PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 186–193 (1981)

Increased Concentrations of Cyclic GMP in Fetal Liver Cells Stimulated by Erythropoietin (41044)

LUANN E. WHITE AND WILLIAM J. GEORGE

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Abstract. The hormone erythropoietin stimulates the processes of cellular proliferation and differentiation in erythroid cells. A possible role for cyclic AMP and cyclic GMP in these events was evaluated in cultures of erythroid cells obtained from the livers of 14-day rat feti. A partially purified preparation of erythropoietin (Ep) stimulated ^{59}Fe incorporation into heme in a dose- and time-dependent manner, indicating that the fetal liver cells were responsive to this hormone. Ep stimulated an increase in tissue cyclic GMP content in these cultures at 4 to 9 hr of incubation. However, exogenous addition of cyclic GMP to cultures did not affect heme synthesis. In correlation studies in which cyclic GMP levels were determined and thymidine incorporation into DNA was monitored, cyclic GMP did not appear to be related to the Ep-induced stimulation of DNA synthesis. Addition of 8-bromo-cyclic GMP to cultures did not increase DNA synthesis and addition of FUdR to cultures did not alter the Ep-induced increase in cyclic GMP. The elevation in tissue cyclic GMP concentrations produced by this Ep was blocked by an antibody to this hormone. Therefore, it appears that cyclic GMP may be associated with Ep-stimulated events in the proliferating fetal liver cell other than heme synthesis.

Erythropoietin (Ep) is a hormone which stimulates the proliferation and differentiation of erythroid cells (1). Ep has been shown to act on erythroid cell precursors to induce the synthesis of RNA, DNA and hemoglobin (2, 3) during maturation. These synthetic events occur in a specific sequence such that if any step in this Ep dependent biochemical series is blocked, all subsequent steps in the pathway are also inhibited. However, the regulatory mechanisms which result in Ep-induced cell division and differentiation have not been elucidated.

The cyclic nucleotides, cyclic AMP and cyclic GMP, have been implicated as possible regulatory agents in cell division and differentiation (4). From studies with a large number of tissues, correlations have emerged between cyclic nucleotide concentrations and changes in the proliferative state. In a number of tissues, direct correlations have been found between increased cyclic GMP concentrations and an increased rate of cell proliferation. On the other hand, inverse correlations have been observed between enhanced cell division and cyclic AMP content (5).

In the lymphocyte, similar correlations have been found between changes in cyclic GMP levels and proliferation induced by mitogens. Phytohemagglutinin and concanavalin A have been shown to increase cyclic GMP content in lymphocytes (6). Furthermore, it has been demonstrated that addition of cyclic GMP to lymphocytes mimics mitogen-induced DNA synthesis (6). Other investigators have suggested that an increase in cellular cyclic AMP content just prior to S is important for the stimulation of DNA synthesis in several tissues (7).

Since Ep acts to induce cell division and differentiation in Ep-sensitive tissues (3), the relationship between Ep and the cyclic nucleotides has been the subject of numerous investigations, Rodgers and co-workers (8) reported that Ep increased the cyclic GMP content in bone marrow cultures. Such Ep-induced elevations in cyclic GMP concentrations were also reported to occur in fetal liver cultures (9) which are an excellent source of erythroid cells.

The present studies were undertaken to clarify further the role of cyclic nucleotides in cell division and to evaluate also the action of Ep on erythroid cells.

Materials and Methods. *Preparation of fetal liver cultures.* Cell cultures from 14-

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day feti of Sprague–Dawley rats were used as the source of Ep-sensitive tissue. At 13–14 days of development, the fetal rat liver is the primary hemopoietic organ and contains a high percentage of erythroid cells (10). The livers of 14-day rat feti were excised and placed in Hanks' balanced salt solution (HBSS). The cells were then mechanically disaggregated and washed twice in HBSS. The cells were placed in culture media which consisted of 90% Waymouth's solution (MB 752/1), 5% fetal calf serum and 5% fetal rat serum. Cultures were then incubated at 37° under an atmosphere of 95% O_2 and 5% CO_2 in a Labline CO_2 incubator. The cells prepared in this manner were used for all subsequent experiments. Viability of the cells in culture was assessed by the dye exclusion method using Trypan Blue. One-tenth milliliter of cell suspension containing 300,000 to 600,000 cells was diluted to 1.0 ml with 1% Trypan blue, and viable cells were monitored microscopically. The viability of cultures was routinely found to be greater than 90%.

Source and purity of erythropoietin and the antibody to erythropoietin. The erythropoietin (Ep) used in these studies consisted of several batches of hormones obtained from the Division of Blood Diseases and Resources of the Department of Health, Education and Welfare. The Ep was collected from urinary concentrates by adsorption using the benzoic acid method of Espada and Gutnisky (17). Resulting partially purified Ep preparations had the following specific activities as determined by the "exhypoxic polycythemic mouse ^{59}Fe incorporation" method:

a. Batch M-3-TaLSL 11,700 mU/mg
b. Batch M-8-TaLSL 16,300 mU/mg
c. Batch H-4H-35 15,300 mU/mg

Each of these preparations was diluted in culture media such that the final concentration of Ep was 100 mU/ml.

Based on a report from Krantz and coworkers (15) it was decided to test the erythropoietin preparations for the presence of bacterial endotoxin. The method employed was the Limulus Amebocyte Lysate endotoxin text (13,14). This test determines the presence of endotoxin at concentrations as low as 10–140 pg/ml by monitoring the formation of a hemolymph plasma gel. When 100 mU of the purified erythropoietin preparation was tested there was no gel formation thus indicating that less than 140 pg of endotoxin was present in 100 mU of the Ep preparation. Negative results were assumed to indicate an endotoxin free preparation.

The antibody to Ep (anti-Ep) in these studies was obtained from rabbits immunized against the M-3 TaLSL batch of Ep. The antiserum was standardized and lyophilized. Anti-Ep sufficient to completely neutralize 100 mU of Ep was used in selected experiments since 100 mU of Ep was used as the test stimulus.

Heme synthesis. Cultures containing 3×10^6 cells were incubated for 20 hr in the presence and absence of Ep (and/or other designated agent). Transferrin-bound ^{59}Fe was added to the cultures and the incubation continued for 4 additional hr (total incubation time = 24 hr). The cells were washed three times with phosphate-buffered saline, after which they were lysed with 2 ml of Drabkins solution. Heme was extracted with cyclohexanone and the percentage ^{59}Fe incorporation into heme was monitored as a measure of heme synthesis.

Cyclic nucleotide content. Cultures containing 7×10^6 cells were incubated in the presence or absence of Ep and/or the presence or absence of 1-methyl-3-isobutylxanthine (MIX). At selected times of incubation, the cultures were frozen in liquid nitrogen. Cyclic AMP was measured by radioimmunoassay (11) and cyclic GMP was measured according to a succinylation modification of the radioimmunoassay (12).

DNA synthesis. Fetal liver cultures containing 3×10^6 cells were pulsed for 30 min with 1 μCi of [^{3}H]thymidine after incubation with various agents. The cells were washed three times with HBSS. DNA was then extracted with hot perchloric acid. DNA synthesis was determined as the percentage [^{3}H]thymidine incorporated into DNA.

Results. *Stimulation of ^{59}Fe incorporation.* Fetal liver cultures exhibit a basal level of ^{59}Fe incorporation into heme in the

absence of added Ep. Addition of the Ep preparation (6.25 to 200 mU) stimulated ^{59}Fe incorporation into heme in these cells in a dose-related manner (Fig. 1). Incubation of fetal liver cultures with 100 mU of Ep increased ^{59}Fe incorporation above basal levels in a time-dependent linear fashion at all times examined (1–6 hr) (Fig. 2). These experiments indicate that fetal liver cells are sensitive to Ep and respond to physiological doses of this hormone.

The possible direct effect of Ep, MIX, and cyclic nucleotides on heme synthesis was evaluated. ^{59}Fe incorporation into heme of fetal liver cells was not significantly different from controls following the addition of lipophilic derivatives (1×10^{-4} *M* to 1×10^{-6} *M*) of cyclic AMP (dibutyryl cyclic AMP) (db cyclic AMP) or cyclic GMP (8-bromo-cyclic GMP) (8-br-cyclic GMP). Table I shows that the percentage ^{59}Fe incorporation in cultures containing 1×10^{-6} *M* db cyclic AMP or 1×10^{-6} *M* 8-br-cyclic GMP was not significantly different from control. The addition of a phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX) (1×10^{-5} *M*), to these cultures was without significant effect on heme synthesis ($P > 0.05$). However, there was a significant difference in ^{59}Fe incorporation between cultures receiving 8-bromo cyclic GMP and those receiving dibutyryl cyclic AMP ($P < 0.05$). When cyclic nucleotides were added to cultures containing Ep, neither dibutyryl cyclic AMP (1×10^{-6} *M*) nor 8-bromo cyclic GMP (1×10^{-6} *M*) changed the stimulation of ^{59}Fe incorporation produced by 100 mU of Ep.

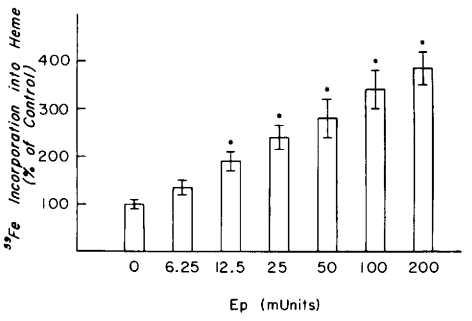

FIG. 1. Effect of erythropoietin (Ep) on ^{59}Fe incorporation into heme. Various doses of Ep ranging from 6.25 to 200 mU/ml were tested in fetal liver cultures incubated for 24 hr. Values represent the mean of three individual experiments, each performed in triplicate. (*) indicates significant difference from control (0 mU) with a $P < 0.05$.

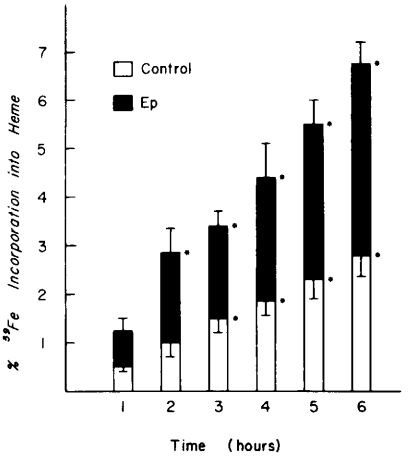

FIG. 2. Time course of erythropoietin (Ep) stimulated heme synthesis in fetal liver cells. Ep was administered to the cultures at a concentration of 100 mU/ml. The ordinate represents percentage ^{59}Fe incorporation into heme at 24 hr of incubation. Each value is the mean ± SEM of three experiments conducted in triplicate. The control values are shown as the open bars whereas the Ep-treated values are the sum of the open and solid bars.

Effects of Ep on cyclic AMP and cyclic GMP levels in fetal liver cells. Cyclic nucleotide levels were monitored in fetal liver cells in control cultures and in cultures containing Ep. Basal levels of cyclic GMP were found to be 0.026 pmole/10^6 cells and those for cyclic AMP were 1.236 pmole/10^6 cells. In cultures incubated with 100 mU of Ep, cyclic AMP levels were not significantly different from controls ($P > 0.05$) (Fig. 3). Addition of MIX (5×10^{-6} *M*) resulted in a modest increase in cyclic AMP levels as would be predicted following exposure of cells to a phosphodiesterase inhibitor. The combination of Ep and MIX produced results on cyclic AMP content similar to that seen when MIX alone was employed.

Ep produced significant elevations in the cyclic GMP content of fetal liver cells beginning at 4 hr after exposure to this hormone (Fig. 4, strippled bars). Increase in cyclic GMP content ranging from 175 to

TABLE I. EFFECT OF Ep, METHYL ISOBUTYL XANTHINE (MIX), AND CYCLIC NUCLEOTIDES ON HEME SYNTHESIS IN FETAL LIVER CULTURES

Agent	Percentage ^{59}Fe incorporation	
	—	Ep
—	100 ± 7	246 ± 29
db-cAMP (1×10^{-6} *M*)	89 ± 3[a]	218 ± 50
8-br-cGMP (1×10^{-6} *M*)	125 ± 17[a]	258 ± 32
MIX (1×10^{-5} *M*)	129 ± 12[b]	294 ± 63
db-cAMP + MIX	86 ± 7[b,c]	238 ± 47
8 br-cGMP + MIX	134 ± 23[c]	304 ± 60
$n = 3$		

[a,b,c] Significant difference from value with corresponding designation ($P < 0.05$). Transferrin-bound ^{59}Fe was added at 20 hr of incubation and was continued for a total of 24 hr.

400% of control was maintained for the duration of the culture becoming maximal at 5–9 hr of incubation. Other experiments (Fig. 4, black bars) were conducted in the presence of MIX (5×10^{-6} *M*) and Ep (100 mU). This combined addition of MIX and Ep potentiated the two- to threefold increase in cyclic GMP content produced by Ep to a value equal to 25–30 times control at 5 hr of incubation.

Studies on relationship of cyclic nucleotide accumulation and DNA synthesis induced by Ep. The time periods (4–6 hr) during which Ep stimulates an elevation of cyclic GMP content in FLC is coincidental with the time during which other investigators have observed that DNA synthesis is stimulated by Ep. Therefore, experiments were conducted to assess a possible relationship between the effects of Ep on

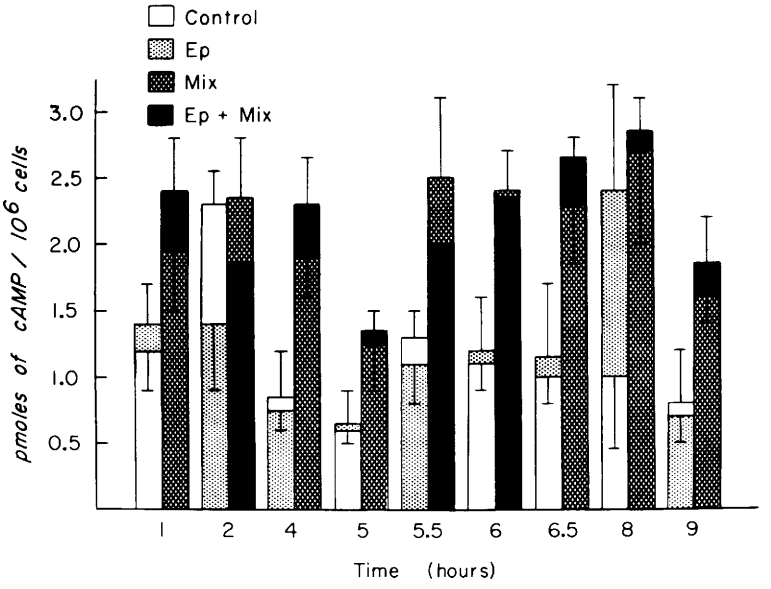

FIG. 3. Effect of erythropoietin (Ep) and/or 1-methyl-3-isobutylxanthine (MIX) on cyclic AMP levels in fetal liver cultures. Ep (100 mU) or MIX (5×10^{-6} *M*) was added at the beginning of the incubation. The ordinate represents the concentrations of cyclic AMP/10^6 cells and the abcissa represents the incubation time when the cells were harvested. Each value represents the mean ± SEM of three individual experiments, each performed in triplicate.

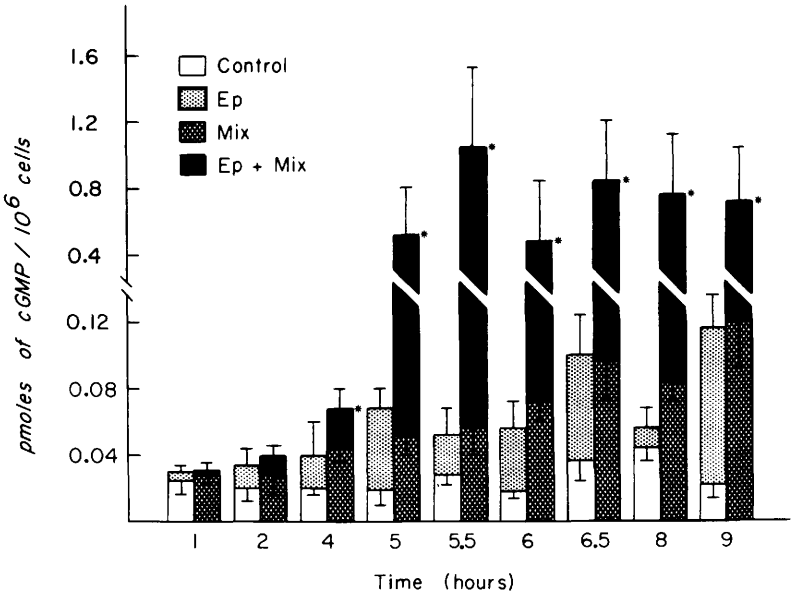

FIG. 4. Effect of erythropoietin (Ep) and/or 1-methyl-3-isobutylxanthine (MIX) on cyclic GMP levels in fetal liver cultures. EP (100 mU) or MIX (5×10^{-6} *M*) was added at the beginning of the incubation. The ordinate represents the concentration of cyclic GMP/10^6 cells. The abcissa represents the incubation time when the cells were harvested. Each value represents the mean ± SEM of three individual experiments each performed in triplicate. (*) indicates significant difference from control ($P < 0.05$).

cyclic GMP concentrations and on DNA synthesis.

Fetal liver cells were incubated for 6 hr with Ep (100 mU), db-cyclic AMP (1×10^{-5} *M*), 8-br-cyclic GMP, or MIX (1×10^{-5} *M*). At selected times, DNA synthesis was monitored (Table II). Ep stimulated DNA synthesis to approximately 185% of control. However, neither db-cyclic AMP, 8-br-cyclic GMP, nor MIX affected the rate of DNA synthesis at this time period. In the same cultures, 5-fluorodeoxyuracil was used to block DNA synthesis at concentrations (10^{-4} *M*) known to be effective for specific inhibition of DNA synthesis (2, 16, 18, 19). In order to assess possible toxic effects of FUdR on FLCs, the cells exposed to FUdR as well as controls were evaluated throughout the course of the incubations with respect to viability and cyclic GMP responsiveness to Ep. Cyclic GMP levels were monitored in cultures in which DNA synthesis was inhibited by FUdR to ascertain if the Ep-stimulated increase in cyclic GMP was a result of the acceleration of DNA synthesis. Table III shows that cyclic GMP concentrations were elevated following treatment of cultures with 100 mU of endotoxin-free Ep and that the combination of Ep and MIX further

TABLE II. EFFECT OF Ep AND CYCLIC NUCLEOTIDES ON DNA SYNTHESIS IN FETAL LIVER CULTURES

	Percentage control
Control	100 ± 4
Ep (100 mU)	185 ± 11*
MIX (1×10^{-5} *M*)	108 ± 5
db-cAMP (1×10^{-5} *M*)	96 ± 4
8-br-cGMP (1×10^{-5} *M*)	106 ± 7
Ep + MIX	176 ± 10*

Note. DNA synthesis is presented as radiolabeled thymidine incorporation into DNA as percentage control. The control value was equal to 8.5% incorporation of total [^{3}H]thymidine. Ep, db-cAMP (dibutyryl cyclic AMP), 8-br-cGMP (8-bromo cyclic GMP), and/or MIX (1-methyl-3-isobutylxanthine), were added to the cultures at the indicated concentrations which were then incubated for 6 hr.

* Indicates significant difference from control with $P < 0.05$.

TABLE III. EFFECT OF FUdR ON Ep STIMULATION OF cGMP CONCENTRATION IN FETAL LIVER CULTURES

	pmole cGMP/10^6 cells
Control	0.037 ± 0.009
Ep (100 mU)	0.096 ± 0.010*
Ep + MIX	0.214 ± 0.016*
MIX (1×10^{-5} M)	0.074 ± 0.012*
FUdR (1×10^{-4} M)	0.035 ± 0.008
Ep + FUdR	0.087 ± 0.009*
FUdR + MIX	0.062 ± 0.008*
Ep + FUdR + MIX	0.234 ± 0.020*

Note. FUdR (5-fluorodeoxyuracil) was added to cultures 30 min prior to addition of Ep (erythropoietin), MIX (1-methyl-3-isobutylxanthine) at the concentrations indicated. Cultures were incubated for 6 hr after the addition of Ep or MIX.

* Indicates significant difference from control ($P < 0.05$).

elevated cyclic GMP levels. FUdR alone had no effect on cyclic GMP concentrations in the fetal liver cells and appears to have no effect on the elevation of cyclic GMP content following treatment with Ep and MIX, alone, or in combination.

Cyclic GMP concentrations were also monitored in fetal liver cultures at 6 hr of incubation with an antibody to Ep (anti-Ep). Anti-Ep, alone, produced no effect on cyclic GMP levels (Table IV). However, anti-Ep did block the increase in cyclic GMP content induced by Ep whereas the enhanced cyclic GMP accumulation stimulated by MIX was not affected. This result was not surprising since the increase in cyclic GMP content produced by MIX is probably due to an inhibition of hydrolysis of this cyclic nucleotide at a site where Ep itself has no effect. Certain cultures were incubated containing various combinations of Ep, anti-Ep, and MIX. In those incubations containing all three additions, the levels of cyclic GMP were 0.068 pmole/10^6 cells, which is approximately the same value as that seen in cultures to which MIX alone was added. Thus, anti-Ep apparently blocks the effect of Ep on cyclic GMP accumulation but does not alter the actions of MIX on the FLC level of this nucleotide.

TABLE IV. EFFECT OF Ep AND ANTI-Ep ON CYCLIC GMP CONCENTRATION IN FETAL LIVER CULTURES

	pmole cGMP/10^6 cells
Control	0.027 ± 0.007
Ep (100 mU)	0.064 ± 0.014*
Ep + MIX	0.235 ± 0.045*
MIX (1×10^{-5} *M*)	0.058 ± 0.009*
Anti-Ep	0.026 ± 0.004
Ep + Anti-Ep	0.035 ± 0.009
Ep + Anti-Ep + MIX	0.068 ± 0.009*
MIX + Anti-Ep	0.064 ± 0.008*

Note. Partially purified endotoxin-free Ep (erythropoietin) and MIX (1-methyl-3-isobutylxanthine) were added to cultures which were then incubated for 6 hr in the absence or presence of the antibody to Ep (anti-Ep). When anti-Ep was included, it was added 30 min prior to the addition of Ep.

* Indicates significant difference from control with $P < 0.05$.

Discussion. Results of these experiments indicate that fetal liver cells in culture are able to incorporate iron into heme; a fact which distinguishes erythroid cells from other types of cells. The time- and dose-dependent increases in ^{59}Fe incorporation into heme of fetal liver cells thus indicate that these cells are erythroid in nature and can be stimulated by Ep. Such a finding is consistent with the published reports of Paul and Hunter (2) and Graber *et al.* (9). The fetal liver cultures employed herein consist of 65–75% erythroid cells as compared with bone marrow cultures which contain less than 35% erythroid cells.

In certain studies, the effect of the partially purified preparation of Ep on levels of cyclic AMP and cyclic GMP was monitored at various times of incubation. Ep produced significant increases in cyclic GMP content in these cultures beginning at 4 hr of incubation with this hormone. This 4-hr time lag is suggestive of Ep producing effects on cyclic GMP indirectly by first affecting other cellular events which must occur prior to the enhancement in the accumulation of cyclic GMP. Ep treatment results in an enhanced rate of synthesis of cyclic GMP as shown by an increased cyclic GMP content. In addition, the incubation of cells with the antibody to Ep prevents this Ep-induced increase in cyclic GMP concentrations. Thus, it is concluded that the hormone EP can stimulate the production or accumulation of cyclic GMP. However, the possibility that the Ep-induced stimulation of cyclic

GMP content is due to some other factor in the preparation cannot at this time be excluded. It has been suggested by Graber *et al.* (15) that small quantities of endotoxin can increase cyclic GMP concentrations in erythroid cells. In consideration of this report, the Ep preparations were tested for endotoxin contamination and found to contain less than 150 pg of endotoxin/ml which is the limit of sensitivity of the assay. However, this does not exclude the possibility of endotoxin being present in other reagents or glassware. Several controls were included in the experiments to minimize such a possibility and thus it appears that the stimulation of cyclic GMP content in erythroid cells is due to the addition of Ep. Nonetheless, because the purest preparation of Ep available for investigational purposes may at best be only 20–25% pure, there may be some other factor in the preparation which exerts effects on cyclic GMP accumulation.

Since Ep is capable of stimulating iron incorporation into heme, and since cyclic GMP content is elevated by Ep administration, it seemed reasonable to postulate that cyclic GMP should also stimulate iron incorporation into heme. This would occur if cyclic GMP were an intracellular mediator of this process. However, this does not appear to be the case. The direct addition of cyclic AMP, cyclic GMP, and/or MIX to cultures does not affect ^{59}Fe incorporation into heme.

The lag in cyclic GMP accumulation following administration of Ep is unlike that seen with other hormonal systems (e.g., epinephrine stimulation of cyclic AMP or acetylcholine stimulation of cyclic GMP) where there is an immediate increase in cyclic nucleotide content upon administration of the hormone. This indicates that the effect of Ep on the cellular content of cyclic GMP does not occur as a result of classical hormonal responses as seen in other systems.

Ep stimulates DNA synthesis in fetal liver cells at the same time that elevated cyclic GMP concentrations are observed. (The stimulation of DNA synthesis by Ep is a necessary prerequisite for heme synthesis (13).) However, the Ep-induced increase in cyclic GMP concentrations appears to be neither a cause nor result of the stimulation of DNA synthesis. The addition of a lipophilic derivative of cyclic GMP, as well as cyclic AMP or MIX, had no effect on DNA synthesis. This indicates that cyclic GMP may not be a traditional intermediate in the stimulation of DNA synthesis. If cyclic GMP were acting as a traditional "second messenger," the addition of such a second messenger would be expected to produce the same effect as the primary hormone, Ep. This does not appear to be the case. Furthermore, the inhibition of DNA synthesis by FUdR has no effect upon the elevation of concentrations of cyclic GMP by Ep. Therefore, it does not seem likely that cyclic GMP is involved in the biochemical events which result in heme synthesis. On the other hand, it is not unlikely that events initiated by Ep require both cyclic GMP elevation and some other cellular event and therefore the possibility exists that a change in the cellular level of cyclic GMP alone may or may not in itself be sufficient to produce changes in heme synthesis or DNA synthesis.

The Ep used in these studies was able to stimulate cyclic GMP content in fetal liver cells, the accumulation of which does not appear to be directly related to heme synthesis. The fact that the antibody to Ep blocks this increase in cellular cyclic GMP content suggests that cyclic GMP may affect Ep-induced cellular events other than those directly responsible for heme synthesis. Ep also stimulates the acceleration of proliferation of erythroid cells and cyclic GMP may be involved in this process. In view of the report by Graber and coworkers (15) that endotoxin contamination of Ep stimulates cyclic GMP accumulation in fetal liver cells as much as 20×, then such contamination may be a significant factor in inducing cyclic GMP concentration changes in these cultures.

However, another possibility is that some of the effects of Ep on cyclic GMP accumulation may be the result of Ep effects which are time-dependent and occur at later times during the incubation. This possibility is supported by the present finding that in our hands cyclic GMP

stimulation is enhanced to approximately 175, 200, 250, and 400% of control at 2, 4, 6, and 9 hr of incubation, respectively. These changes may be related to some cell cycle event.

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Received February 4, 1980, P.S.E.B.M. 1980, Vol. 166.