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Adenosine, AMP, Cyclic AMP, Theophylline and the Action and Production of Erythropoietin (39005)

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Cyclic adenosine 3′:5′-monophosphate (cyclic AMP) mediates the effects of many hormones at the cellular level. The injection of exogenous cyclic AMP or its dibutyryl derivative stimulates erythropoiesis in the plethoric mouse, as measured by an increased uptake of ^{59}Fe into circulating red blood cells, thereby seemingly mimicking the action of the hormone, erythropoietin, on erythropoiesis (1–4). This *in vivo* action of cyclic AMP is, however, partially or completely prevented by the simultaneous administration of anti-erythropoietin (3, 4), indicating that the action of cyclic AMP is erythropoietin dependent and suggesting that cyclic AMP acts by stimulating erythropoietin production rather than by mediating directly an action upon erythropoietin-sensitive cells in the bone marrow.

The present *in vivo* experiments were undertaken to explore further the effect of cyclic AMP and other nucleotides on the action and particularly on the production of erythropoietin.

Materials and methods. Female LAF_1/JAX mice were made plethoric by a 3-week exposure to increasing concentrations of carbon monoxide. The mice were used 1 week after the end of this exposure when their hematocrits were greater than 70% and their 72-hr ^{59}Fe uptake averaged 0.62 ± 0.09%. Reticulocytes were not found in the peripheral blood or bone marrow, and nucleated erythroid cells were rarely seen in bone marrow smears.

Adenosine, adenosine 5′-monophosphate (AMP), N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP), adenosine 3′:5′-cyclic phosphate (cyclic AMP), cytidine, cytidine 5′-triphosphate (CTP), cytidine 5′-diphosphate (CDP), cytidine 5′-monophosphate (CMP), and guanosine 3′:5′-cyclic monophosphoric acid, sodium salt (cyclic GMP) were all purchased from Calbiochem. They were injected intravenously (iv) at a dose of 6 μmoles/mouse in 0.2 ml of Gey's solution. Theophylline was injected intraperitoneally (ip) at a dose of 60 mg/kg body weight. The dosage of theophylline used in these experiments is reported to have pronounced effects upon lipolysis and glycogenolysis in rats *in vivo* (5, 6). Rabbit antihuman erythropoietin sera was injected iv (0.2 ml). One milliliter neutralizes 25 units of human urinary erythropoietin. Sheep plasma erythropoietin (Connaught step I) was injected iv.

Groups of plethoric mice were exposed for 6 hr to a simulated altitude of 22,000 ft, and theophylline was injected immediately before or after the hypoxic exposure. Theophylline was injected at the same time as exogenous erythropoietin. Radioiron (0.5 μCi as the citrate) was injected iv 56 hr after injection of the test substances or after the beginning of the 6-hr hypoxic exposure. Seventy-two hours after the ^{59}Fe injection, the mice were bled and the radioactivity in 0.5 ml of blood determined. The results are expressed as the percent of injected ^{59}Fe in the calculated blood volume, which is assumed to be 7% of the body weight. The mice weighed about 22–25 g and had hematocrits >60% at the end of the assay. Estimations of units of erythropoietin were made from the 72-hr ^{59}Fe uptakes by reference to a standard curve prepared using the International Reference Preparation (IRP units).

Male rats weighing about 350 g and female LAF_1 mice weighing about 25 g were placed at a simulated altitude of 22,000 ft (321 Torr; PO_2 62.4) for 2 hr. Theophylline was injected ip at a dose of 60 mg/kg body weight either immediately before or after the brief hypoxic exposure. Other groups of mice were injected with 120 or 180 mg/kg body weight of theophylline. Rats were bled from the dorsal aorta at the indicated times under ether anesthesia. Mice were bled by cardiac puncture. The blood from 2–4

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TABLE I. EFFECT OF THEOPHYLLINE ON ERYTHROPOIESIS IN PLETHORIC MICE.

Group[a]	Treatment[b]	72-Hr ^{59}Fe uptake (%)
1	Erythropoietin (0.2 unit)	8.8 ± 0.49[c]
2	Erythropoietin (0.2 unit) + theophylline	8.6 ± 0.30
3	Theophylline	0.59 ± 0.06
4	Saline	0.91 ± 0.10
5	6 Hrs at 22,000 ft	9.4 ± 0.68
6	Theophylline + 6 hr at 22,000 ft	7.6 ± 0.83
7	6 Hr at 22,000 ft + theophylline	7.2 ± 0.69

[a] Six to eight mice per group.
[b] Erythropoietin injected iv, theophylline ip.
[c] Standard error of the mean.

TABLE II. EFFECT OF ADENINE NUCLEOSIDE AND VARIOUS NUCLEOTIDES ON ERYTHROPOIESIS IN THE PLETHORIC MOUSE

Group[a]	Treatment[b]	72-Hr ^{59}Fe uptake (%)[c]
1	ATP	1.1 ± 0.19[d]
2	ADP	1.5 ± 0.17
3	AMP	3.5 ± 0.33
4	Cyclic AMP	7.7 ± 0.52
5	Dibutyryl cyclic AMP	5.0 ± 0.59
6	Adenosine	3.2 ± 0.41
7	Saline control	0.91 ± 0.10

[a] Six to eight mice per group.
[b] Six micromoles of each compound injected iv.
[c] Groups 3, 4, 5, 6 vs 7, $P < 0.001$; Group 4 vs 5, $P < 0.005$.
[d] Standard error of the mean.

similarly treated rats or 20 mice was pooled, allowed to clot, and the serum removed. All sera were dialyzed against several changes of distilled water in the cold for 24 hr to remove theophylline. The dialyzed sera were lyophilized, redissolved in saline to the initial predialysis volume and stored frozen until used for erythropoietin assay. One milliliter of the serum was injected sc into plethoric mice, six to eight mice per group, for the bioassay of erythropoietin. The time of sampling and radioiron injection was the same as previously described (4). It is assumed that the stimulation of ^{59}Fe uptake in plethoric mice by the dialyzed sera is caused by erythropoietin.

Results. The results shown in Table I indicate that the erythropoietic response initiated by the iv injection of 0.2 IRP units of sheep erythropoietin is not significantly increased by the simultaneous administration of theophylline (Group 1 vs 2). Neither saline nor theophylline significantly stimulated erythropoiesis in the plethoric mice (Group 3 vs 4); indeed, the response to theophylline is somewhat less than the control value ($0.025 > P > 0.02$). Furthermore, the erythropoietic response of plethoric mice to a 6-hr exposure to hypoxia is not significantly altered by the injection of theophylline, either before or after the hypoxic stimulus (Groups 6 and 7 vs 5).

The results in Table II show that equimolar concentrations of adenosine and AMP, as well as of cyclic AMP and its dibutyryl derivative, significantly stimulate erythropoiesis in the plethoric mouse. The magnitude of the stimulation has varied in different experiments with similar qualitative results. ATP and ADP had no effect on erythropoiesis at this dose. The stimulation of erythropoiesis as measured by ^{59}Fe uptakes by adenosine, AMP, cyclic AMP, and dibutyryl cyclic AMP was completely neutralized by the simultaneous injection of anti-erythropoietin with ^{59}Fe uptakes less than 1%. In other experiments the same concentration of cytidine, CTP, CDP, CMP, and cyclic GMP did not stimulate erythropoiesis; the 72-hr ^{59}Fe uptakes were 0.30 ± 0.01, 0.37 ± 0.05, 0.20 ± 0.02, 0.24 ± 0.02, and 0.49 ± 0.05% respectively.

The results shown in Fig. 1 indicate that only a small amount of erythropoietin occurs in the serum of male rats immediately after a brief 2-hr hypoxic exposure, but during the next 2 hr the erythropoietin levels are markedly increased (Group 1 compared to Group 2, $P < 0.001$), even though the rats are no longer in an hypoxic environment. In rats surviving the injection of theophylline immediately before the brief hypoxic exposure (Group 3 compared to Group 2), the serum erythropoietin levels observed 2 hr after the end of the hypoxic exposure were not greater than those observed in

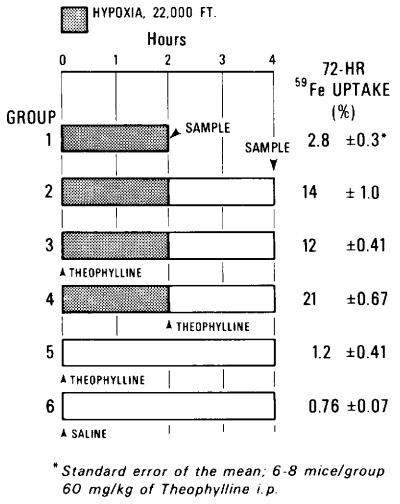

FIG. 1. Effect of theophylline given either before or after a brief hypoxic exposure in the rat.

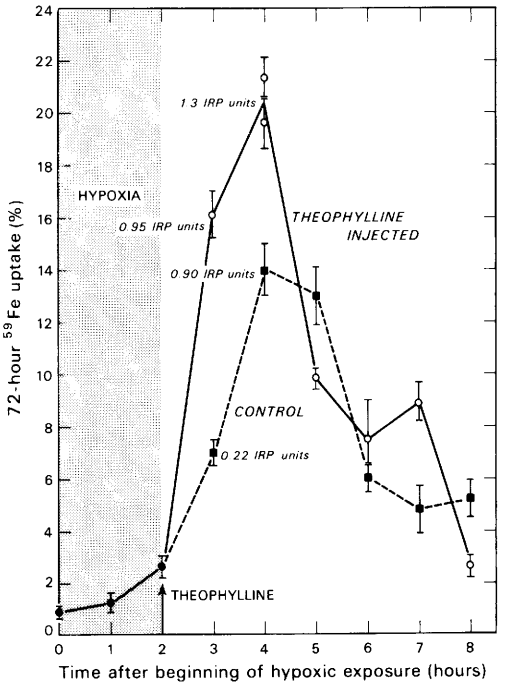

FIG. 2. Comparison of temporal pattern of erythropoietin production in control and theophylline-injected rats. Theophylline was injected (60 mg/kg, ip) immediately after a brief 2-hr hypoxic exposure.

control rats. However, the injection of theophylline after the brief hypoxic exposure does result in a significant increase in the serum erythropoietin level compared to controls measured 2 hr after the end of the hypoxia (Group 4 compared to Group 2, $P < 0.005$). This amounts to about a 30% increase in units of erythropoietin produced. Injection of theophylline alone to rats (or mice) without an hypoxic exposure did not increase the serum erythropoietin levels 4 hr later (Group 5) or at any earlier time.

The entire temporal pattern of serum erythropoietin changes for an 8-hr period in rats exposed to 2 hr of hypoxia and then injected with theophylline is compared to control rats in Fig. 2. The data for control rats has been previously reported. The serum erythropoietin levels of theophylline-injected rats increase more rapidly and reach higher levels than in control rats after the end of the hypoxic exposure.

In contrast to male rats the temporal pattern of serum erythropoietin changes in female mice exposed to 2 hr hypoxia and then injected with theophylline (60 mg/kg) was indistinguishable from the pattern observed in control mice. The maximal increase in serum erythropoietin in mice was observed 2 hr after the end of hypoxia, but the increase was much less than that seen in rats. Increasing the dosage of theophylline to 120 or 180 mg/kg in mice did not alter the temporal pattern of serum erythropoietin changes; for all doses of theophylline used, about 0.18 IRP units/ml of serum were observed 2 hr after the end of hypoxia.

It should be noted that mortality rates in rats and mice given theophylline just prior to hypoxia exposure was about 80% at a dose level of 60 mg/kg in male rats and at 120 mg/kg in female mice. No animals receiving the drug at any of the doses used died when injected immediately after the hypoxic exposure.

Discussion. Cyclic AMP has been found to mediate the action of a large number of hormones at the cellular level. It is considered that these hormones act on the surfaces of their target cells, promoting the formation of an increased concentration of cyclic AMP through the action of adenyl cyclase. Cyclic AMP in turn is destroyed by the action of phosphodiesterase, resulting in the formation of 5′-AMP. Robison *et al.* (8) have discussed four criteria neces-

sary to provide presumptive evidence that a hormone acts through stimulation of adenyl cyclase. Briefly, (a) the hormone should stimulate adenyl cyclase activity in broken-cell preparations of the target cells; (b) the hormone should elevate the intracellular level of cyclic AMP in target cells; (c) the physiological effect of the hormone should be mimicked by exogenous cyclic AMP; and (d) the action of the hormone should be potentiated by simultaneous injection of the phosphodiesterase inhibitor, theophylline.

The primary action of erythropoietin in the regulation of erythropoiesis appears to be the initiation of a number of biochemical changes in erythropoietin-sensitive cells resulting in their differentiation into recognizable erythroid cells. The morphological identity of erythropoietin-sensitive cells is conjectural, and separation of these cells from the other cells of the bone marrow has not been achieved, so that ideal *in vitro* studies to satisfy the first two criteria for the mechanism of action of erythropoietin are difficult to perform. Graber *et al.* (9), using cell suspensions of fetal rat liver that were very sensitive to erythropoietin, could not demonstrate an increase in cyclic AMP by erythropoietin in the presence of theophylline and concluded that cyclic AMP does not mediate the action of erythropoietin. The observations that injection of cyclic AMP or its dibutyryl derivative stimulated erythropoiesis in the plethoric mouse appears to satisfy the third criterion given above, that the action of erythropoietin is normally mediated by cyclic AMP. However, the failure of erythropoietic stimulation to occur in the presence of anti-erythropoietin implicates cyclic AMP and the other adenine nucleotides in the production rather than in the action of erythropoietin. The action of exogenous erythropoietin in the plethoric mouse is not potentiated by theophylline, and therefore the fourth criterion listed above does not appear to be fulfilled and is further evidence against the view that the action of erythropoietin on its target cells is mediated by cyclic AMP.

The fact that equimolar concentrations of adenosine and AMP, as well as cyclic AMP, stimulate erythropoiesis is clear, but the mechanism of action is unknown. They may stimulate adenyl cyclase activity or be converted into cyclic AMP and therefore stimulate erythropoietin production in the kidney by a cyclic AMP-dependent protein kinase, as suggested by Rodgers *et al.* (10), or they may act less specifically by altering the renal blood flow, through a depressor activity described by Gordon (11), producing an hypoxic kidney, which in turn gives rise to erythropoietin production. The multitude of physiological effects mediated by these compounds makes interpretation of the mechanism of action of these compounds difficult, particularly in intact animals; however, the results with anti-erythropoietin clearly indicate that the ultimate stimulation of erythropiesis by these compounds is erythropoietin dependent.

We have previously demonstrated (6) that the elevation of erythropoietin after a brief hypoxic exposure is the result of *de novo* protein synthesis that is not actinomycin D sensitive, whereas the initial inductive phase of the hormone's production occurring during the hypoxic exposure is actinomycin D sensitive. The present experiment implicates cyclic AMP or adenyl cyclase in the synthetic phase of erythropoietin production, as theophylline only increases the hormone's production when given after the hypoxic exposure. This increase in the synthesis of the hormone may be a direct action of cyclic AMP, but the possibility that the nucleotide stimulates other physiological processes, which indirectly increase the synthesis of erythropoietin, cannot be excluded. The failure of theophylline to increase erythropoietin production in the mouse even at high doses is not understood and must be further studied. The possibility of a sex difference in the action of cyclic AMP is being investigated.

The experiments on the effects of adenine nucleotides and theophylline on the action and production of erythropoietin in the intact animal must be considered cautiously. The results do not favor the concept that the action of erythropoietin on its target cell is mediated by cyclic AMP, but they do indicate that processes involved in the biogenesis of erythropoietin may be either directly or

indirectly influenced by cyclic AMP or adenyl cyclase. We would emphasize that the results from bioassay clearly indicate that the injection into plethoric mice of substances suspected of containing erythropoietin, which could also contain cyclic AMP, AMP, and/or adenosine, may be interpreted falsely as indicating the presence of the hormone. Simultaneous injection of anti-erythropoietin or the *in vitro* destruction of erythropoietin by neuraminidase or trypsin can be used to determine the presence or absence of substances used in the bioassay.

Summary. Erythropoiesis, as measured by the uptake of ^{59}Fe into plethoric mice, is stimulated by adenosine, AMP, cyclic AMP, and dibutyryl cyclic AMP, but not by cytidine, its nucleotides or cyclic GMP. This stimulation is erythropoietin dependent, because it is prevented by anti-erythropoietin. Theophylline neither stimulates erythropoiesis nor potentiates the action of erythropoietin on bone marrow cells in plethoric mice. Theophylline does potentiate the production of erythropoietin in rats following a brief hypoxic exposure but does not cause a similar increase in mice.

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