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Publication Date
2023-09-06

ERYTHROPOIETIN PRODUCTION IN THE ANEPHRIC RAT. I. RELATIONSHIP BETWEEN
NEPHRECTOMY, TIME OF HYPOXIC EXPOSURE, AND ERYTHROPOIETIN PRODUCTION

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ABSTRACT

The serum erythropoietin levels of adult male and female rats exposed immediately after nephrectomy to a simulated altitude of 22,000 ft. for 5 hours is measurable, but decreased to about 15 per cent of normal. Erythropoietin is not detected in the serum of adult female and male rats when the interval between nephrectomy and the beginning of the hypoxic exposure is increased to 8 and 16 hours, respectively. The ability of an anephric rat to respond to this hypoxic stimulus progressively decreases with increasing time after nephrectomy. The serum erythropoietin levels of rats exposed to hypoxia immediately after nephrectomy decreases when the duration of the hypoxic exposure is increased from 5 to 18 hours. The ability of young anephric rats to increase their serum erythropoietin levels is little altered if the rats are exposed to hypoxia immediately after nephrectomy, but exposure to the same hypoxic stimulus 24 hours later results in a significant reduction in erythropoietin production. The ability of both normal and anephric rats to produce erythropoietin is reduced or abolished by Actinomycin D. The serum erythropoietin produced in hypoxic anephric rats is immunologically indistinguishable from normal erythropoietin.

Functioning renal tissue is necessary for the regulation of normal erythropoiesis, and the available evidence suggests that this regulation results from the renal production or activation of the hormone erythropoietin. This evidence has been reviewed by several authors.[1-4] Equally well-established is the finding that erythropoiesis occurs at a reduced rate in the anephric human,[5] and that erythropoietic activity can be demonstrated in his serum.[6,7]

Since the initial observations of Jacobson and co-workers[8] on the importance of the kidney in rodent erythropoiesis, the production of erythropoietin in anephric animals, as a result of various types of erythropoietic stimuli, has been studied by a number of investigators in several mammalian species. The results have been somewhat conflicting even when the same species is studied. For example, some investigators report elevated plasma erythropoietin levels in nephrectomized rats exposed to hypoxia,[9-10] whereas, other workers find either no elevation[11-14] or only a slight increase.[15] Some of these differences, particularly in the older work, are probably related to the assay system used for erythropoietin determinations, but in the more recent work other variables are of importance. The age and sex of the rats used by different workers has varied as well as the interval between nephrectomy and the hypoxic exposure. Also, the length and the magnitude of the hypoxic exposure have varied. The present work is primarily concerned with a systematic study of some of these variables on erythropoietin production in the anephric rat.

MATERIALS AND METHODS

Groups of male and female Sprague-Dawley rats of varying ages were bilaterally nephrectomized under methoxyflurane (Metofane, Pitman-Moore) anesthesia. The kidneys were removed through a midline incision after ligating the renal vessels. The adrenals were left intact. Sham-operations

were performed, the adrenal separated from the kidney, and the kidneys left in situ. The rats were placed in an altitude chamber at 22,000 ft. (321 torr. PO_2 62.4) at varying times after the operation. Immediately upon removal from the altitude chamber, the rats were bled from the dorsal aorta under ether anesthesia. The blood was allowed to clot, and the serum was removed and stored frozen until used. The sera from varying numbers of similarly treated rats were pooled.

Actinomycin D, obtained from Calbiochem, was injected intravenously at a dose of 0.5 µg./Gm. body weight into groups of sham-operated and nephrecto- mized rats before the hypoxic exposure. Aliquots of serum collected from these rats were dialyzed against saline in the cold for 24 hours.

The serum of rats demonstrating erythropoietin activity was reacted against anti-erythropoietin immune sera as follows: Ten ml. aliquots of the various sera, previously diluted 1:4 with normal rat sera were placed in separate tubes, and 1.5 ml. of rabbit anti-erythropoietin or normal rabbit sera was added. The normal rat and rabbit sera had no detectable erythro- poietic activity. After incubation for 0.5 hour at $37^{\circ}C$, 3 ml. of goat anti-rabbit γ-globulin were added to each tube, and the tubes were reincubated. Each tube contained a total of 14.5 ml. The immune precipitate formed in each tube was removed by centrifugation, and the erythropoietic activity in 1 ml. of the supernatant was measured. The goat anti-rabbit γ-globulin contained no erythropoietic activity, and was added to the tubes to insure that no rabbit anti-erythropoietin was injected into the assay mice. Addition of the same amount of goat anti-rabbit γ-globulin to anti-erythropoietin before the addition of erythropoietin containing sera removed the anti- erythropoietin, and neutralization of the erythropoietic activity did not occur. One ml. of the particular immune sera used in these experiments can

neutralize the biological activity of 25 I.R.P. units of human urinary erythropoietin or about 2.5 units of sheep or mouse erythropoietin. The method of preparation and properties of anti-erythropoietin have been summarized before.[16]

Erythropoietin (EPO) was assayed in female LAF_1/JAX mice made plethoric by exposure to increasing amounts of carbon monoxide for three weeks as described by Fogh.[17] The mice were used one week after removal from the CO chamber. At this time the hematocrits were about 70 per cent, and the 72-hour ^{59}Fe uptake was 0.62 ± 0.09 per cent. Reticulocytes were not found in the peripheral blood, and nucleated erythroid cells were rarely seen in bone marrow smears. One ml. of test sera was injected subcutaneously, and 56 hours later 0.5 μCi of ^{59}Fe citrate was injected intravenously. Seventy-two hours after the radio-iron injection the mice were bled and the radioactivity in 0.5 ml. of blood determined. The results are expressed as the per cent of the injected radio-iron in the calculated blood volume which was assumed to be 7 per cent of the body weight. The mice weighed 22.3 ± 0.12 g. and had a hematocrit of 61.8 ± 0.26 at the end of the assay. Estimations of the units of erythropoietin were made from the ^{59}Fe uptakes by reference to a standard curve prepared using the International Reference Preparation (I.R.P.).

RESULTS

One ml. of serum taken from adult male rats (350 g.) 0, 1, 2, 3, 4, and 5 hours after a hypoxic exposure of 22,000 ft. stimulates erythropoiesis in plethoric mice with a 72-hour ^{59}Fe uptake of 0.76 ± 0.07 per cent, 0.88 ± 0.21 per cent, 6.8 ± 0.41 per cent, 23 ± 1.5 per cent, 21 ± 1.9 per cent, and 28 ± 0.66 per cent respectively. Therefore, a standard exposure of five hours at this altitude was used in most experiments.

In the first experiment adult male and female rats were nephrectomized and sham-operated, and immediately after recovery from the anesthesia

(0.5 - 1 hour) placed at 22,000 ft. for five hours. The erythropoietic activity of their sera is shown in Table 1. It is evident that detectable erythropoietic activity is found in the sera of both male and female rats. The serum from sham-operated females stimulated the ^{59}Fe uptake to about 40 per cent of the level observed with male rats. This means that a more than 2-fold difference in the erythropoietin levels of male and female rats occurs as a result of the hypoxic exposure, since the relationship between ^{59}Fe uptake and erythropoietin titers is logarithmic. Thus, nephrectomy decreases the ability of the male rat to respond to hypoxia more than the female, i.e., from more than 1.9 I.R.P. units/ml. to 0.22 units in the male compared to 0.80 to 0.12 units/ml. in the female.

In the next experiment the time between nephrectomy and the 5-hour hypoxic exposure was varied in both female and male rats, and the erythro-poietic activity of the sera determined. The data are shown in Table 2. It is evident that the longer the interval between nephrectomy and the hypoxic exposure the less erythropoietic activity is found in the serum. Detectable erythropoietic stimulation is not found in female rats when the interval is eight hours and in the male when the interval between nephrectomy and hypoxic exposure is 16 hours.

When male rats were exposed to hypoxia immediately after nephrectomy for increasing periods of time, the erythropoietic activity in their sera pro-gressively declines, and by 18 hours just detectable levels of erythropoietin are found. These data are shown in Table 3.

A highly significant depression in the erythropoietin levels of male rats exposed to 5-hour hypoxia immediately after nephrectomy is not ob-served until the 38th day of age. At 14 and 22 days of age nephrectomy has no discernable effect, whereas, in older rats the effect is pronounced. These data are given in Table 4. However, as shown in Table 5, when 22-day old rats

were exposed to 5-hour hypoxia 24 hours after nephrectomy, a decided de-
crease (< 0.001) in the erythropoietin levels of their sera was observed.

If sham-operated or nephrectomized rats were exposed to hypoxia im-
mediately after operation, but injected with Actinomycin D prior to the
hypoxic exposure, the erythropoietin levels of their sera decreased sig-
nificantly (P < 0.001). As indicated in Table 6, the erythropoietic
activity of the sera of nephrectomized rats receiving the drug was completely
abolished. No difference in the erythropoietic activity was observed between
serum samples dialyzed 24 hours against saline and undialyzed sera.

The data shown in Table 7 indicate that the erythropoietic activity of
the serum obtained from rats exposed to hypoxia immediately after nephrectomy
is indistinguishable immunologically from erythropoietin. These data also
indicate that the erythropoietin was present in the serum itself, and the
erythropoietic stimulation observed in the assay mouse was not the result of
endogenous erythropoietin production caused by some unknown substance in the
rat sera. Anti-erythropoietin was not injected into the assay mouse.

DISCUSSION

A number of variables involved in studying erythropoietin production in
the anephric rat have been investigated. It is evident that the sex and
age of the rat, the interval between nephrectomy and the hypoxic exposure,
as well as the length of the hypoxic exposure, are all important. Fried and
co-workers[10] have shown that the degree of hypoxia is also important. Con-
sideration of these variables can reconcile many of the apparent differences
observed in studies of the anephric rat in the recent literature.

The relationship between age and nephrectomy, in general, agrees with
the data of Carmena and co-workers,[14] but disagrees in some particulars. We
consistently find detectable levels of erythropoietin in 1 ml. of young 14-
and 22-day old rat serum assayed either in the transfusion-induced or post-

CO plethoric mouse, whereas, they do not detect erythropoietin at similar ages. They concluded that erythropoietin production is independent of the kidney during the first 15 days of life. Certainly our data, indicating that nephrectomy and immediate exposure to hypoxia has almost no effect of erythropoietin production at 22 days of age, could be similarly interpreted. However, we feel that since similar animals exposed to the same hypoxic stimulus, 24 hours after nephrectomy, make very little erythropoietin, the above interpretation is not necessary.

Giger[18] previously found that the serum erythropoietin levels of rats exposed to hypoxia (about 19,500 ft.) for 16 hours were significantly depressed by the injection of Actinomycin D. It has been suggested that this depression could have occurred because of a decrease in oxygen demand by the organism.[2] In our experiments Actinomycin D injected rats were exposed to hypoxia for only 5 hours, and although the oxygen demand may have decreased, we could not detect, with continuous montoring, any differences in oxygen consumption or CO_2 production between normal and injected rats during the 5-hour period. We tentatively conclude, in agreement with Giger[18] that no readily available storage form for erythropoietin exists in the nephrectomized or normal rat, and that hypoxia induces the biogenesis of erythropoietin through processes, one step at least, requiring a DNA dependent-RNA synthesis. If this conclusion is warranted, it is evident that this DNA dependent control does not reside in the kidney.

The finding that the erythropoietic activity of serum from nephrectomized rats is neutralized by anti-erythropoietin corroborates the findings of Fried et al.[10] that the stimulation is erythropoietin-dependent, and further indicates that the erythropoietin occurring in the assay serum is immunologically indistinguishable from normal erythropoietin. The possibility that endogenous erythropoietin production is stimulated in the assay mice by

some unknown factor in the anephric rat serum is excluded. The progressive decrease after operation in the ability of a nephrectomized rat to respond to a constant hypoxic stimulus with erythropoietin production raises several fundamental questions regarding the role of the kidney in erythropoietin production. Is the erythropoietin produced when the rat is exposed to hypoxia immediately after operation actually derived from extra-renal sources? Is the failure of the anephric rat to produce erythropoietin with increasing time after nephrectomy due to the disappearance of some kidney produced component involved in the synthesis of erythropoietin? How is the failure of the anephric rat to produce erythropoietin with increasing time after nephrectomy related to the profound physiological alterations in acid-base balance and hemodynamics occurring as a result of the absence of the kidney?

The kidney may normally produce and release into the blood some component involved in the biogenesis of erythropoietin, and after nephrectomy this component slowly disappears. If this component is the renal erythropoietic factor (REF) studied by Gordon and co-workers,[1] it is evident that it does not react with its substrate in the absence of a hypoxic stimulus. REF substrate is present in the plasma of normal rats,[1] and presumably is present in the plasma of anephric rats immediately after nephrectomy. It is possible that the REF substrate also disappears after nephrectomy, contributing to the failure of erythropoietin production; however, some preliminary observations do not favor this view. It appears, therefore, that if the present experiments are interpreted in terms of the REF-REF substrate concept of erythropoietin production, a third condition or component produced as a result of the hypoxic exposure must be present for erythropoietin production to occur in vivo.

The complex physiological changes in hemodynamics and acid-base balance,

occurring as a result of the removal of the kidney, probably play a more important role in the failure of the anephric rat to respond to hypoxia with the production of erythropoietin than has been suspected. It has recently been shown that nephrectomized rats, in addition to developing hypertension, have an increased cardiac output and stroke volume as well as a slight but significant decrease in oxygen consumption.[19] We have evidence, to be presented in a subsequent paper, that the hypoxic tolerance of anephric rats is far greater than normal rats.

Table 1

Erythropoietic activity of plethoric LAF_1 mice injected with serum taken from nephrectomized or sham-operated rats exposed to hypoxia.

Treatment	72-hr ^{59}Fe incorporation.	I.R.P. units EPO/ml serum.
Uninjected	0.67 ± 0.06	
Males		
Sham-operated	27 ± 0.65*	1.9
Nephrectomized	7.3 ± 0.82	0.22
Females		
Sham-operated	11 ± 0.55	0.80
Nephrectomized	4.5 ± 0.45	0.12

* Standard error of the mean. Rats exposed to a simulated altitude of 22,000 ft (321 torr.) for 5 hours.

Table 2

Erythropoietic activity of plethoric LAF_1 mice injected with serum
taken from nephrectomized rats exposed to hypoxia
at different times after operation

Time between nephrectomy and hypoxic exposure	Male		Female	
	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum
0 hours	7.3 ± 0.82*	0.22	4.5 ± 0.45	0.12
4 hours	4.3 ± 0.42	0.12	1.4 ± 0.13	detectable
8 hours	3.4 ± 0.31	0.09	0.89 ± 0.09	NS
16 hours	0.92 ± 0.10	NS	0.91 ± 0.07	NS
24 hours	0.67 ± 0.07	NS	0.77 ± 0.09	NS
uninjected	0.67 ± 0.06			

* Standard error of the mean. All rats exposed to a simulated altitude of 22,000 ft for 5 hours.

Table 3

Erythropoietic activity of plethoric LAF$_1$ mice injected
with serum taken from nephrectomized male rats exposed
immediately after operation to varying periods of hypoxia

Treatment	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum
Nephrectomy + 5 hrs. hypoxia	7.3 ± 0.82*	0.22
Nephrectomy + 11 hrs. hypoxia	5.8 ± 0.38	0.18
Nephrectomy + 18 hrs. hypoxia	1.9 ± 0.27	Detectable
Normal serum	0.54 ± 0.09	-

* Standard error of the mean. Rats exposed to a simulated altitude
of 22,000 ft (321 torr).

Table 4

Erythropoietic activity of plethoric LAF$_1$ mice injected with
serum taken from male rats of various ages
after nephrectomy and hypoxic exposure

Age of rat	Treatment	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum
14 days	Normal	2.1 ± 0.26*	0.06
	Sham-operated + 5 hrs. hypoxia	19 ± 1.2	1.1
	Nephrectomy + 5 hrs. hypoxia	17 ± 1.6	1.0
22 days	Normal	1.3 ± 0.25	Detectable
	Sham-operated + 5 hrs. hypoxia	24 ± 1.1	1.5
	Nephrectomy + 5 hrs. hypoxia	19 ± 1.8	1.1
38 days	Normal	0.44 ± 0.06	NS
	Sham-operated + 5 hrs. hypoxia	33 ± 0.78	3.0
	Nephrectomy + 5 hrs. hypoxia	16 ± 0.55	0.9
50 days	Normal	0.37 ± 0.03	NS
	Sham-operated + 5 hrs. hypoxia	29 ± 1.5	2.2
	Nephrectomy + 5 hrs. hypoxia	8.7 ± 1.3	0.26
70 days	Normal	0.82 ± 0.11	NS
	Sham-operated + 5 hrs. hypoxia	23 ± 0.96	1.5
	Nephrectomy + 5 hrs. hypoxia	2.2 ± 0.11	0.06
85 days	Normal	0.54 ± 0.09	NS
	Sham-operated + 5 hrs. hypoxia	27 ± 0.65	1.9
	Nephrectomy + 5 hrs. hypoxia	7.3 ± 0.82	0.22
	Saline	0.45 ± 0.03	-

* Standard error of the mean. Rats exposed to a simulated altitude of
22,000 ft. (321 torr). NS = not significant.

Table 5

Erythropoietic activity of plethoric LAF_1 mice
injected with serum taken from 22-day old
male rats after nephrectomy and hypoxic exposure

Treatment	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum
Normal	1.3 ± 0.25[*]	Detectable
Sham-operated + 5 hrs. hypoxia	24 ± 1.1	1.5
Nephrectomized + 5 hrs. hypoxia	19 ± 1.8	1.1
24 hrs. post-nephrectomy + 5 hrs. hypoxia	2.3 ± 0.33	0.06

[*] Standard error of the mean.

Table 6

Erythropoietic activity of plethoric LAF_1 mice injected with
serum taken from nephrectomized male rats exposed
to hypoxia and injected with Actinomycin D

Treatment	72-hr ^{59}Fe incorporation	I.R.P. units EPO/ml serum
Sham-operated +[**] 5 hrs. hypoxia	27 ± 0.65[*]	1.9
Sham-operated + 5 hrs. hypoxia + Actinomycin D	2.4 ± 0.35	0.06
Nephrectomy + 5 hrs. hypoxia	7.3 ± 0.82	0.22
Nephrectomy + 5 hrs. hypoxia + Actinomycin D	0.78 ± 0.14	NS
Normal rat serum	0.54 ± 0.09	

[*] Standard error of the mean. Actinomycin D injected intravenously
with a dose of 0.5 µg/gm body wt.
[**] Simulated altitude of 22,000 ft. (321 torr.)

Table 7

Erythropoietic activity of plethoric LAF_1 mice injected with serum
taken from nephrectomized rats exposed to hypoxia
after in vitro incubation with anti-erythropoietin

Treatment	72-hr ^{59}Fe incorporation
Males	
Sham-operated + normal rabbit sera	7.7 ± 0.92[*]
Sham-operated + anti-erythropoietin	0.99 ± 0.20
Nephrectomized + normal rabbit sera	5.9 ± 0.52
Nephrectomized + anti-erythropoietin	0.64 ± 0.07
Females	
Sham-operated + normal rabbit sera	2.9 ± 0.26
Sham-operated + anti-erythropoietin	0.68 ± 0.05
Nephrectomized + normal rabbit sera	4.2 ± 0.57
Nephrectomized + anti-erythropoietin	0.82 ± 0.12
Uninjected	0.67 ± 0.06

[*] Standard error of the mean. All rats exposed to a simulated altitude of
22,000 ft (321 torr.) for 5 hours.

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ACKNOWLEDGMENTS