# Increased Kidney Cyclic AMP Levels and Erythropoietin Production Following Cobalt Administration[1] (36593)

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Adenosine 3′,5′-monophosphate (cyclic AMP) has been considered as the common mediator or "second messenger" of certain hormonal actions at the cellular level (1, 2) and indeed, considerable evidence supports this hypothesis. Hormones that have been demonstrated to elevate intracellular cyclic AMP levels in their target tissues include catecholamines, adrenocorticotropic hormone, and vasopressin. These hormones have also been shown to stimulate the production of erythropoietin (3, 4), the humoral agent which controls the rate of production of erythrocytes in the bone marrow. More recently, dibutyryl cyclic AMP was reported to produce an increase in radioactive iron ($^{59}Fe$) incorporation in red cells of polycythemic mice (5, 6) and in bone marrow cultures (7), indicating a stimulatory effect on erythropoiesis.

Gordon suggested in 1959 that a renal enzyme acts on a plasma protein to form erythropoietin (ESF). This enzyme, which is referred to as the renal erythropoietic factor (REF) or erythrogenin, was subsequently extracted from the light mitochondial fraction of kidneys from hypoxic rats (8) and shown to be capable of generating ESF. He furthermore showed that hypoxia increased the level of this enzyme in the kidney. Such an increase in REF may explain the well-known observation that hypoxia stimulates erythropoietin production.

The ability of cobalt to stimulate erythropoiesis has been demonstrated to be mediated through increased kidney erythropoietin production but the precise mechanism by which cobalt exerts this renal effect is not known (9, 10). The present studies attempt to determine if the erythropoietic effects of cobalt correlate with enhanced production of renal cyclic AMP, renal eythropoietic factor and/or plasma erythropoietin.

*Materials and Methods. Materials.* Pyruvate kinase and myokinase were obtained from the Boehringer Mannheim Corporation and cobaltous chloride hexahydrate was obtained from the Mallinckrodt Company. The antiserum to human urinary erythropoietin was produced in New Zealand albino rabbits with the use of complete Freund's adjuvant. One-tenth milliliter of this antiserum (anti-ESF) was capable of completely blocking the erythropoietic effects of 0.2 units of human urinary ESF.

*Methods. Preparation of normal dialyzed rat plasma.* Blood was obtained from the abdominal aorta of normal male Sprague Dawley rats (250 g) in syringes rinsed in heparinized saline containing 100 units heparin/ml. Plasma was obtained by centrifugation at 200*g* for 15 min. This plasma was dialyzed against cold 0.005 *M* EDTA for 24 hr, and then dialyzed against cold distilled water for 48 hr. The dialyzed plasma was stored at −20° until used in the polycythemic mouse assay.

*Preparation of REF.* Male Holtzman albino rats (200–250 g) were injected with cobaltous chloride hexahydrate (250 μmoles/kg, sc) or saline. Groups of three rats were sacrificed at 3 hr intervals up to 15 hr after injections and their kidneys were rapidly excised. These kidneys were used for the preparation of a light mitochondrial fraction containing the renal erythropoietic factor (REF) and for cyclic AMP determinations. In preparing the kidney light mitochondrial fraction, a modification of the method of Contrera, Gordon and Weintraub (8) was

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used.

In this procedure the kidneys were minced, and then homogenized with an electrically driven Potter Elvehjem homogenizer in freshly prepared 0.25 *M* sucrose. Ten grams of tissue were used for each 100 ml of 0.25 *M* sucrose. The resulting homogenate was centrifuged at 6000*g* for 10 min and the supernatant was recentrifuged at 21,000*g* for 30 min. The pellet obtained was resuspended in 20 ml of cold distilled water. This suspension was frozen and thawed three times before a final centrifugation at 35,000*g* for 30 min. The resulting supernatant which contained the REF was diluted in cold distilled water such that each milliliter of the final preparation contained the light mitochondrial fraction from 0.33 g of kidney. The REF was incubated for 30 min at 37° with an equal volume of normal rat plasma which had been dialyzed against 0.005 *M* EDTA. Two milliliters of this incubation mixture were injected ip in the polycythemic mice.

*Determination of renal cyclic AMP concentrations.* Renal cyclic AMP levels were determined in extracts of rat kidneys rapidly excised at various time intervals following cobalt administration. These kidneys were quick-frozen in a liquid nitrogen bath, scraped free of excess blood and powdered in a mortar at —20°. Following extraction with 10% trichloroacetic acid, the nucleotides were purified with thin layer chromatography and cyclic AMP concentrations were determined by the method of Goldberg *et al.* (11).

*Measurement of erythropoietic activity.* A modification of the exhypoxic polycythemic mouse assay of Cotes and Bangham (12) was used to determine the erythropoietic activity of the various preparations. HAM/ICR female mice (22–25 g) were made polycythemic by exposure in a hypobaric chamber to 0.42 atm for 2 weeks. Samples (1.0 ml) to be tested were injected subcutaneously in divided doses on the fourth and fifth day after removal from the chamber. An exception of this was the procedure followed in the determination of renal erythropoietic factor (REF). In this case 1.0 ml of REF was added to 1.0 ml of normal dialyzed rat plasma and incubated for 30 min at 37°. The REF–plasma incubation mixture (2.0 ml) was injected intraperitoneally into the test animal on the fourth day following removal from the chamber.

In all cases each mouse received 0.5 μCi of $^{59}$Fe citrate intravenously on the sixth posthypoxic day. On the eighth posthypoxic day, the mice were exsanguinated via cardiac puncture, hematocrits were determined and the percent $^{59}$Fe incorporation into newly formed red cells was determined. The significance of differences between experimental data was determined by the use of Dunnett's test for multiple comparisons with a single control (13).

*Results.* In testing the effect of cyclic AMP on erythropoiesis, the dibutyryl derivative of cyclic AMP (dBcAMP) was used because of its greater lipid solubility and its resistance to degradation by phosphodiesterase.

Figure 1 is a semilog dose–response regression line illustrating the effect of various doses of dBcAMP on $^{59}$Fe incorporation in red cells in the polycythemic mouse. As shown, the response to dBcAMP is linear between the concentrations of 300 to 1000 μmoles/kg. Each point on this plot represents the mean $^{59}$Fe incorporation in RBC of 7 animals. The dose–response regression line was fitted by the method of least squares

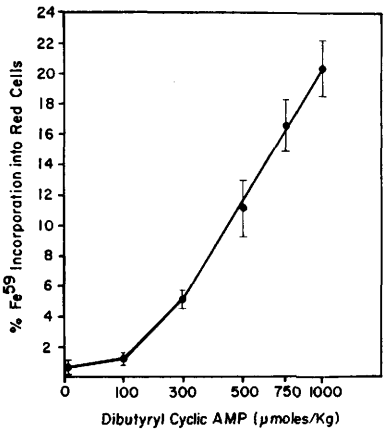


FIG. 1. Mean radioactive iron incorporation in red cells of polycythemic mice injected with dibutyryl cyclic AMP (dBcAMP). The amount of iron incorporation in red cells is represented as the mean ± the standard error of the mean ($n = 7$). The dBcamp is expressed as the log dose.

(14). In order to determine whether this erythropoietic effect of dBcAMP was directly on the bone marrow or indirectly through erythropoietin, the effects of the antibody to erythropoietin (anti-ESF) on the action of dBcAMP in polycythemic mice was studied. When anti-ESF (0.1 ml) was injected concomitantly with dBcAMP (500 μmoles/kg), a significant inhibition of the effects of dBcAMP on $^{59}Fe$ incorporation in RBC was seen (Table I). These data (Table I) suggest that the erythropoietic effects of dBc AMP are at least partially erythropoietin dependent.

In an attempt to correlate the erythropoietic effects of cobalt with enhanced production of renal cyclic AMP, renal erythropoietic factor and ESF, rats were treated with cobaltous chloride hexahydrate (250 μmoles/kg, sc) and the levels of renal cyclic AMP, REF, and plasma ESF were determined at 3 hr intervals following treatment. Figure 2 shows the results of renal cyclic AMP levels, REF levels, and plasma ESF levels following cobalt treatment. REF and ESF values are expressed as units of the International Reference Preparation of erythropoietin.

Renal cyclic AMP levels in the control rats were 9.22 ± 0.97 × $10^{-7}$ moles/kg of tissue, whereas 45 min following the administration of cobalt, cyclic AMP levels were rapidly and significantly elevated ($p < .05$) to 13.27 ± 0.43 × $10^{-7}$ moles/kg of tissue. Cyclic AMP levels in the kidney were also significantly elevated 1.5 and 3 hr following cobalt administration with a maximum concentration generated at the 3 hr period (23.05 ± 3.96 × $10^{-7}$ moles/kg). Renal cyclic AMP levels returned to control values 6 hr following cobalt administration. Control REF levels were 0.07 ± 0.01 units of ESF as compared to the 9 hr values which were equivalent to 0.18 ± 0.05 units of ESF. Tissue and incubation blanks were determined with plasma or the REF-saline mixture. When 2.0 ml of normal dialyzed rat plasma was given to the mice ip, no detectable erythropoietic activity was found. When REF was incubated with saline at 37° for 30 min instead of normal dialyzed rat plasma, the values for each time interval never exceeded 0.08 units of activity.

Plasma ESF levels were rapidly elevated to a 17-fold increase over control at 12 hr following cobalt administration. Control ESF values were 0.06 ± 0.01 units, whereas 12 hr

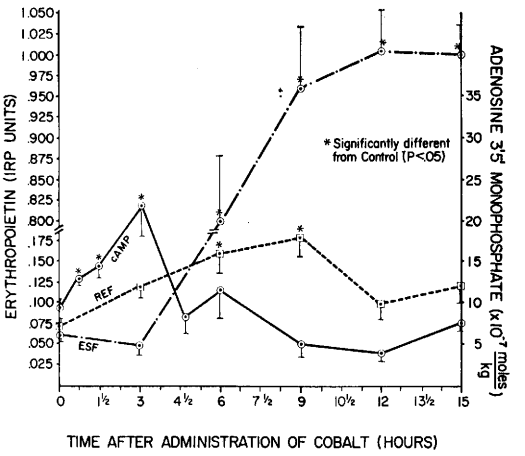


FIG. 2. Effect of cobalt administration on the levels of kidney cyclic AMP, renal erythropoietic factor and plasma erythropoietin. Measurements were performed at various times following the administration of cobaltous chloride (250 μmoles/kg). Cyclic AMP concentrations (⊙—) are in moles/kg kidney, whereas REF (□—) and ESF (⊙- - -) values are both expressed in IRP units ESF. Asterisks denote a significant difference from values at zero time ($p < .05$).

TABLE I. Effect of the Antibody to Erythropoietin on the Erythropoietic Effect of dBcAMP.

| Treatment | No. of mice | Mean % $^{59}Fe$ incorporation into RBC ± SE[a] |
|---|---|---|
| Saline | 8 | 1.73 ± 0.31 |
| ESF, 0.2 units | 10 | 13.09[c] ± 1.19 |
| Dibutyryl cyclic AMP, 500 μmoles/kg | 9 | 15.86[c] ± 2.33 |
| Dibutyryl cyclic AMP + Anti ESF, 500 μmoles/kg | 9 | 9.39[b,c] ± 1.49 |

[a] Standard error of mean.

[b] Significantly different from dibutyryl cyclic AMP alone ($p < .05$).

[c] Significantly different from saline control ($p < .05$).

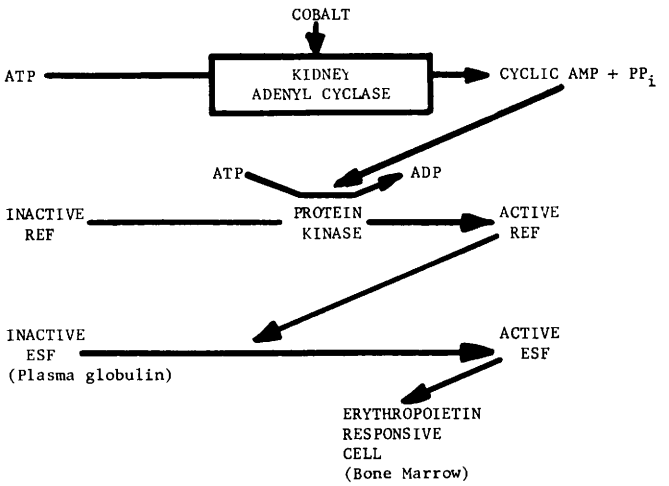


FIG. 3. Hypothesis for erythropoietic action of cobalt.

values were 1.05 ± 0.05 units. Thus, sequential increases in renal cyclic AMP, renal REF, and plasma ESF activity following cobalt administration were seen.

*Discussion.* In the present studies cobalt was found to produce a sequential rise in kidney cyclic AMP and renal erythropoietic factor which was followed by an elevation in plasma levels of erythropoietin. These data suggest that cyclic AMP may mediate the erythropoietic response to cobalt. We postulate from the model shown in Fig. 3 that cobalt stimulates kidney adenyl cyclase to produce cyclic AMP. Cyclic AMP then activates an enzyme, presumably a protein kinase, which in turn activates kidney REF leading to the production of ESF through an action on a plasma protein. ESF is postulated to stimulate an erythropoietin responsive cell in the bone marrow (15). Such an hypothesis is supported by the demonstration that cobalt is capable of stimulating certain purified cardiac adenyl cyclase preparations (16). Furthermore, the conversion of inactive REF to active REF by a cyclic AMP-dependent protein kinase is based on the ubiquitous occurrence of protein kinases in many tissues, including kidney (17). In addition, the conversion of an inactive ESF by REF has been reported by others (8). Our hypothesis is further supported by the finding (Table I) that anti-ESF partially blocked the erythropoietic action of dBcAMP. Although the action of dBcAMP may be related in part to a direct effect on the bone marrow, the results obtained in the present investigation are also consistent with our hypothesis that dBcAMP affects the production and/or release of erythropoietin.

*Summary.* The possible role of adenosine-3′,5′-monophosphate (cyclic AMP) in mediating the effect of the hormone erythropoietin (ESF) was evaluated. The administration of dibutyryl cyclic AMP (dBcAMP) to exhypoxic polycythemic mice resulted in an increase in radioactive iron ($^{59}$Fe) incorporation in RBC. This $^{59}$Fe incorporation in RBC was linear with doses of dBcAMP ranging from 300 to 1000 μmoles/kg. This erythropoietic response was partially blocked by the antibody to ESF. Three hours after cobalt administration, kidney cyclic AMP levels were significantly increased over control values. This elevation in cyclic AMP preceded a rise in renal REF activity which reached a maximum 9 hr after cobalt administration. Plasma ESF levels were also elevated reaching a maximum 12 to 15 hr after cobalt treatment. These data suggest that cyclic AMP is involved in the activation and/or production of ESF following cobalt administration.

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