pressure (mm Hg)</th>
<th>Hematocrit (%)</th>
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<td>Control</td>
<td>7</td>
<td>A</td>
<td>60</td>
<td>32</td>
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<td>7.36</td>
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<td>170</td>
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<td></td>
<td>V</td>
<td>87</td>
<td>55</td>
<td>0</td>
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<td>3.5</td>
<td>125</td>
<td>36 - 33</td>
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<td>101</td>
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<td>70</td>
<td>3</td>
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<td>A</td>
<td>88</td>
<td>49</td>
<td>5</td>
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<td>240</td>
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<td>45</td>
<td>38</td>
<td>1</td>
<td>7.00</td>
<td>3.0</td>
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<td>44 - 30</td>
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<tr>
<td>Fluoxymest</td>
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<td>A</td>
<td>79</td>
<td>102</td>
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<td>3α0H5β11.20</td>
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<td>A</td>
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<td>A</td>
<td>87</td>
<td>32</td>
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<td>7.19</td>
<td>3.0</td>
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occurred with 5β-DHT, 3αOH-5β-preg, 3β-OH-5β-preg, fluoxymest, and oxymeth. The significance of this decrease in blood pO₂ is not known. From our previous hypoxemic perfusion experiments this level of blood pO₂ would not appear to be sufficient alone to stimulate kidney ESF production. This may be related to the metabolic changes in the red cell induced by these steroids to modify oxygen uptake or delivery to the tissues.

Table 3 attempts to correlate the anabolic, androgenic, and erythropoietic effects of the various steroids used in these studies. The comparisons between anabolic (levator ani muscle) and androgenic (ventral prostate and seminal vesicles) potencies were obtained from the Androgenic and Myogenic Endocrine Bioassay Data Bulletin of the Cancer Chemotherapy National Service Center of the National Institutes of Health. The data on oxymetholone were obtained from the work of Sanchez-Medal et al. and Suchowsky et al. The erythropoietic activities of the various steroids were compared on erythropoietin production in the isolated perfused kidneys (present studies) and on radioactive iron incorporation in red cells of polycythemic mice. It is of interest that the 5α-di hydrotestosterone was the most potent in its androgenic and erythropoietic potencies of any of the steroids examined when compared to testosterone. Even though 3β-hydroxy-5β-pregnane, 20-one was almost devoid of anabolic and androgenic potency it still retained significant activity in stimulating kidney production of erythropoietin. Fluoxymesterone was the most potent anabolic agent and was more active in its androgenic activity than testosterone but was less active than testosterone in stimulating ESF production. Oxymetholone is only slightly active anabolically, less than half the anabolic activity of testosterone, and moderately active in stimulating kidney production of erythropoietin. Both oxymetholone and fluoxymesterone were more active in

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Anabolic *</th>
<th>Androgenic *</th>
<th>Erythropoietin (Exhypoxic Polycythemic Mice)</th>
<th>ESF Production (Kidney)</th>
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<tr>
<td>Testosterone</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>19-Nor</td>
<td>100</td>
<td>80</td>
<td>127</td>
<td>50</td>
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<tr>
<td>5α-DHT</td>
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<td>220</td>
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<td>57</td>
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<td>&lt;5</td>
<td>17</td>
<td>68</td>
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<td>&lt;5</td>
<td>93</td>
<td>82</td>
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<td>14</td>
<td>124</td>
<td>64</td>
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<td>Fluoxymest</td>
<td>235</td>
<td>123</td>
<td>116</td>
<td>80</td>
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</tbody>
</table>

* Estimated from parenteral studies on the ventral prostate and seminal vesicles (androgenic) and levator ani muscle (anabolic).
† Based on studies of the effects of steroid metabolites on radioactive iron incorporation in red cells of polycythemic mice injected daily (subcutaneously) for 4 days with dosages of 0.015, 0.0625 and 0.25 mg steroid/g (steroid suspended in steroid diluent). The total activity (three dosages of steroids) is expressed as per cent increase in ⁵⁹Fe incorporation in RBC.
Derived from studies on ESF production in the isolated perfused dog kidney when compared to testosterone (total activity expressed as percent increase in plasma ESF levels after 1, 2, 3, 4, and 5 hr perfusion).
stimulating erythropoiesis in polycythemic mice than testosterone. Both 5β-
dihydrotestosterone and 3α-hydroxy-5β-pregnane 11,20-dione have only negl-
igible anabolic and androgenic activity and a slight effect in stimulating kidney
production of erythropoietin. It is of interest that 19-nortestosterone, which is
similar to testosterone in its anabolic and androgenic activity, was more potent
than testosterone in stimulating erythropoiesis in the exhypoxic polycythemic
mouse assay system. However, 19-nortestosterone was only slightly active
in stimulating ESF production in the isolated perfused kidney. Thus, it would
appear that there is not a correlation between the anabolic, androgenic, and
erthropoietic activities of the steroids studied. Some of the steroids which were
not as active in stimulating ESF production may act directly on the bone
marrow erythroid cells to potentiate the action of erythropoietin at this recep-
tor site.

DISCUSSION

In the present studies, testosterone, 5α-DHT, 3β-hydroxy-5β-pregnane-20-
one, fluoxymesterone, and oxymetholone were found to be erythropoietically
active in the isolated perfused dog kidney. A slight increase in ESF levels oc-
curred in the perfusates with 5β-DHT after 2 hr perfusion. 19-Nortestosterone
and 3α-hydroxy-5β-pregnane, 11,20 dione were only slightly active in stimulat-
ing ESF production in the isolated perfused kidney.

The present finding that 5α-DHT stimulates ESF production is the first re-
port of a direct effect of this steroid to stimulate kidney production of ESF.
Therefore, the previous observations that testosterone and 5α-DHT stimulated
erthropoiesis in polycythemic mice are probably related in part to an effect
of these two androstanes in stimulating the kidney in vivo to increase elabora-
tion of ESF. The report by Gordon et al. that pretreatment with testosterone
potentiates ESF production in hypoxemic perfusions in isolated perfused rab-
bbit kidneys may indicate that steroids act through the renal erythropoietic fac-
tor (erythropoietin).

The results reported in this study indicate that there is an increased sensi-
tivity of the isolated perfused posthypoxic kidney to the erythropoietic effects
of steroids, as compared to the low sensitivity found in normal kidneys. A
similar enhancement of the effect of testosterone on ESF production was seen
in posthypoxic kidneys perfused with hypoxemic blood, suggesting that a cer-
tain threshold of renal erythropoietic factor (REF) must be reached before
hypoxia can increase ESF production. Kidneys from dogs previously exposed
to hypoxia probably have reached this threshold level of REF, and further
stimulation with steroid metabolites may produce a greater amount of ESF
than that seen with subthreshold levels of REF such as in normal kidneys. It is
also possible that 5α-DHT, Test, 3β-OH5βpreg, Oxymeth, and Fluoxymest
stimulate REF directly and/or accelerate the action of REF in converting the
plasma substrate to ESF. In this regard, it is of interest that Schooley has
demonstrated that the posthypoxic phase of erythropoietin production can be
inhibited by puromycin, a well-known inhibitor of protein synthesis. REF is
presumably a protein with enzyme characteristics. Thus, the increased
erythropoietin production in the isolated perfused kidneys seen with the
steroids may be due to induction of protein synthesis, perhaps REF, in the kidney by these steroid metabolites.

These studies indicate that the 19 methyl group and the saturation of the 4-5 double bond between rings A and B might possess some functional significance regarding the erythropoietic effects of testosterone-like steroids. The 5αH compounds caused an enhancement of ESF production while the 5βH metabolite had only negligible erythropoietic activity indicating the importance of the stereo configuration of the hydrogen at the 5 position of the androstane nucleus.

19-Nor, which differs from Test in that it lacks the 19-methyl group, was found to be almost completely devoid of erythropoietic activity in the kidney perfusion studies but more active than testosterone in stimulating erythropoiesis in polycythemic mice. This finding may indicate that the 19-methyl group is necessary for these compounds to stimulate kidney production of erythropoietin, but may not be necessary for a direct action of this steroid derivative on the bone marrow. Even though it is very difficult to draw any definite conclusions from our studies on the two 5α-pregnanes, it is of interest that compounds with a hydroxyl in the beta position and lacking an 11-ketone are active in stimulating kidney production of ESF and increasing erythropoiesis in polycythemic mice whereas the 5β-pregnane with an alpha-hydroxyl in the 3 position and a ketone at the 11 position was inactive in polycythemic mice or in stimulating kidney ESF production.

ACKNOWLEDGMENT

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