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High glucose enhances cAMP level and extracellular signal-regulated kinase phosphorylation in Chinese hamster ovary cell: Usage of Br-cAMP in foreign protein β-galactosidase expression

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Glucose is a carbon source for Chinese hamster ovary (CHO) cell growth, while low growth rate is considered to enhance the production of recombinant proteins. The present study reveals that glucose concentrations higher than 1 g/L reduce the growth rate and substantially increase in cAMP (~300%) at a high glucose concentration (10 g/L). High glucose also enhances the phosphorylation of extracellular signal-regulated kinase (ERK) and $p27^{kip}$ by Western blot analysis. To determine whether the phosphorylation of ERK is involved in the mechanism, a cyclic-AMP dependent protein kinase A (PKA) inhibitor (H-8) or MEK (MAPKK) inhibitor (PD98059) was added to block ERK phosphorylation. We show that both the high glucose-induced ERK phosphorylation and growth rate return to baseline levels. These results suggest that the cAMP/PKA and MAP signaling pathways are involved in the abovementioned mechanism. Interestingly, the direct addition of 8-bromo-cAMP (Br-cAMP), a membrane-permeable cAMP analog, can mimic the similar effects produced by high glucose. Subsequently Br-cAMP could induce β-galactosidase (β-Gal) recombinant protein expression by 1.6-fold. Furthermore, Br-cAMP can additionally enhance the β-Gal production (from 2.8- to 4.5-fold) when CHO cells were stimulated with glycerol, thymidine, dimethyl sulfoxide, pentanoic acid, or sodium butyrate. Thus, Br-cAMP may be used as an alternative agent in promoting foreign protein expression for CHO cells.

[**Key words:** Chinese hamster ovary cells; High glucose; cAMP; Br-cAMP; Recombinant protein production]

The expression system of Chinese hamster ovary (CHO) cell is one of the leading platforms for recombinant protein production used in therapeutic applications and viral vaccines (1). Various cellular processes including metabolic regulation (2), cell growth control (3,4), low culture temperature (5), and anti-apoptosis (6) in mammalian cells have also been used for manipulating the production. Any increase in protein yield is an energy-intensive process and is most likely accompanied by an increasing burden on the energy capacity of the cell. Therefore many cell engineering strategies have focused on enhancing energy metabolism (7). In a typical cell culture medium, glucose concentrations are usually in some excess to meet cell requirements for growth and energy production.

In general, high-density cell cultures can be obtained by the addition of appropriate amounts of glucose. In endometrial cancer cells (8) and pancreatic cancer cells (9), high glucose leads to cell proliferation, but the opposite effect is seen in endothelial (10) and mesangial cells (11). Thus, the effects of glucose on cell growth vary with the cell type. Previous reports have indicated that high glucose in CHO cells reduces their growth rates, which may be related to the metabolic events involved in the glycolysis pathway and TCA cycles (12,13). In these events, the accumulation of excessive lactate (cytotoxicity) leads to a subsequent inhibition of cell growth (12). However, the possible involvement of a signaling pathway other than those related to metabolic regulation in the growth control of CHO cells is not yet clear.

In cell signal transduction, cyclic AMP (cAMP) acts as second messenger for many biological processes. The role of cAMP involves cell growth, cell morphology, and nutrient uptake in CHO cells (14,15). The growth rates of some mammalian cells are decreased under high glucose conditions (12,16). Given that glucose is one of the most essential carbon sources for maintaining mammalian cells (17), we attempted to elucidate the mechanism involved in the growth rate-lowering effect of high glucose on CHO cells in the present study.

In this report, we demonstrate that under high glucose conditions, cAMP plays a key role in the phosphorylation of extracellular signal-regulated kinase (ERK) and $p27^{kip}$, which subsequently lowers the specific growth rate of CHO cells. Given that the reduced growth rate was accompanied by increased cAMP, we investigated whether cAMP could directly regulate the growth rate and recombinant protein production of the cells. A membrane-permeable cAMP analog, bromo-cAMP (Br-cAMP), was supplemented in the medium and recombinant CHO cells that produce intracellular β-galactosidase (β-Gal) were used. We show that the addition of Br-cAMP not only decreases cell growth, but also significantly promotes β-Gal production. These results provide additional reference for the application of cellular metabolites to enhance a given recombinant expression in CHO cells.

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MATERIALS AND METHODS

Cell lines and CHO cell culture CHO K1 cells (BCRC 60006) used for studying the effects of high glucose on growth rates and phosphorylations, as well as CHO-β-Gal-SF cells (BCRC 60378) used for monitoring the β-Gal production, were obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute in Hsinchu, Taiwan. Both cell types were first grown in a minimum essential medium containing standard 1 g/L glucose supplemented with 1.5 g/L sodium bicarbonate and 10% (v/v) fetal bovine serum (Gibco/Invitrogen, Grand Island, NY, USA). Cells in the log phase were initially used to seed at 1×10^5 cells/mL in a six-well plate (3 mL per well) for the determination of the growth rate of cells, in 10 cm cell culture dishes (10 mL per dish) for the extraction of cell lysate, or in 6 cm-diameter dishes (5 mL per dish) for cell cycle analysis and β-Gal expression. In general, the cells were maintained in a humidified incubator supplied with 5% CO_2 at 37°C for 18 h, and then washed with phosphate-buffered saline (PBS). Subsequently, cells were grown in a fresh medium supplemented with various concentrations of glucose or cAMP analog for another 15 h (about one doubling time) before harvesting for the assays.

Cell number, specific growth rate and MTT assay Cell viability was first determined by trypan blue exclusion procedures. The specific growth rate of CHO cells in the exponential phase of growth was calculated (18) using the equation

$$\mu = \frac{d\ln(n)}{dt} \quad (1)$$

where μ is the specific growth rate (d^{-1}), n is total number of cells, and t is time (in days).

We performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to confirm the number of cells at each end point. We seeded 3000 cells/100 μL per well in 96-well plates and cultured them for 18 h, after which the medium was changed to the tested condition (glucose or chemicals). Each experiment was conducted at least in triplicate. After 15 h, MTT was added to a final concentration of 0.5 mg/mL. The reaction mixture was incubated for 3 h at 37°C and the supernatant aliquot containing the purple crystal pallet was dissolved with 200 μL of dimethylsulfoxide per well. Absorbance was then measured at 570 nm with a plate reader.

Determination of intracellular cAMP levels The assay for cAMP levels was performed using an Amersham cAMP Biotrak Enzymeimmunoassay System (code RPN 225; GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Cells were washed twice with PBS and treated with 2 mL of lysis reagent containing 0.25% dodecyltrimethylammonium bromide, which was slowly added by successive pipetting. After incubating the cells for 5 min, a 100 μL aliquot of cell lysate was added to a well of an enzyme-linked immunosorbent assay plate pre-coated with anti-rabbit IgG mixed with 100 μL of rabbit anti-cAMP. After incubation at 4°C for 2 h, 50 μL of cAMP-peroxidase conjugate in 0.05 M acetate buffer containing 0.02% (w/v) bovine serum albumin (pH 5.8) was added. Incubation at 4°C for another 60 min followed. Finally, each well was washed four times with a washing buffer and developed by adding 150 μL of substrate (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in 20% (v/v) dimethylformamide) for 60 min at room temperature. The reaction was terminated by the addition of 100 μL of 1.0 M sulfuric acid. The optical density was monitored using a plate reader at 450 nm.

Cell lysate extraction and Western blot analysis The harvested cells from the 10 cm culture dishes were washed twice with PBS and homologized in 500 μL of radioimmunoprecipitation (RIPA) buffer. The cell lysates were passed several times through a 27.5-guage needle. Insoluble debris was removed by centrifugation at 12,000 ×g for 30 min. Aliquots of the supernatant were stored at −80°C before use. The protein concentrations were determined by the Bradford method (19) using BSA as a standard. Western blot analysis was conducted according to procedures previously described (20). Cell lysates with equal protein masses (20 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% or 12% polyacrylamide gel) and then electrically transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Piscataway, NJ, USA). Each membrane was blocked with PBS containing 5% dried milk and 0.2% Tween 20, and incubated for 1 h at room temperature. The membranes were then incubated with the respective primary rabbit antibodies against phospho-ERK1/2, ERK1/2 (both from Cell Signaling Technology, Beverly, MA, USA), phospho-$p27^{kip}$, or $p27^{kip}$ (both from Upstate Biotechnology, Lake Placid, NY, USA) at 4°C for 16 h with appropriate dilutions according to the manufacturer's instructions. After three washes with PBS containing 0.02% Tween-20 (PBST), the blots were developed at room temperature for 1 h using secondary antibody goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000 dilution) (Chemicon, Temecula, CA, USA). The membranes were washed twice with PBST and twice with PBS alone. The immune signal was then detected by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The blots were exposed to Kodak X-ray films (Eastman Kodak, Rochester, NY, USA).

Cell cycle analysis Cells were grown in 6 cm culture dishes containing 5 mL of media with standard (1 g/L) or high glucose (10 g/L) as described above. Approximately 1×10^6 cells were harvested and resuspended in 200 μL of PBS. The cells were gently mixed with 3 mL of 70% ice-cold ethanol and incubated at −20°C for at least 1 h. After centrifugation at 180 ×g for 5 min, the cells were washed with PBS twice and resuspended in 1 mL of DNA staining solution containing 200 μg/mL RNase A, 200 μg/mL propidium iodide, and 0.1% Triton X-100 in PBS. Incubation for 30 min at room temperature in the dark followed. Flow cytometry analysis was conducted using a FACSCalibur System (Becton Dickinson, Mountain View, CA, USA). The data acquired from the FACS were analyzed by the CELL QUEST software. Cellular debris and doublets were gated out using a bitmap surrounding the DNA-stained single cells. The relative portion of cell population residing in each cell cycle phase was calculated by the simple linear gating of a DNA content histogram.

Determination of β-Gal expression Recombinant CHO-β-Gal-SF cells were prepared using the same procedures indicated above and then seeded onto a 24-well plate. Each well contains 5×10^4 cells, which were incubated for 18 h in MEM with 10% FBS to reach the exponential phase. Afterward, the cells were washed with PBS and incubated at 37°C for 30 h in MEM with 10% FBS plus a sterilized chemical reagent sodium butyrate (NaBu), pentanoic acid, dimethyl sulfoxide (DMSO), thymidine, glycerol, or Br-cAMP (0.5 μM–250 μM) which were obtained from Sigma (St. Louis, MO, USA). For the mixture experiment, Br-cAMP (250 μM) was added to each reagent with the dosage that induced optimal enhancement.

β-Gal expression in CHO cells was determined using a commercial kit (Cat. 200710, Agilent Technologies, La Jolla, CA, USA). The cells prepared using the same procedure indicated above were washed twice with PBS and treated with 100 μL of lysis buffer provided in the kit. After the insoluble debris was removed by centrifugation at 12,000 ×g for 30 min at 4°C, aliquots of the supernatant were used for β-Gal activity (21) and total protein (19) assays. β-Gal activity was calculated and corrected by the total protein concentrations in each batch.

Statistical evaluations Results are given as means ± SD. Statistical evaluation was done by Student's t test for paired observations. Means were considered significantly different when p value was less than 0.05.

RESULTS

High glucose concentrations reduce the specific growth rate of CHO cells and elevate intracellular cAMP levels In general, the optimized and standardized glucose concentration in a medium for cell growth is 1 g/L (22). We show that glucose concentrations exceeding 1 g/L significantly reduce the specific growth rates of CHO cells in a dose-dependent manner (1–10 g/L) (Fig. 1).

As aforementioned, glucose is a key carbon source in glycolysis and the TCA cycle for ATP generation. We found that high glucose (10 g/L) with a 10-fold excess elevates the intracellular ATP levels by ~20% relative to the standard concentration (1 g/L) (data not shown). Since ATP levels are tightly linked with the outcome of cAMP levels in previous studies (17,23). We also examined the cAMP level upon the addition of high glucose (10 g/L) and found that intracellular cAMP levels were markedly increased by ~300% (Fig. 1). Our results showed that cAMP levels were in fact correlated with the glucose concentrations over the medium in a dose-dependent manner (Fig. 1). Meanwhile, the cAMP levels were inversely correlated with the growth rate induced by glucose.

High glucose elevates ERK phosphorylation Given that changes in cAMP levels in mammalian cells may affect their signaling pathway(s) (24–27), we next addressed whether the

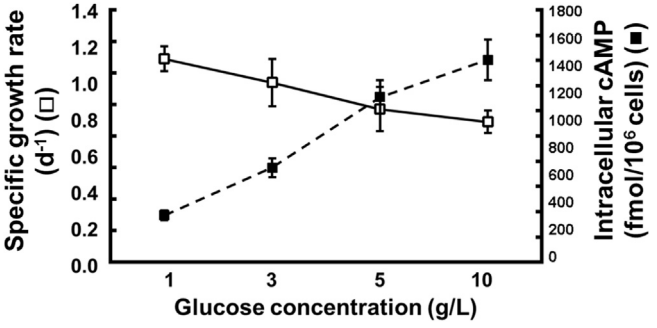

FIG. 1. Effect of glucose on the growth rate and intracellular cAMP levels of CHO cells. Inverse relationship between specific growth rate and cAMP level over glucose concentration. Each value represents the mean ± SD of triplicate determinations.

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