Title

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STIMULATION OF ERYTHROPOIESIS IN THE PLETHORIC MOUSE BY CYCLIC-AMP AND ITS INHIBITION BY ANTI-ERYTHROPOIETIN

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Cyclic-AMP stimulates steroidogenesis and mediates the effects of a number of other hormones and drugs (1,2,3). Androgens, such as testosterone, stimulate erythropoiesis, whereas estrogens are inhibiting (4). The erythropoietic stimulatory effect of testosterone appears to be erythropoietin-dependent since the effect is abolished by anti-erythropoietin (5,6). The present in vivo studies were undertaken to determine whether cyclic-AMP stimulates erythropoiesis in the rodent without the participation of erythropoietin.

Materials and Methods. Female LAF_1/Jax mice weighing about 25 g were used. Mice were made plethoric by exposure to increasing amounts of carbon monoxide for 3 weeks as described by Fogh (7). The mice were used 7 days after removal from the CO chambers. At this time the 72-hr ^{59}Fe uptake was 0.62 ± 0.09%, and reticulocytes were absent from the peripheral blood. Nucleated erythroid cells were rarely seen in bone marrow smears.

N^6-2'-O-dibutyryl cyclic-AMP (db-cyclic AMP), obtained from Calbiochem, was dissolved in Gey's solution. Mice were injected intravenously with 0.2 ml containing 6 μM db-cyclic AMP. Some mice received 24 μM of db-cyclic AMP in 4 injections given at 2-hr intervals.

Anti-erythropoietin immune serum obtained from rabbits immunized with human urinary erythropoietin was injected intravenously in a volume of 0.2 ml immediately after the first db-cyclic AMP injection. One ml of this particular immune serum can neutralize the biological activity of 25 I.R.P. units of human erythropoietin or about 2.5 units of sheep or mouse erythropoietin. The method of preparation and properties of anti-erythropoietin have been summarized elsewhere (8).

Two groups of plethoric mice were exposed to a simulated altitude of 22,000 ft (321 torr.) for 6 hr; one group was injected with db-cyclic AMP immediately before the hypoxic exposure, and the other group received saline.

Fifty-six hours after the first db-cyclic AMP injection the plethoric mice were injected intravenously with 0.5 μCi of ^{59}Fe as the citrate. One group of mice was injected with ^{59}Fe 80 hr after the injection of db-cyclic-AMP. All mice were bled 72 hr after the ^{59}Fe injection, and the radioactivity in 0.5 ml of blood was measured. The results are expressed as the percent of the injected ^{59}Fe in the calculated blood volume. The blood volume of these plethoric mice was assumed to be 7% of the body weight. All hematocrits were above 60% at the time of sacrifice. Each experimental group contained 6-8 assay mice.

One group of normal mice received ^{59}Fe 24 hr after db-cyclic AMP injections, and the 6-hr ^{59}Fe uptake into the 2 femurs and spleen was determined and compared to normal mice injected with saline.

Results. The data shown in Table I indicates that db-cyclic AMP significantly stimulates erythropoiesis, as measured by ^{59}Fe incorporation, in plethoric mice. Interestingly, 1 injection of 6 μM of db-cyclic AMP stimulated more than 24 μM ($P < 0.02$). The stimulatory effect of db-cyclic AMP is completely abolished if anti-erythropoietin is also injected. The 72-hr ^{59}Fe incorporation, observed when ^{59}Fe was injected 80 hr after db-cyclic AMP, was 4.9 ± 0.89% which is not significantly greater than the 4.25 ± 0.47% value observed when ^{59}Fe was injected 56 hr after db-cyclic AMP. Thus, the maximum stimulation is not temporally displaced from that observed after erythropoietin injection.

The data presented in Table II indicates that a brief hypoxic exposure significantly stimulates erythropoiesis in the plethoric mouse as previously reported by other workers (9). This erythropoietic activity is completely abolished if anti-erythropoietin is injected before the hypoxic exposure, suggesting that the response is due to the production or release of endogenous

erythropoietin. The erythropoietic response was significantly ($P < 0.02$) potentiated when db-cyclic AMP was injected prior to the hypoxic exposure.

The 6-hr uptake of ^{59}Fe into the spleens of normal mice was significantly increased ($P < 0.005$) 24 hr after db-cyclic AMP, whereas the uptake in the 2 femurs were the same as control mice. This data is shown in Table III.

Discussion. Sandler and Hall (10) found that cyclic-AMP significantly increases the biogenesis of testosterone from endogenous precursors in the rat testis in vitro. Imura et al. (11) have shown that intravenous infusion of the dibutyryl derivative of cyclic-AMP, at dosages similar to those used in the current experiments, rapidly increased corticosterone secretion by the rat adrenal in vivo. Testosterone significantly stimulates erythropoiesis in the plethoric female mouse (12), but the evidence indicates that this stimulation is erythropoietin-dependent (5,6). Gorshein and Gardner (13) have shown that certain nonandrogenic steroid metabolites of the 5β-H configuration significantly stimulate erythropoiesis in the post-hypoxic plethoric Swiss Webster mouse. Gordon et al. (14) have concluded that these 5β-H steroids have a direct influence on erythropoietic tissues since simultaneous administration of anti-erythropoietin into normal CF-1 mice receiving the 5β-H steroid, 11-ketopregnanolone, only slightly depressed the erythropoietic stimulation. Unfortunately, these workers did not inject 5β-H steroids and anti-erythropoietin simultaneously into plethoric mice to determine whether the steroid stimulation was erythropoietin-dependent. We have attempted this experiment but have been unable in 3 separate experiments to stimulate erythropoiesis in either post-CO plethoric or transfusion-induced plethoric mice using the same steroids, the same dosages (10 μM/mouse), and the same route of injection as the above workers used in their studies with post-hypoxic (altitude) plethoric mice (unpublished observations). The injected LAF_1 mice sleep for periods of 6-8 hr after steroid injection. Regardless of

the explanation of these differences, the available evidence does not clearly indicate whether these 5β-H steroids trigger the differentiation of precursor cells into erythroid cells or increase hemoglobin synthesis in identifiable erythroid cells.

The present experiments clearly indicate that db-cyclic AMP stimulates erythropoiesis, as measured by the incorporation of ^{59}Fe into the red blood cells, in both normal and plethoric mice. It is equally clear that the stimulation of erythropoiesis in plethoric mice by db-cyclic AMP is erythropoietin-dependent, since it does not occur in the presence of anti-erythropoietin antibody. If increased steroidogenesis with the production of testosterone and possibly nonandrogenic 5β-H steroids is involved in this erythropoietic stimulation of plethoric mice, the effect is mediated by erythropoietin. It is unlikely that thyroid hormones,produced as a result of the cyclic AMP injections, stimulate erythropoiesis in these severely plethoric mice since, in our experience, injections of thyroid hormones themselves or TSH do not stimulate erythropoiesis in such mice. Even if they were involved, it is evident that the stimulation is erythropoietin-dependent.

A direct effect of db-cyclic AMP on hemoglobin synthesizing cells is not excluded by the present experiments. The increased ^{59}Fe uptake observed in normal mice 24 hr after db-cyclic AMP could be the result of an increased heme synthesis in nucleated erythroid cells already present at the time of db-cyclic AMP injection and/or an erythropoietin-dependent stimulation of erythropoietin-sensitive-cells. Gorshein and Gardner (15) have briefly reported that db-cyclic AMP and 5β-H steroids stimulate heme synthesis in human marrow cultures, and the 5β-H steroid stimulation occurred in the presence of anti-erythropoietin. Further work is required to determine whether cyclic-AMP has a direct effect upon differentiated erythroid cells.

We conclude that db-cyclic AMP stimulated erythropoiesis in the plethoric

mouse either by stimulating the production of erythropoietin itself and/or by increasing the sensitivity of erythropoietin-sensitive cells to small amounts of erythropoietin already present in the plethoric mouse. In our experience, injections of anti-erythropoietin into plethoric mice always decrease the ^{59}Fe incorporation below the uninjected or saline-injected control value. This occurs regardless of the method used in producing the plethoric state. The observed decrease is always of doubtful significance, i.e., in these experiments $0.10 > P > 0.05$. The consistency of the finding, however, does suggest that some erythropoietin is always present in plethoric mice. We believe, however, on the basis of other experiments (to be published), that the primary effect of db-cyclic AMP is an increased production of erythropoietin.

Summary. The dibutyryl derivative of cyclic-AMP significantly stimulates erythropoiesis in plethoric mice. Anti-erythropoietin completely prevents the erythropoietic stimulation caused by cyclic-AMP, indicating that the observed erythropoietic stimulation is erythropoietin-dependent. Significant stimulation of radioiron uptake was observed in the spleens of normal mice after injection of cyclic-AMP. In addition, cyclic-AMP potentiated the erythropoietic response of plethoric mice exposed to brief periods of hypoxia.

Table I

Erythropoietic response of plethoric mice to the intravenous injection of db-cyclic AMP

	72-hr ^{59}Fe incorporation
Uninjected	0.57 ± 0.05*
6 μM db-cyclic AMP	4.25 ± 0.47
6 μM db-cyclic AMP + anti-erythropoietin	0.34 ± 0.03
24 μM db-cyclic AMP**	2.67 ± 0.36
24 μM db-cyclic AMP + anti-erythropoietin	0.34 ± 0.03
Anti-erythropoietin	0.40 ± 0.07

* Standard error of the mean.

** Each mouse received 4 injections of 6 μM db-cyclic AMP at 2-hour intervals.

Table II

Erythropoietic response of plethoric mice exposed to hypoxia and injected with db-C-AMP

	72-hr ^{59}Fe incorporation
Untreated	0.57 ± 0.05*
6 hrs. hypoxia**	5.56 ± 1.0*
Anti-erythropoietin + 6 hrs. hypoxia	0.56 ± 0.09
6 μM db-cyclic AMP + 6 hrs. hypoxia	9.22 ± 0.7

* Standard error of the mean.

** Simulated altitude of 22,000 ft.

Table III

Effect of db-cyclic AMP on the 6-hr ^{59}Fe uptake of normal mouse spleen and bone marrow

	Normal	db-cyclic AMP
Femurs	5.06 ± 0.20*	4.99 ± 0.19*
Spleen	2.25 ± 0.38	6.26 + 1.0

* Standard error of the mean.

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