ELEVATED CYCLIC GMP CONCENTRATIONS IN RABBIT BONE MARROW CULTURE AND MOUSE SPLEEN FOLLOWING ERYTHROPOIETIC STIMULATION

by

George M. Rodgers, James W. Fisher and William J. George

Department of Pharmacology
Tulane Medical School
New Orleans, Louisiana 70112

Received February 9, 1976

SUMMARY

The effects of erythropoietin and hypoxia on cyclic nucleotide concentrations in erythroid tissue were evaluated. A rabbit bone marrow culture system and a mouse spleen model provided evidence that erythropoietin and an hypoxic stimulus which increases erythropoietin production may enhance erythropoiesis by initiating reciprocal changes in erythroid cell cyclic nucleotide levels. Cyclic GMP appears to be the active signal in mediating the response to erythropoietin, whereas cyclic AMP may be a passive signal allowing full expression of the cyclic GMP response. Whether the type of response mediated by cyclic nucleotides is proliferative, differentiative or both is not clear, but our data and that of other investigators suggest that cyclic GMP mediates the proliferative actions of erythropoietin.

Cyclic nucleotides (cAMP[1], cGMP) have been demonstrated to mediate hormonal responses by acting as "second messengers" in many tissues (1). We have previously described roles for these cyclic nucleotides in the production of Ep (2,3), the renal hormone that regulates erythropoiesis (4). The present investigation is an extension of our earlier studies involving cyclic nucleotides and the response to Ep (5). Two different models that were employed in these experiments to test the effects of Ep or erythropoietic stimuli on cAMP and cGMP levels in Ep-responsive tissue are: (a) the rabbit bone marrow culture to which Ep was added and (b) spleens of mice exposed to hypoxia.

---

[1] Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP; Ep, erythropoietin

Bruce and McCulloch (6) observed that the spleen was an important erythropoietic organ in mice, and this tissue has since been used to study erythropoiesis by many investigators. In our experiments, the spleen was selected because it represented an in vivo erythropoietic tissue that is easily accessible for cyclic nucleotide analysis. In order to determine whether the cyclic nucleotide pattern seen in mouse spleens following erythropoietic stimulation was also present in other erythroid tissue, a rabbit bone marrow culture was utilized. The data from these studies suggest that the target organ response to Ep involves reciprocal changes in the tissue concentrations of both cyclic nucleotides; elevated cGMP concentrations may represent the active signal for Ep stimulation, while decreased cAMP levels may represent a passive signal for this hormone.

METHODS

Rabbit bone marrow culture. An in vitro rabbit bone marrow culture system was used in these studies as previously described (7). Each culture dish contained 2.5×10^6 nucleated cells. Following addition of Ep (0.05 units), cultures were frozen in liquid nitrogen and stored at -50°C until extracted and assayed as previously described (8). Separate cultures in each experiment were prepared to measure heme synthesis (7) to insure erythropoietin responsiveness of the culture system.

In vivo experiments. Normal female CD-1 mice (23-25 g) were exposed to 0.42 atmospheres in a hypobaric chamber for up to 7 hours. Hypoxia has previously been shown to be a potent stimulus for Ep production (8). After several time intervals of hypoxic exposure, mice were removed from the chamber, anesthetized with ether and their spleens excised and quickly frozen in freon cooled by liquid nitrogen. Tissues were then extracted in preparation for cyclic nucleotide assays as previously described (8).

Cyclic nucleotide analysis. Determination of cAMP and cGMP concentrations were carried out with a protein-binding method (9) and radioimmunoassay (10), respectively.

Standard Ep. Human urinary Ep with a specific activity of 11.7 units/mg protein was obtained from the National Institutes of Health.*

Statistics. The statistical significance of experimental data was analyzed according to Dunnett's test for comparing several treatments with a single control (11).

---

*Human urinary erythropoietin was supplied by the Department of Physiology, University of the Northeast, Corrientes, Argentina, further processed and assayed by Hematology Research Laboratories, Childrens Hospital of Los Angeles, under Research Grant HE 10880 (National Heart and Lung Institute).

TABLE I

EFFECTS OF Ep ON CYCLIC NUCLEOTIDE LEVELS
OF RABBIT BONE MARROW CULTURE

Time of incubation with Ep (0.05 units)	% of Control at Each Interval	
	Cyclic AMP	Cyclic GMP
1 hour	80 ± 61	343 ± 24*
6 hours	100 ± 24	180 ± 60
12 hours	76 ± 12*	46 ± 26*
24 hours	68 ± 18*	350 ± 55*

The results from 4 experiments are expressed as per cent of control at each interval. Basal marrow cAMP and cGMP levels were 7 pmoles/10^6 cells and 1 pmole/10^6 cells, respectively. Asterisks denote values significantly ($p < .05$) different from control.

RESULTS

Bone marrow culture. Table I depicts the effects of Ep on cyclic nucleotide levels, expressed as per cent of control at each time interval, in rabbit bone marrow cultures. After 1 hour of incubation with Ep (0.05 units), marrow cGMP concentrations were significantly ($p < .05$) increased to more than 300% of the control values, while cAMP levels were not significantly changed. After 12 hours of incubation with Ep, both cAMP and cGMP levels were significantly decreased reaching minimal values equivalent to 76% and 46% of control, respectively. After 24 hours of incubation with Ep, marrow cGMP levels were again significantly elevated to over 300% of control values, while cAMP levels were still below control values at the same interval.

Other experiments were performed to test the effects of cAMP, cGMP and their dibutyryl or 8-bromo derivatives on ^{59}Fe incorporation into heme of rabbit bone marrow cultures. In concentrations ranging from $10^{-5}M$ to $10^{-12}M$, neither cyclic nucleotide nor their derivatives exhibited any effect on heme synthesis.

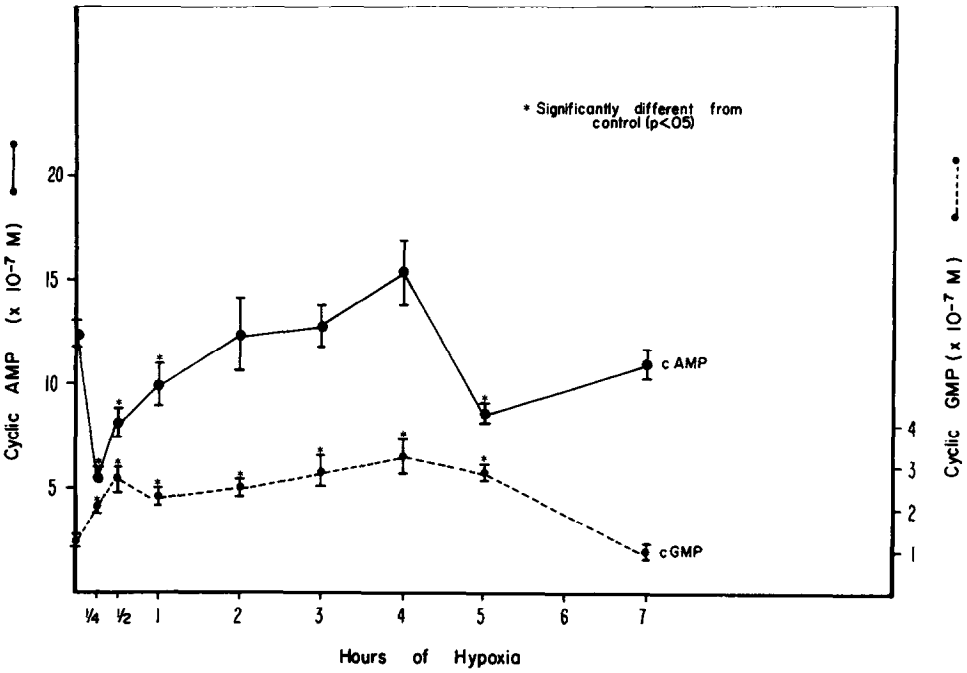

Figure 1. Cyclic nucleotide levels in mouse spleen following exposure to hypoxia. Concentrations of cAMP and cGMP were determined as described in the text. Each value represents the mean ± standard error of 4 experiments. Asterisks denote experimental values significantly ($p < .05$) different from control.

Studies with mouse spleens. Normal mice were exposed to hypoxia and their spleens were removed after several time intervals of hypoxic exposure and assayed for cAMP and cGMP. Figure 1 illustrates changes in splenic cyclic nucleotide levels after hypoxia. Control levels of cAMP and cGMP were $12.4 \pm 0.6 \times 10^{-7}$M and $1.5 \pm 0.10 \times 10^{-7}$M, respectively. As little as 15 minutes of hypoxic exposure induced reciprocal changes in splenic cyclic nucleotide levels, with an increase in cGMP to 188% of control and a decrease in cAMP content to 55% of control. As the hypoxic interval was prolonged, elevated splenic cGMP levels were maintained for up to 5 hours of hypoxia.

DISCUSSION

Bürk (12) in 1968 initially implicated cyclic nucleotides in cellular proliferation and differentiation by reporting that cAMP inhibited cell

division. Since that time, many investigators have presented conflicting evidence concerning the role of cAMP in these processes (13). The evidence for a cGMP role in influencing cell proliferation was first presented by Hadden et al. (14) in which they found that cGMP was associated with lymphocyte mitogenesis. Goldberg and coworkers (15) have postulated that cGMP represents an active signal for these processes, while cAMP is a passive signal. Reciprocal changes in both nucleotides may be necessary to express the full cGMP-linked response.

Although the effect of cyclic nucleotides on Ep production has been studied (2,3), the role of cAMP and cGMP in the target organ response to Ep is unclear. Most of these studies have concerned a possible cAMP effect on erythroid tissue. Bottomley et al. (16) demonstrated a cAMP stimulation of δ-aminolevulinic acid synthetase. Additionally, Brown and Adamson (17) and Congote et al. (18) reported an in vitro stimulation of heme synthesis by cAMP in cell culture. However, other investigators (19,20) including those from our own laboratory have been unable to stimulate heme synthesis in vitro by cAMP in various systems. Additionally, Ep was shown to lack a stimulatory effect on rat fetal liver adenylate cyclase (21) while in mouse spleen, Ep decreased both cAMP levels and adenylate cyclase activity (22).

Reports of cGMP effects on Ep-mediated processes in vitro or in vivo have also been conflicting. Studies with the polycythemic mouse bioassay, an in vivo model sensitive to erythropoietic agents which act directly on erythroid cells, indicate that cGMP is without effect (2). Additionally, we have failed to observe any in vitro stimulation of heme synthesis in bone marrow cultures with cGMP or its derivatives. However, Byron has demonstrated that cGMP does exert an effect on hematopoietic stem cells of spleen to trigger these cells into cell cycle and increase DNA synthesis (23).

Our data in the two models tested (rabbit bone marrow culture and normal mouse spleen) indicate that cGMP is the important cyclic nucleotide mediating the erythroid cell response to Ep. However, the cGMP mediated effect may

only be expressed when there is a reciprocal decrease in cAMP concentration (15). Although the extent to which the cyclic nucleotide levels varied was different in each model, the pattern of increased cGMP and decreased cAMP levels was consistently observed.

The key question raised by these studies concerns the role of cGMP in the mediation of the Ep response. Ep has been demonstrated to exert both proliferative (24) and differentiative (25) effects on erythroid cells. The previous data concerning cGMP effects on erythropoiesis have uniformly failed to demonstrate a direct stimulation of heme synthesis by this nucleotide (2,20). These studies would appear to rule out a primary effect of cGMP on erythroid differentiation. On the other hand, studies with lymphocytes (14,16), hematopoietic stem cells (23) and other tissues (15) would suggest that cGMP may mediate erythroid proliferation in response to Ep. Additionally, other investigators have studied DNA synthesis in erythroid models similar to those in our experiments. Dukes (26) reported that in rat bone marrow culture, DNA synthesis increased 3-4 hours after the addition of Ep. In our marrow experiments (Table I), elevations in cGMP concentrations occurred 1 hour after Ep addition, indicating a temporal relationship between increased marrow cGMP levels and DNA synthesis. Hodgson (27) observed that mouse splenic DNA synthesis after exogenous Ep injection was increased 12 hours following treatment. In our mouse model, splenic cGMP levels were elevated for up to 5 hours (Figure 1), again correlating increased cGMP levels with DNA synthesis. In other studies, associations of cGMP changes with stages of the cell cycle have been reported (28), providing further evidence of a cGMP role in cell proliferation.

The exact cGMP mechanism in Ep-stimulated tissue is still unclear. However, experiments using a rat fetal liver culture may elucidate the correct role for this nucleotide. This culture system has been reported by White *et al.* (29) and Bomboy *et al.* (30)to be extremely sensitive to Ep and to respond to this hormone by increasing levels of cGMP. At this

point, our data implicate cGMP as a mediator of the erythroid cell response to Ep and suggest that this nucleotide may act to stimulate erythropoiesis by primarily enhancing erythroid proliferation.

ACKNOWLEDGEMENTS

This work was supported in part by USPHS grants HL-14976 and HL-13776 and a grant from the Edward G.Schlieder Foundation. The authors gratefully acknowledge the excellent technical assistance of Mrs. Phyllis Howley and Miss Patricia Dargon.

REFERENCES

1. Robison, G.A., Butcher, R.W. and Sutherland, E.W. (1971) In: Cyclic AMP, Academic Press, New York and London.
2. Rodgers, G.M., Fisher, J.W. and George, W.J. (1975) Amer. J. Med. 58,31-38.
3. Rodgers, G.M., Fisher, J.W. and George, W.J. (1974) Biochem. Biophys. Res. Comm. 59,979-984.
4. Fisher, J.W. (1972) Pharmacol. Rev. 24,459-508.
5. Rodgers, G.M., Fisher, J.W. and George, W.J. (1974) Abstracts of the Amer. Soc. Hematol. 17,64.
6. Bruce, W.R. and McCulloch, E.A. (1964) Blood 23,216-232.
7. Krantz, S.B., Gallien-Lartigue, O. and Goldwasser, E. (1963) J. Biol. Chem. 238,4085-4090.
8. Rodgers, G.M., Fisher, J.W. and George, W.J. (1974) J. Pharmacol. Exp. Ther. 190,542-550.
9. Gilman, A.G. (1970) Proc. Natl. Acad. Sci. USA 67,305-312.
10. Steiner, A.L., Parker, C.W. and Kipnis, D.M. (1972) J. Biol. Chem. 247,1106-1120.
11. Dunnett, C.W. (1964) Biometrics 20,482-490.
12. Bürk, R.R. (1968) Nature 219,1272-1275.
13. Whitfield, J.F., Rixon, R.H., MacManus, J.P. and Balk, S.D. (1973) In Vitro 8,257-278.
14. Hadden, J.W., Hadden, E.M., Haddox, M.K. and Goldberg, N.D. (1972) Proc. Natl. Acad. Sci. USA 69,3021-3027.
15. Goldberg, N.D., Haddox, M.K., Dunham, E., Lopez, C. and Hadden, J.W. (1974) In: Control of Proliferation in Animal Cells. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 1 (ed. by B. Clarkson and R. Baserga), pp. 609-625, Academic Press, New York.
16. Bottomley, S.S., Whitcomb, W.H., Smithee, G.A. and Moore, M.Z. (1971) J. Lab. Clin. Med. 77,793-801.
17. Brown, J.E. and Adamson, J.W. (1974) Abstracts of the Amer. Soc. Hematol. 17,53.
18. Congote, L.F., Stern, M.D. and Solomon, S. (1974) Biochem. 13,4255-4263.
19. Morley, A., Quesenberry, P., Garrity, M. and Stohlman, F. (1971) Proc. Soc. Exp. Biol. Med. 138,57-59.
20. Graber, S.E., Carrillo, M. and Krantz, S.B. (1972) Proc. Soc. Exp. Biol. Med. 141,206-210.
21. Graber, S.E., Carrillo, M. and Krantz, S.B. (1974) J. Lab. Clin. Med. 83,288-295.

22. Winkert, J. (1973) Fed. Proc. 32,535.
23. Byron, J.W. (1973) Nature 241,152-154.
24. Chui, D.H.K., Djaldetti, M., Marks, P.A. and Rifkind, R.A. (1971) J. Cell Biol. 51,585-595.
25. Goldwasser, E.(1966) In: Current Topics in Developmental Biology (ed. by A.A. Moscona and A. Monroy), pp. 173-211, Academic Press, New York.
26. Dukes, P.P. (1968) Ann. N.Y. Acad. Sci. 149,437-444.
27. Hodgson, G. (1967) Proc. Soc. Exp. Biol. Med. 124,1045-1047.
28. Seifert, W. and Rudland, P.S. (1974) Proc. Natl. Acad. Sci. USA 71, 4920-4924.
29. White, L.A., Rodgers, G.M., Howley, P.S., Fisher, J.W. and George, W.J. (1975) Pharmacologist 17,269.
30. Bomboy, J.D., Graber, S.E., Salmon, W.D. and Krantz, S.B. (1975) Clin. Res. 23,269A.