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C-phycocyanin transcriptionally regulates uPA mRNA through cAMP mediated PKA pathway in human fibroblast WI-38 cells

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Abstract

We have previously demonstrated the efficacy of c-phycocyanin in up-regulation of urokinase-type plasminogen activator (uPA) in bovine endothelial cell line. However, the mechanism of action and pathway elucidation in uPA regulation is unclear. In experiments reported here, we have investigated the mechanism of action of c-phycocyanin (c-pc) induced uPA gene modulation in human fibroblast (WI-38) cell line. ELISA test confirmed that c-pc increased the uPA antigen whereas PAI-1 antigen level was unaffected. Treatment of cells with c-pc significantly ($P<0.05$) enhanced the uPA mRNA level in a dose (50 μg/ml) and time dependent (up to 4 h) manner. This effect of c-pc was abolished by treatment with dichloro-1-β-D-ribofuranosyl benzamidazole (DRB) (10 μg/ml). Co-treatment of c-pc with 200 μg/ml cycloheximide (CHX), translation inhibitor, resulted in over accumulation of uPA mRNA. These results suggest that uPA induction by c-pc is transcriptionally regulated and does not require *de novo* protein synthesis. We also provide evidence that c-pc stimulates uPA gene through cAMP dependent pathway as adenylyl cyclase (AC) inhibitor, dideoxyadenosine (DDA) significantly inhibited the uPA mRNA expression and co-treatment with adenylyl cyclase analogue, dBcAMP recovered the effect of c-pc on gene activity. Furthermore, the present investigation provides evidence on the regulatory pathway involved in the c-pc stimulus. C-pc induced uPA expression was completely inhibited by PKA inhibitor (KT 5200), indicating the regulation is dependent on PKA pathway. Elimination of PKC pathway components by prolonged incubation with excess amount of phorbol 12-myristate 13-acetate (PMA) failed to abolish the c-pc effect on uPA expression indicating the regulation is independent of PKC pathway. Taken together, our data indicate that uPA gene regulation by c-pc is transcriptionally controlled through cAMP mediated PKA pathway.

Keywords: *Spirulina fusiformis*; C-phycocyanin; uPA; Fibroblast; PKA pathway

1. Introduction

Natural compounds from blue-algae have attracted a great deal of interest as physiologically functional food supplements and several bioactive metabolites have been successfully developed into clinically useful drugs [1]. More than 60% of the anticancer and 70% of the anti-infective antibiotics currently in clinical use are natural products or natural product-based. C-phycocyanin (c-pc), a fluorescent protein present in the blue-green algae, *Spirulina fusiformis,* is one such molecule which has promising medicinal value. It has recently been reported to exhibit a variety of pharmacological properties like antioxidant, antiinflammatory, neuroprotective and hepatoprotective in both *in vitro* and *in vivo* experimental models [2–4] and may serve as an alternate compound with high efficacy towards vascular fibrinolysis.

Urokinase-type plasminogen activator (uPA) plays a key role in cell migration and tissue invasion by regulating both cell-associated fibrinolysis and proteolysis [5] and other physiological functions in human body. uPA is a serine protease that converts inactive proenzyme, plasminogen to its active form, plasmin. Plasmin generated by uPA promotes matrix degradation and remodeling by activating matrix metalloproteinases. uPA activity is regulated by a specific inhibitor, plasminogen activator inhibitor-1 (PAI-1) [5]. Regulation of uPA expression by different agents in human

Abbreviations: c-pc, c-phycocyanin; uPA, urokinase-type plasminogen activator; CHX, cycloheximide; DRB, dichloro-1-β-D-ribofuranosyl benzamidazole; PMA, phorbol 12-myristate 13-acetate; DDA, dideoxyadenosine; dBcAMP, dibutyryl cyclicAMP

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fibroblasts is well documented since migration of fibroblasts promotes wound healing [6,7].

We have previously isolated, purified, characterized and demonstrated that c-pc induces uPA in bovine endothelial cell line [8]. However, the mechanism of action underlying uPA regulation by c-pc was not investigated. This study examined the role of c-pc in the regulation of uPA gene expression and the mechanism of action in human fibroblast cells.

2. Materials and methods

Spirulina fusiformis dry powder was kindly provided by Parry Agro Industries, Chennai, India. Minimum Eagle's Medium (MEM), dideoxyadenosin (DDA), dibutyryl cAMP (dBcAMP), cycloheximide (CHX) and dichloro-1-β-D-ribofuranosyl benzamidazole (DRB) were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin, streptomycin and amphotericin B were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from JRIA Bioscience (Lenexa, KS, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from PL-Biochemicals Inc. (Milwaukee, USA). KT-5270 was purchased from Calbiochem (Novabiochem, CA, USA). β-actin cDNA probe was purchased from Clontech (Palo Alto, CA, USA).

2.1. Isolation, purification and characterization

C-pc was isolated from the *Spirulina fusiformis* dry powder according to methods of Boussiba and Richmond [9]. The detailed procedures for purification and characterization were published [8]. In brief, c-pc was isolated by Na-phosphate buffer (pH 7.5), purified by hydroxylapatite column chromatography and gel filtration chromatography with superose 12 HR 10/30 column and characterized by SDS-PAGE to check the purity. Gel filtration process yielded the highest purity value of R620/280, i.e., 3.8 equivalents to pharmaceutical and clinical grade. This value was confirmed by the standard purity value of published work [10,11].

2.2. Cell culture

WI-38 fibroblast cells were purchased from Health Science Research Resource Bank (Osaka, Japan). Cells were cultured in MEM with heat inactivated 20% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified environment with 5% CO_2 at 37 °C. Experiments were conducted using sub-confluent cells in serum free medium. C-pc was isolated and purified from the dry powder of *Spirulina fusiformis* as described previously [8]. After incubation of cells with various concentrations of c-pc, the conditioned medium was collected, centrifuged at 5000g and stored at −70 °C until assayed.

2.3. Fibrin zymography

Fibrin zymography was carried out according to the standard method. Conditioned medium of treated cells was subjected to SDS-PAGE electrophoresis (10% polyacrylamide gel, non-reducing conditions) and the gels were washed twice for 1 h each with 500 ml of 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.2% v/v Triton X-100 at room temperature with gentle agitation. Following a brief wash with distilled water, the gels were overlaid on fibrin-agar plates. Each fibrin-agar plate was prepared by pouring 8.5 ml of 1.2% w/v bovine fibrinogen in 0.1 M phosphate buffer (pH 7.4), 8.5 ml of 1.2% w/v agar in water and 1 ml of 20 U/ml thrombin on to a plate (13.5×9.5 cm) at 42 °C. After incubation of the plates at 37 °C, fibrinolytic activities of enzymes were visualized as lytic bands.

2.4. uPA ELISA test

The levels of uPA and PAI-1-1 antigens in the conditioned medium were measured by ELISA using Technoclone uPA kits (Mullnergasse, Vienna, Austria) and biopool PAI-1-1 kits (IMULYSE™, Meditech, Ventura, CA, USA) according to manufacturer's instructions. The results of antigen assays are expressed as mean±SD.

2.5. Northern blot analysis

Total RNA was isolated from treated cells by phenol–chloroform extraction. Total RNA (10 μg), as determined spectrophotometrically, was subjected to electrophoresis, transferred to Hybond N filters (Amersham Pharmacia Biotech Ltd, Little Chalfont, UK) and immobilized by UV exposure in a UV cross linker (UVP-CL-1000, San Gabriel, CA, USA). The filters were hybridized with flourescein-labeled cDNA probes using the Gene Images Random prime Labeling and Detection System (Amersham, South Clearbrook, IL, USA) according to the manufacturer's instructions. uPA primers used were, (forward) 5′-GGCACAAGCTGTGAGATCAC-3′ and (reverse) 5′CTCAGC-TGCTTACAGTTCCT-3′ which were designed based on the well-conserved regions of human uPA. mRNA signals obtained were quantified with a Luminescent Image Analyzer LAS-3000 Plus (Fuji, Tokyo, Japan). uPA mRNA signals were normalised against corresponding β-actin signals. Values were plotted as the ratio of normalized uPA levels to either control or time zero, as the case may be.

2.6. Translation and transcription inhibition assays

Cells were pretreated for 2 h with serum free medium followed by 10 μg/ml of the translational inhibitor, cycloheximide (CHX). The cells were then treated with or without c-pc (50 μg /ml) and incubated further for 6 h. Total RNA was isolated and uPA expression was measured with β-actin as control. DRB, a transcriptional inhibitor, was used for transcription inhibition study. Cells were pretreated with 10 μg /ml of DRB for 2 h in serum free medium, to prevent new RNA synthesis. Then c-pc (50 μg/ml) was added. Zero h represents the time of c-pc addition. Total RNA was isolated at various time intervals (up to 8 h) and uPA mRNA levels were measured against β-actin.

2.7. cAMP regulation study

Cells were pretreated with 200 μM DDA, an adenyl cyclase inhibitor, dissolved in dimethyl sulphoxide (DMSO) for 30 min. Later 50 μg/ml c-pc was added and incubated for 16 h. After incubation, RNA was isolated and processed for Northern blot analysis. In cAMP recovery experiments, 250 μg/ml of dbcAMP was added along with c-pc (50 μg/ml) after treatment with DDA. Total RNA was isolated after 16 h of incubation and uPA signals were detected using Northern blot technique.

2.8. PKA and PKC pathway study

PKC inhibition assay was performed using PMA as per the method of Thalacker and Hamilton [12]. Briefly, cells were treated with (a) 0.1% DMSO or (b) PMA (100 ng/ml) in 0.1% DMSO for period of 48 h as initial pre-incubation period. At the end of pre-incubation period, medium was removed; cells were washed three times with PBS and incubated in fresh serum free medium. After 1 h the cells were treated with 50 μg/ml of c-pc with 10 ng/ml PMA, to reactivate the PKC system and further incubated for 16 h. After reactivation, RNA was isolated and subjected to Northern analysis for detection of uPA signals with β-actin as internal control. PKA pathway study was carried out by PKA inhibition assay with KT-5720. Cells were pre-incubated with KT-5720 for 30 min following which, c-pc (50 μg/ml) was added and incubated for 16 h. Total RNA was isolated and subjected to Northern blot analysis with β-actin as internal control.

2.9. Statistical analysis

Data were collected from three independent experiments. Results were analyzed using one way ANOVA test with $P<0.05$ of significance. Data are presented as mean±SD.