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CALCIUM MODULATORY EFFECTS OF CROCIN FROM SAFFRON (CROCUS SATIVUS L.) IN C6 GLIOMA CELLS

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ABSTRACT

Objective:

To explore the effects of crocin treatment on intra cellular and extracellular calcium levels in C6 glioma cells.

Material and methods:

To elucidate the calcium modulatory effects of crocin in C6 glioma cells. Intracellular and extracellular analysis of calcium levels was carried out by atomic absorption spectroscopy and Fura-2 AM fluorescent assay.

Results:

Crocin treatment strongly modulates both intracellular and extracellular calcium levels.

Disscussion:

Crocin treatment inhibits proliferation in number of malignancies. Calcium is known to modulate a number of processes in gliomagenesis. Crocin modulates both intracellular and extracellular calcium levels thus could target cell signaling cascades involved in gliomagenesis with lower drug induced resistance.

Keywords Crocin, Calcium levels. Drug Therapy, Glioma.

I. INTRODUCTION

Gliomas comprise the largest group of the central nervous system tumors (45-55% in hospital series) [1,2]. Due to the limitations of current treatment modalities, among all the newly developed anti-tumor agents, natural extracts with effective anticancer activities have been researched [3,4]. Saffron plant (Crocus sativus L.) a flowering plant species of the Iridaceae family is best known for its spice product saffron, which is produced from parts of the flowers. Saffron contains many plant derived chemical compounds that are known to have several biological activities including antioxidant, anti-inflammatory, and antiproliferative [5]. Among various saffron extracts crocin has shown various biological activities [6]. Crocin a diester formed from dicarboxylic acid crocetin and a disaccharide gentiobioses in addition to number of biological activities has potent antioxidant properties [7,8].

This study was carried out with the aim to explore calcium modulatory effects of crocin in C6 glioma cells by two different methods. In the present study, we addressed the question whether crocin can modulate calcium levels in C6 glioma cells or not. Since calcium is involved in number of processes in glioma growth, proliferation and development it was imperative to evaluate its effect on calcium levels while studying effect of crocin on various cellular processes involved in proliferation, cell motility, angiogenesis, and invasion.

II. MATERIALS AND METHODS

2.1 Reagents

All the chemicals were of ultra pure grade and obtained from Sigma-Aldrich, or otherwise indicated. 100% HPLC purified crocin was provided by Dr. syed sana et al, centre for research and development, University of Kashmir.

2.2 Cell Culture

The studies were performed on C6 cell line; Rattus Norvegicus glioma-derived cell line. The cells were cultured, subcultured and cryopreserved as per instructions (ATCC/ CCL-107) in DMEM supplemented with penicillin-streptomycin and 10% FBS. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. IC -50 dose of crocin in C6 glioma cells was 15mg/ml as was evaluated through a series of assays (unpulished data).

2.3 Detection of calcium concentrations by atomic absorption spectroscopy

Role of calcium has been implicated in control of different responses in brain during tumorigenesis, one of the important attributes of calcium being glutamate excitotoxicity .Intra and extra cellular levels of calcium were determined by aid of an atomic absorption spectrophotometer (AAS 800 Perkin Elmer, USA) equipped with a model 2200 HGA graphite furnace. In brief logarithmically growing cells were treated as per the experimentation. Culture media was collected from treated dishes, cell monolayers were washed twice with PBS and detached from the culture flasks and pelleted. Washed packed cells and culture media were separately digested and diluted with digestion buffer (12 mM/L HCl and 120 ml/L Triton X-100). After centrifugation clear solutions were aspirated into a flame and wavelength of the light was selected corresponding to an electron transition of calcium. The amount of radiation absorbed was related to the concentration of calcium in the solution using Beer's Law. Intensity was measured at 422.7 nm radiation, which corresponds to a prominent calcium atom electron transition. The 423 nm radiation emitted by the calcium atoms in the cathode lamp is selectively absorbed by the calcium atoms in the solutions fed into the AA flame. Standard solution dilutions of 0.1, 1, 5 and 10 ppm of calciumwere made. Each solution was aspirated into the flame of the AA spectrophotometer and its absorbance measured. From this data a calibration curve of absorbance versus ppm of calcium was obtained. The unknown calcium solutions were aspirated into the flame and absorbance was determined. Then the concentration of calcium in the unknown solutions were determined with pre-installed AAS software (Win Lab Software). At least five determinations were made for each sample.

2.4 Fura-2 AM fluorescent assay

Fura-2 AM is a fluorescent calcium indicator, which exhibits calcium-dependent fluorescence excitation

shift. The ability to make ratiometric measurements helps in understanding the role of calcium in cellular regulation. Fura-2 AM fluorescence can usually be made over a period of an hour without significant loss of fluorescence resulting from either leakage or bleaching. The cytoplasmic level of calcium was examined using fura-2 AM. The cells for these experiments were cultured in six well plates at a density of 2×10^4 cells/ml in complete culture medium under normal conditions before analysis. Cells were treated and next, washed once with PBS and once with fura-2 AM buffer (137 mM NaCl, 2.7 mM KCl, 1 mM Na_2 HPO_3 , 25 mM glucose, 20 mM Hepes). Cells were then incubated at 37°C in the above buffer with 5 µM fura-2 AM for 30 min in dark. After incubation, the cells were washed three times with the same buffer solution but without fura-2 AM at 37°C. To determine the maximum calcium signal, 0.1% triton X-100 was added along with fura buffer. Triton X- 100 liberates calcium into fura-2 AM containing buffer and under these conditions fura-2 AM saturates with calcium. Similarly, to determine the minimum calcium signal, cells were treated with 4.5 mM ethylene glycol tetra-acetic acid [EGTA] to cheleate all available calcium. Fluorescence emission was captured using the Floid Cell Imaging Station (Life Technologies). Fluorescence changes within the cells were converted to reflect the changes in calcium levels. Quantification to facilitate statistical comparisons was based on the means of the fluorescence response in six randomly chosen fields of view in three separate experiments after exposure to the condition.

2.5 Statistical analysis

All the assays were repeated independently three times and values were expressed as Mean ± SD of three independent experiments. All data were analyzed by using One Way ANOVA by Dunnett's multiple comparisons tests using GraphPad Prism 5.

III.RESULTS

2.6 Calcium Depletion in C6 Glioma Cells

Calcium as a signaling molecule regulates cellular processes like proliferation and invasion, so effect of drug treatment on intracellular and extracellular calcium concentrations in C6 cells was evaluated by AAS. The calcium content of C6 glial cell was reduced on treatment with crocin. When intra and extracellular preparations of same group were examined, it was observed that the calcium content of the intracellular samples was lower in comparison with corresponding extracellular samples (Table 1). Results suggest that crocin affects both intracellular and extracellular calcium concentrations hence have significant effect on the invasive and proliferative ability of cells.

Table1: Intracellular and Extracellular calcium levels as detected by AAS.

Calcium Content		
	Intracellular	Extracellular
Control	3.975µM	49.95µM
PBS	3.190µM	48.65µM
Crocin	0.175µM	0.135µM

2.7 Fura-2 AM staining: Corroborative to inhibitory effect on intracellular calcium

An additional approach of labeling with fura-2 AM was taken to ascertain the indicated inhibitory effect of

drug treatment on intracellular calcium levels. Staining with fura-2 AM indicated high calcium induced fluorescence in C6 cells without treatment. Fluorescence gradually decreased in cells treated with crocin (Fig 1).

The observed results were consistent with those obtained in AAS experiments.

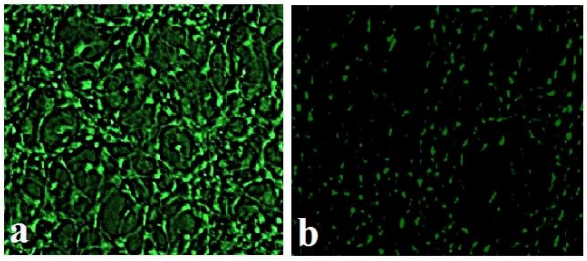

Figure 1: Representative fluorescent photomicrographs showing calcium induced fluorescence after staining with fura-2AM(scale bar :100μM): (a) Control cells showed maximum fluorescence which gradually decreased in (b) cells treated with crocin indicating attenuation of intracellular calcium levels in C6 glioma cell culture

IV.DISCUSSION

Glioma is one of the most common brain tumors accounting for 30 to 40% of all intracranial tumors. They are one of the most aggressive tumors with less than 5% of patients surviving five years post diagnosis; such a clinical behavior of these tumors is attributed to invasive properties of glial cells. Despite best efforts, outcome for gliomas are still very poor even with the best treatments currently available. In light of the poor treatment outcomes there is significant room for researching a new therapeutic drug. Due to the limitations of current treatment modalities, among all the newly developed anti-tumor agents, natural extracts with effective anticancer activities are seen as potential adjuvants to the treatment of glioma. Chemotherapeutic drugs work by targeting multiple mechanisms of tumorigenesis hence dwindling the possibility of drug resistance. Crocin has been used in medicine since ages as anti-oxidative agent with a certain anticancer activity. It is known to have anti-mitogenic, anticarcinogenic activities in various malignancies [8]. In the present study, crocin was tested for its ability to modulate the intracellular and extracellular free calcium levels in C6 glioma cells. Role of calcium has been implicated in control of different responses in brain during tumorigenesis, one of the important attributes being glutamate excitotoxicity [9]. Calcium is a highly versatile intracellular signaling molecule that regulates many different cellular processes, like cellular metabolism, proliferation and invasion [10]. Hence, the concentration of intracellular calcium in the cytosol needs to be permanently constant [11]. We observed that the treatment with crocin strongly decreases the expression of calcium which would influence a multitude of cellular reactions due to diversity of calcium binding proteins via calcium signaling. Being an important player in neuronal transmission and glial physiology, changes in calcium signaling would play a crucial role in the behavior of transformed glioma cells. Studies have shown migration of glioma cells involve glutamate activation of calcium permeable Amino-3- Hydroxy-5- Methyl-4- Isoxazol-Propionic Acid Receptors [AMPARs]. Glioma cells invade and migrate into the adjacent area by a unique process of

cell condensation which utilizes chloride channel-3 (ClC-3)- type channels, these channels are also activated downstream of intracellular calcium activated AMPARs [12]. This highly orchestrated interplay of ion transporters and ion channels allows a cell to shrink its cell volume rapidly, facilitating the pre-mitotic rounding of the cell and maximizes the chance of the cell to advance past a barrier [12]. Beside studies show that calcium entering the cells preferentially activates nNOS via calmodulin, which supports. Thus a decrease in intracellular Cacium would put to halt all the processes promoting proliferation and invasion of glioma cells as well as their highly altered mechanisms of division and migration. The combination of drugs when used, act onto calcium signaling pathways, decreasing the intracellular calcium which would have effect on glioma development.

Calcium ions control different responses in the brain during tumorigenesis and modulate glutamate excitotoxicity which influences a multitude of cellular reactions due to the diversity of calcium binding proteins Crocin treatment decreases the expression of calcium in C6 glioma cells; imperatively it would influence the whole process of gliomagenesis via calcium signaling. Crocin works by targeting multiple mechanisms of tumorigenesis hence dwindling the possibility of drug resistance. Thus crocin can be used at its optimal dose, without unendurable side effects and be an effective strategy for treating resistant gliomas.Further work needs to be carried out to

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