Renal Erythropoietic Factor: Role of Ions and Vasoactive Agents in Erythropoietin Formation

Abstract. *Evidence is provided for the existence of a renal erythropoietic factor, devoid of vasopressor activity, which upon interaction in vitro with normal serum yields erythropoietin. When undialyzed serum is used, erythropoietin inactivation develops in the incubation mixtures, and this inactivation appears to be dependent on an enzymatic component in preparations of the factor and on ions in serum.*

The light-mitochondrial extract (*1*) of kidneys from hypoxic rats contains a factor (REF) which, although not erythropoietic when given alone, induces rapid production of erythropoietin (ESF, erythropoietic stimulating factor) when incubated with normal rat serum (*2*). Considerable loss of ESF activity occurs when the incubation time is prolonged (*3*). This inactivation phenomenon precludes the obtaining of maximum yields of ESF in such a system and renders difficult an accurate kinetic analysis of the ESF-generation reaction.

We now describe some of the properties of this ESF-inactivation reaction and a procedure that prevents its occurrence. Since it seemed possible that the inactivation process stemmed from the presence of substances in the incubation mixture that are ion-dependent, some of the incubations were conducted with serum that had been dialyzed against disodium ethylenediaminetetraacetate (EDTA). The possibility that vasoactive agents from the kidney, including renin, might be associated with the REF was also investigated.

Serum from male Long-Evans rats (250 to 280 g) was used in all cases. Light-mitochondrial extracts containing the REF were prepared by differential centrifugation from the kidneys of rats rendered hypoxic by exposure to 0.42 atm of air for 16 to 19 hours (*2*). One milliliter of extract was equivalent to 0.5 g of kidneys from hypoxic rats.

A batch of normal rat serum was dialyzed for 24 hours in cellulose tubing against 50 to 100 volumes of 0.005*M* EDTA at 4°C, and then immediately against 50 to 100 volumes of deionized water for 24 hours at 4°C. This latter dialysis procedure removed essentially all of the excess EDTA in the serum. The dialyzed serum was centrifuged at 10,000*g* for 10 minutes, the precipitated euglobulins were removed, and the supernatant was used for our experiments. A similar procedure was used to prepare EDTA-dialyzed REF. Another batch of normal rat serum was dialyzed only against 50 to 100 volumes of deionized water for 24 hours at 4°C.

The incubation system consisted usually of 6 ml of undialyzed or dialyzed serum and 6 ml of the REF preparation. All incubations were open to the air and were conducted in a water-bath incubator shaken at 37°C. The incubation mixtures, administered as a single, 2-ml intraperitoneal injection, were assayed for ESF content in mice which were made polycythemic by hypoxia (*4*). Incorporation of radioactive iron in the erythrocytes (percent) was expressed as ESF international units (*5*). Six mice were used for each sample tested. The effect of the REF and incubation mixtures, administered intravenously, on blood pressure was determined indirectly with the use of the microphonic method on the caudal artery of anesthetized, warmed adult rats and mice (*6*). The influence of Ca^{++}, Mg^{++}, and Cu^{++} was tested to investigate which divalent ions might be involved in the inactivation reaction. Five micromoles of each of these ions in chloride form were added respectively to each of three incubation mixtures containing 6 ml of EDTA-dialyzed serum and 6 ml of REF. Purified hog renin (*7*), synthetic angiotensin II (*8*), and synthetic bradykinin triacetate (*9*) were also tested for erythropoiesis-stimulating and pressor activity. All experiments were repeated three to five times.

Intraperitoneal administration of 2 ml of REF alone or 2 ml of untreated normal rat serum alone did not increase erythropoietic activity in polycythemic mice. However, the incubation of REF for 10 minutes with untreated normal rat serum resulted in the formation of ESF (*2*). When the incubation time was extended to 60 minutes, no activity could be detected in the mixtures (Table 1). The incubation of the REF with serum dialyzed against EDTA for 10 minutes produced twice the amount of ESF noted at this time with undialyzed serum. Whereas no detectable ESF activity was found after 60 minutes when undialyzed serum was used, incubation mixtures containing the EDTA-dialyzed serum and REF exhibited significantly more erythropoiesis-stimulating activity at 60 than at 10 minutes. The generation of ESF with the serum dialyzed against only deionized water (EDTA not used at all) was greater than that seen with undialyzed serum, but was significantly less, at both the 10- and 60-minute incubation periods, than that observed with the EDTA-dialyzed serum (see Table 1).

Table 1. Effect of dialyzing serum against EDTA on generation of ESF in incubation mixtures containing serum and REF. N.D., activity not detected.

Serum (ml)	REF (ml)	Erythropoiesis stimulating activity (ESF unit* ± S.E.M.)† at	
		10 min	60 min
Serum not treated			
6	6	0.48 ± 0.02	N.D.
Dialysis against EDTA			
6	6	0.96 ± 0.03	1.20 ± 0.03
12	0	N.D.	N.D.
Dialysis against deionized water only			
6	6	0.72 ± 0.02	0.24 ± 0.04

* Units of erythropoietic stimulating factor produced in the 12 ml of incubation mixture. † Mean of five assays.

Table 2. Factors influencing production of ESF in vitro. The reaction mixtures were incubated for 60 minutes.

Addition to incubation mixture	EDTA-dialyzed serum (ml)	REF (ml)	Erythropoiesis stimulation (ESF unit ± S.E.M.)*
None	6	6	0.96 ± 0.02
None		12	N.D.
5 μM $CaCl_2$	6	6	1.68 ± 0.04
5 μM $CaCl_2$	12		N.D.
5 μM $CaCl_2$			N.D.
5 μM $MgCl_2$	6	6	1.08 ± 0.04
5 μM $MgCl_2$	12		N.D.
5 μM $MgCl_2$			N.D.
5 μM $CuCl_2$	6	6	0.60 ± 0.02
5 μM $CuCl_2$	12		N.D.
5 μM $CuCl_2$			N.D.
EDTA-dialyzed REF	6	6	0.36 ± 0.02
EDTA-dialyzed REF		12	N.D.
1 μM Na_2-EDTA	6	6	0.48 ± 0.02

* Mean of three assays.

Table 3. Effects of REF, renin, angiotensin II, and bradykinin on erythropoiesis in polycythemic mice and on blood pressure in normal rats and mice. The incubation time was 60 minutes.

Composition of incubation mixture	Erythropoiesis stimulation (ESF unit ± S.E.M.)	Mean change in blood pressure (mm-Hg)	
		Rats (3)	Mice (3)
6 ml saline + 6 ml REF	N.D.	0	0
6 ml EDTA-dialyzed serum + 6 ml REF	1.16 ± 0.03	−10	0
12 ml saline + 20 units renin	N.D.	+50	+30
12 ml EDTA-dialyzed serum + 20 units renin	N.D.	+35	+15
12 ml saline + 54 μg angiotensin II *	N.D.	+30	+20
12 ml saline + 12 μg bradykinin acetate	N.D.	− 5	
12 ml saline + 120 μg bradykinin acetate	N.D.	−20	

* 180 rat units.

Table 2 indicates that when 5 μmole of Ca^{++} was added to the 12 ml of incubation mixture, a significant increase of approximately 75 percent occurred in the amount of erythropoiesis-stimulating activity generated in the 60-minute incubation period. The same quantity of Mg^{++} exerted no effect, and Cu^{++} caused a 38 percent decrease in activity. Table 2 also shows that incubation of EDTA-dialyzed REF with similarly dialyzed serum resulted in the appearance of 63 percent less erythropoiesis-enhancing activity than observed when undialyzed REF was used. The addition of 1 μmole of EDTA to the incubation mixture caused a 50 percent decline in the generation of erythropoiesis-stimulating activity (Table 2).

Intraperitoneal administration to each of five assay mice of 3.7 Goldblatt units of purified renin, incubated for 60 minutes in saline or in dialyzed serum, did not result in stimulation of erythropoiesis (Table 3). Likewise, angiotensin II (9 μg or 30 rat units per mouse) failed to augment erythropoiesis in polycythemic mice. However, similar quantities of both renin and angiotensin II increased blood pressure when administered intravenously to normal rats and mice (Table 3). Neither the REF nor the incubation mixture of REF and serum exerted any pressure-elevating action. On the contrary, in rats a slight drop in blood pressure was observed with the REF-serum mixture (Table 3). This mild vasodepressor action of the mixture of REF and serum in rats and the report of a kinin-forming enzyme in the light-mitochondrial fraction of rat kidneys (*10*) prompted us to test bradykinin for erythropoietic activity. Intravenous injections of 2.0 and 20 μg of bradykinin triacetate evoked no detectable erythropoietic response in polycythemic mice (Table 3). Injection of this agent into rats produced the expected decrease in blood pressure.

Our study supports and extends our earlier observations that an ESF-inactivating substance is present in the REF-serum mixture (*2*). The destructive factor seems to require for its activation a cation or cations present in serum. The ESF-inactivating agent is probably not a component of serum since incubation of relatively purified ESF with undialyzed serum results either in potentiation of or in no effect on erythropoietic activity of the hormone rather than its destruction (4, 11). Therefore the inactivating factor is probably present in the REF-containing extracts of kidney.

Published data indicate that kidney homogenates can inactivate exogenous ESF (*12*). Thus the REF-containing extracts may have an enzyme which, in the presence of a serum-borne metal ion, destroys newly formed ESF in the incubation mixture. The nature of the ion is not known. Although Cu^{++} caused a decrease in erythropoietic activity of the mixture, it is not believed to be the ion essential for the operation of the ESF-inactivating enzyme. A concentration of 5 μmole of Cu^{++} in the incubation mixture exceeds the amount normally present in 6 ml of serum. Moreover, even with these high concentrations of Cu^{++} in the incubation mixture, complete inactivation of ESF was not achieved even after 60 minutes of incubation (Table 2).

The plasma enzyme angiotensinase that destroys angiotensin (*13*) requires calcium ions for activity (*14*), and therefore their removal by chelation with EDTA results in the inactivation of this enzyme (*15*). Because our system shares features of the renin-angiotensin complex, it was thought that the ESF-inactivating factor might be angiotensinase. However, the finding that calcium not only failed to activate this destructive factor but actually stimulated the production of ESF in vitro suggests that angiotensinase and the ESF-inactivating enzyme are not the same. This is further supported by our observation that the REF does not contain any detectable renin-like material. Also, both renin and angiotensin exhibit no erythropoiesis-stimulating activity (Table 3) (*16*). The observed stimulatory effect of calcium on the production of ESF may be due to enhancement of ESF-generating activity of the REF. In this regard, the fact that either the dialysis of the REF against EDTA or the addition of EDTA directly to the reaction mixture caused a decrease in the erythropoiesis-stimulating activity of the incubation mixtures (Table 2) suggests that the REF may also require divalent or multivalent cations (or both) for its operation, although in concentrations lower than those needed for activity of the erythropoietin inactivator.

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References and Notes

1. The light-mitochondrial fraction, from which the extract was derived, contains particles intermediate in size between the heavy mitochondria and the microsomes. It is a heterogeneous mixture which is known to contain lysosomes and other cellular granules, but few or no true mitochondria.
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