Erythroid Differentiation Inducer, Hemin Inhibits Cyclic AMP Production in K562 Cells

K562 Hücrelerinde Eritroid Farklılaşmayı Uyaran Hemin, Siklik AMP Oluşumunu Baskılar

■ Bu çalışma, "Hemin İle İndüklenen K562 Hücrelerinde cAMP Konsantrasyonlarının Kolorimetrik İncelenmesi" başlığı ile 6-9 Eylül 2006'da XVIII. Ulusal Biyofizik Kongresi'nde poster olarak sunulmuştur.

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ÖZET

Amaç: Bu çalışmada heminle indüklenen ve indüklenmeyen K562 hücrelerinde, hücre içi siklik AMP düzeyleri incelendi

Yöntemler: K562 hücreleri %10 fetal dana serumu, 100 IU/ml penisilin, 100 μg/ml streptomisin, 25 μg/ml amfoterisin B ve 2 mM L-glutamin içeren RPMI-1640 içerisinde ve nemli % 5'lik karbondioksit etüvünde, 37 °C'da çoğaltıldı. Çoğaltılan bu hücrelerde Tripan mavisi boyama canlılık testi yapıldı. Hemin ile muamele edilen (deney grubu) ve hemin ile muamele edilmeyen (kontrol grubu) hücrelerden birinci günden itibaren altıncı güne kadar hücre peletleri elde edildi. Elde edilen bu hücre peletlerinde siklik AMP konsantrasyonları, siklik AMP Enzim İmmuno Analiz sistemi kullanılarak ölçüldü. Tüm veriler Student's *t*-testi' ni takiben tek-yol ANOVA ile istatistiksel olarak analiz edildi.

Bulgular: K562 hücrelerinin heminle muamelesi, hücre çoğalmasının baskılanmasına neden olmuştur. Heminle muamele edilen K562 hücrelerinin çoğalmasına rağmen siklik AMP düzeyleri zamana bağlı olarak azalmıştır. Heminle indüklenen K562 hücrelerinin hücre içi siklik AMP düzeyleri azalırken, kontrol hücrelerinin siklik AMP düzeyleri kararlı kalmıştır.

Sonuç: Bu sonuçlara dayanarak, diğer eritroid indükleyiciler veya hemin ile muamele edilen K562 hücrelerinde hücre içi siklik AMP düzeylerinin hangi mekanizmayla düzenlendiğini anlamak için daha çok bilgiye gerek vardır.

Anahtar Kelimeler: cAMP, hemin, K562, eritroid farklılaşma, hücresel sinyal iletimi

ABSTRACT

Objective: This study investigated the intracellular cyclic AMP concentrations in hemin-induced and uninduced K562 cell line.

Methods: K562 cell line were grown in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 2 mM L-glutamin at 37 °C in humidified air containing 5% CO_2. A Trypan blue stain viability test was performed for produced K562 cells, and those used to obtain cell pellets from 1st day to 6th day, under the the following conditions: without hemin treatment as a control group and with hemin treatment as an experimental group. All data were statistically analyzed using one-way ANOVA, followed by Student`s *t* test.

Results: Treatment of K562 cells with hemin leads to inhibition of cell proliferation. Cyclic AMP levels of K562 cells that were treated with hemin are decreased in a time dependent manner, although the hemin-induced cells proliferated. Intracellular cyclic AMP levels of hemin-induced K562 cells decreased, while the control cells had stable cyclic AMP concentrations.

Conclusion: On the basis of these results, it is recommended that further data collection is needed to analyze the mechanism by which intracellular cyclic AMP levels are regulated in K562 cells treated with hemin or other erythroid inducers.

Key Words: cAMP, hemin, K562, erythroid differentiation, cellular signaling

INTRODUCTION

Perhaps the earliest research into signal transduction pathways focused upon the regulation of the cellular concentration of second-messenger molecules by cell-surface receptors. In 1958, Sutherland & Rall identified cyclic AMP as a key intermediate in the hepatic glycogenolytic response to adrenaline and glucagon; subsequent biochemical studies quickly identified the enzymatic activities that synthesize (adenylate cyclase (AC)) and degrade (cyclic nucleotide phosphodiesterase (PDE)) cyclic AMP(1).

The second messenger cyclic AMP controls growth and differentiation in a variety of organisms and cell types(2,3). In eukaryotes, cAMP binds to and directly activates protein kinase A (PKA, cAMP-dependent protein kinase) as well as the cAMP binding guanine nucleotide exchange factors Epac1 and –2, which, in turn, stimulate the small GTPases Rap1 and Rap2. Together, PKA and Epacs appear to mediate the majority of effects of cAMP in mammalian cells(4-7). At present cAMP is known to also directly regulate cyclic nucleotide gated ion channels(8). However, the mechanism by which cAMP utilizes these effectors to induce distinct biological responses is unknown(5,9).

One target of cAMP that is associated with cell proliferation is the mitogen-activated protein (MAP) kinase, also called extracellular signal-regulated kinase, or ERK-cascade (10). The activation of ERKs is a key event in many cellular processes, including proliferation, differentiation, and apoptosis (11,12). ERK can be activated or inhibited by cAMP, in a cell-specific manner, to dictate the growth effects of cAMP (10). ERK can stimulate either proliferation or differentiation depending on the stimulus and cell type. Hormonal stimulation of cells can activate $G\alpha_s$ and adenylyl cyclase to stimulate the production of cAMP. cAMP activates the cAMP-dependent protein kinase, PKA. In some cells, PKA activation inhibits growth factor-dependent activation of ERKs. In other cells, PKA activation stimulates ERKs (12).

The precise role of cAMP in regulating cell growth and proliferation remains a matter of considerable debate (13,14). Pituitary adenylate cyclase-activating polypeptide is one of the most potent activators of adenylate cyclase in pituitary cells(15), and its receptor is expressed in the subventricular zone, an area that gives rise to both neurons and glia, around the onset of gliogenesis(16,17). It was recently shown that cAMP-elevating stimuli, including pituitary adenylate cyclase-activating polypeptide, can induce astrocytogenesis of rat cortical precursors (18). The role of cAMP in differentiation of chicken erythroblast cell line HD3 was associated with an increase in cAMP (19). Treatment of K562 cells with hemin leads to erythroid differentiation, as evidenced by inhibition of cell proliferation and induction of hemoglobin synthesis (20,21). The present study investigated the role of intracellular cyclic AMP concentrations in hemin-induced and uninduced human erythroleukemia cell line, K562 cells.

MATERIAL AND METHODS

Reagents

Hemin, RPMI 1640, and penicillin/streptomycin solutions were obtained from Sigma (St Louis, MO, USA) Fetal Calf Serum was from Biochrom. All other chemicals and enzymes were obtained from commercial sources. A direct colorimetric immunoassay kit for detection of cAMP was purchased from Calbiochem (La Jolla, CA).

Cell Culture

K562 cell line obtained from ATCC (MD, USA) were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B and 2 mM L-glutamin at 37 °C in humidified air containing 5% CO_2. Hemin was added at the stated concentrations to a culture media containing $1x10^5$ cells/ml (20,22). This density allowed log-phase growth of cells for the time periods tested. The survival of the K562 cells was determined by trypan blue dye exclusion assay (23).

Measurement of cAMP in K562

Intracellular cAMP concentrations were determined as described previously(24). A number of signals are known to stimulate the production of cAMP through the action of adenylate cyclase converting ATP to cAMP. Intracellular cAMP concentrations were measured using a colorimetric direct immunoassay, in accordance with the manufacturer's instructions. K562 cells were collected by low speed (400 g) centrifugation and washed twice with phosphate-buffered saline (PBS), pH 7.5. The resulting pellets ($2.5x10^6$ cells) were re-suspended and lysed in 0.1 N HCl (250 μl) at room temperature for approximately 10 min. Cell debris was pelleted by centrifugation at 600 g for 10 min and the resulting supernatant was used for assay. In the capture microtitre plate provided, 100 μl lysate and controls were added per well, along with 50 μl conjugate and 50 μl antibody solution, and incubated for 2 hours at room temperature on a plate shaker. The plate was then emptied and washed three times in wash buffer provided. Colour development was detected using 200 μl pNpp substrate solution and incubated for 1 hour at room temperature, and stopped by the addition of 50 μl stop solution. These assays were read and quantified on a Multiskan EX plate reader at 405 nm (Thermo). All results are expressed as the mean concentration of cAMP obtained per condition.

Statistical Analysis

Statistical analyses was conducted using SPSS for windows (version 8.0). All data were analyzed using one-way ANOVA, followed by Student`s *t* test. A value of $P<0.05$ was considered to be statistically significant. Values were expressed as the mean±SD.

RESULTS

In this study, K562 cells were induced with hemin. Cell viability was >95%, as determined by the trypan blue dye exclusion assay. Treatment of K562 cells with hemin leads to inhibition of cell proliferation (Fig.1). Cell proliferation was declined from the 1st to the 6th day after treatment. To determine whether the cAMP-dependent pathway is activated in K562 cells following hemin treatment, intracellular cAMP concentrations were measured in K562 cells treated with hemin for six days, and also in their untreated controls. Following the addition of hemin, intracellular cAMP concentrations were determined using the cAMP enzyme immunoassay system. Table I. shows statistical value of total intracellular cAMP concentrations in hemin-induced and uninduced K562 cells. No statistically significant difference was found between uninduced K562 cells' cAMP concentrations base compared with different day samples of uninduced K562 cells. There was a significant difference in cAMP concentration between 5th and 6th day samples between uninduced (control) and hemin-induced cells, as a result of decreasing intracellular cyclic AMP concentrations in hemin-induced K562 cells. In hemin-induced K562 cells, comparison of 2nd, and 3rd day samples showed no statistical differences. However, comparing cAMP concentrations in hemin-induced cells between the 1st and 5th or 6th day's values indicated statistically significant differences ($p<0.05$, $p<0.02$).

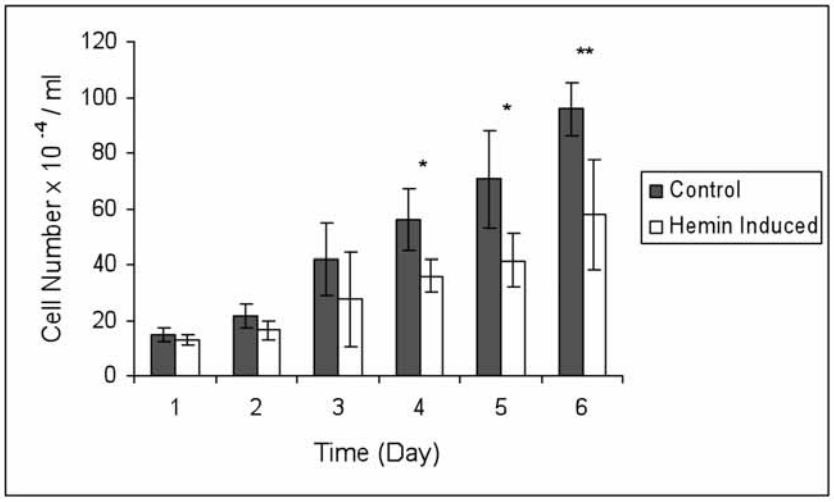

Fig 1. Time course of antiproliferative effects of hemin. Daily cell counts were performed for the 6 days after no treatment (control, uninduced), and hemin treatment (induced). Viable cells were counted at 1 day intervals as indicated. Each point represents the mean of six determinations. The eror bars represent standart deviation. *: p< 0.05, **: p< 0.001

Table 1. Cyclic AMP Concentrations in K562 Cells in Response to Treatment with Hemin
Note. Values represent the means (±SD) of three independent experiments.

Days	uninduced (Control) [cAMP] (pmol/ml)/10^6 cell ± SD n=3	Hemin induced [cAMP] (pmol/ml)/10^6 cell ± SD n=3
1.	70.00±0.00[a]	102.5±38.89[b]
2.	87.50±17.67[c]	105.00±22.91[d]
3.	89.33±41.00[e]	87.50±45.96[f]
4.	91.66±33.29[g]	54.33±24.00[h]
5.	91.66±33.29[j]	32.66±10.78[k]
6.	105.00±22.91[l]	1.66±1.15[m]

d-k p < 0.01 h-m p < 0.05 k-m p < 0.01
d-m p < 0.001 b-m p< 0.02 j-k p < 0.05
f-m p < 0.05 b-k p < 0.05 l-m p < 0.001

DISCUSSION

cAMP is a soluble second messenger that regulates various cellular functions, including cell motility, growth, metabolism, ion channel conductivity, and synaptic plasticity (25,26). The K562 cell line is a commonly-used model system for investigating cellular and molecular events involved in cell proliferation and differentiation (27-29). Erythroleukemia cells differentiate into more mature erythroid cells and accumulate hemoglobin by various low molecular-weight compounds including dimethylsulfoxide, hexamethylene bisacetamide, butyric acid, and hemin (30). In this study, treatment of K562 cells with hemin leads to inhibition of cell proliferation. cAMP levels of K562 cells that were treated with hemin decreased in a time-dependent manner, although the hemin-induced cells proliferated. Intracellular cyclic AMP levels of hemin-induced K562 cells decreased, while the control cells had stable cAMP concentrations.

The adenyl cyclase-cAMP second messenger pathway has been implicated in the regulation of growth and development in several cell types, including Schwann cells, the major glial element in peripheral nerves (31). The effect of the adenyl cyclase-cAMP signalling pathway on proliferation is dependent on the cell type. In certain cells, cAMP inhibits growth and can even suppress transformation, but in others it exerts a proliferative effect (32-34). Receptors have been shown to either increase or decrease cyclic AMP accumulation within the cell, and that the receptor-AC linkage was not direct, but occurs via a family of heterotrimeric guanine nucleotide-binding proteins, termed G proteins (35). The somatostatin analog octreotide appears to directly stimulate CaCo-2, and proliferation of human intestinal epithelial cells by decreasing cAMP. These proliferative effects modulate Caco-2 differentiation but do not affect cell-matrix interactions (36). Inoue et al. showed that intracellular cGMP and cAMP levels increase within a few hours in K562 cells treated with hemin (37,24). In contrast, in the present study, hemin treatment did not increase the cyclic AMP content of K562 cells. Cokic *et al*. reported that high cAMP levels continuously decrease during erythroid differentiation (38). We have previously shown that hemin induced erythroid differentiation and also shown that hemin stimulates hemoglobin production (39). These results suggest that hemin-promoted differentiation can be regulating by cyclic AMP-independent pathway. The role of cAMP on hemin-induced erythroid differentiation and proliferation is as yet unexplained in K562 cell line. Further studies are needed to better understand the effect of cyclic AMP on hemin-induced proliferation and differentiation of K562 cells.

Conclusion: On the basis of these results, it is recommended that further data are collected on the mechanism by which intracellular cAMP levels are regulated in K562 cells treated with hemin or other erythroid inducers.

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