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Down-regulation of immediate early gene *egr-1* expression in rat C6 glioma cells by short-term exposure to high salt culture medium

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Abstract

Influence of high salt culture conditions on the expression of immediate early gene *egr-1* in rat C6 glioma cells was investigated by measuring both Egr-1 mRNA and protein levels in the cells exposed to the medium containing high concentrations of NaCl. The exposure to high salt medium reduced Egr-1 mRNA and protein levels, while Egr-1 mRNA levels were not altered by the medium containing either sucrose or glycerol. Veratridine and monensin also reduced Egr-1 mRNA levels, similar in extent to that induced by high salt medium. Imaging analysis indicated that the exposure to high salt medium induced the elevation of Na^+ levels within the cells. These results indicate that neither hyperosmotic pressure nor ionic strength of high salt medium contribute to the reduction of Egr-1 expression, and suggest that the elevation of intracellular Na^+ concentration is closely associated with the down-regulation of *egr-1* gene expression.

Keywords: High salt culture conditions; Egr-1 expression; Down-regulation; Intracellular Na^+ concentration; Rat C6 glioma cells

1. Introduction

Pharmacological studies on a possible relationship between Na^+ flux and cell proliferation have been carried out by analyzing the effects of various ion flux modulators on DNA synthesis and cell growth in a variety of cell lines, and considerable evidence for a possible relation of Na^+ influx to the rate of cell proliferation has been presented. Potent mitogenic agents, such as platelet-derived growth factor and bradykinin have been shown to stimulate Na^+ influx through an amiloride-sensitive transport system, resulting in the enhancement of DNA synthesis in human fibroblast HSWP cells and vascular smooth muscle cells (Owen, 1984; Owen and Villereal, 1983). In contrast, Na^+ flux inhibitors, such as amiloride and its analogs, have been shown to inhibit DNA synthesis in HSWP cells and neuroblastoma-glioma hybrid NG108-15 cells at the concentrations required for the inhibition of Na^+ influx into these cells (O'Donnell et al., 1983). Moreover, many classic vasoconstrictors have been reported to enhance the proliferation rate of vascular smooth muscle cells, which may be in part due to the stimulation of Na^+ influx through Na^+/H^+ antiporter (Huckle and Earp, 1994; LaPointe and Batlle, 1994). Further studies on the influence of ion channel modulators on the proliferation of human chondrocytes have provided evidence for suggesting a possible connection between ion channel activity and cell proliferation (Wohlrab

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et al., 2002). Thus, it seems conceivable that the stimulation of Na^+ influx, and hence the elevation of intracellular Na^+ concentration, may contribute in part to the enhancement of cell proliferation.

Previous studies have shown that Na^+/H^+ and $Na^+/K^+/Cl^-$ exchange systems may contribute to the elevation of cytoplasmic Na^+ concentration in response to serum growth factors, suggesting a possible implication of these ion cotransport systems in the mitogenic effects of serum growth factors in different types of the cells (Amsler et al., 1985; Canessa et al., 1994; Delvaux et al., 1990; Panet and Atlan, 1991). Based on these findings, it seems interesting to investigate a potential role of Na^+ cotransport systems in the mechanism of mitogenic signaling within the cells, and the effects of ion cotransport inhibitors, such as amiloride and bumetanide, on the expression of genes related to cell growth have been investigated. Consequently, these ion cotransport inhibitors have been shown to block the transition of the cells into the S-phase of the cell cycle, but failed to significantly alter the expression of *c-fos*, *c-myc* and *ornithin decarboxylase* genes in response to serum growth factors, thus suggesting that, although the stimulation of Na^+ cotransport systems may be essential for cell proliferation, Na^+ influx by itself seems to play no apparent role in the transduction of growth signal from the cell surface to the interior of nucleus (Panet et al., 1989).

On the other hand, the infusion of artificial cerebrospinal fluid containing a high concentration of NaCl into rat supraoptic nucleus has previously been shown to increase both c-fos protein and its mRNA levels in glial cells, suggesting that the exposure to high salt solution may affect the expression of immediate early genes in glial cells in this brain region (Ludwig et al., 1997). However, the influence of high salt artificial cerebrospinal fluid on glial cells has not yet been fully characterized, and the critical question of whether the stimulation of *c-fos* gene expression by high salt solution may be due to its osmotic and/or ionic effects still remains to be elucidated. Therefore, the direct influence of high salt concentration in the culture medium on the expression of immediate early genes in glial cells seems interesting, and may be worthy of investigation.

As one of the immediate early genes related to cell growth, *egr-1*, also known as *NGFI-A*, *zif268*, *Krox-24* and *TIS-8*, has been shown to be implicated in the regulation of cell growth by facilitating the transition of the cells from the G1-phase of the cell cycle to the S-phase (Hallahan et al., 1995; Meyyappan et al., 1999), and hence it seems possible that a high salt solution may be able to affect the expression of the *egr-1* gene in glial cells. In the present study, the effect of high salt culture medium on Egr-1 expression in rat C6 glioma cells was examined as one of the in vitro model experiments for investigating the influence of high salt culture conditions on the expression of immediate early genes in glial cells. Short-term exposure to high salt medium was shown to induce the reduction of Egr-1 mRNA levels and its protein contents, and elevation of the intracellular Na^+ concentration was suggested to be responsible for the down-regulation of *egr-1* gene expression in glioma cells.

2. Materials and methods

2.1. Cell culture and treatment

Rat C6 glioma cells (CCL-107; the American Type Culture Collection, Rockville, MD, USA) were seeded onto a 60-mm culture dish at a density of $1.5-2\times10^6$ cells/dish, and maintained in 5 ml of DMEM supplemented with 10% heat-inactivated bovine calf serum, 2 mM L-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 50 μg/ml of gentamycin sulfate at 37 °C for 48 h in a humidified incubator containing 95% air–5% CO_2 atmosphere. Then, the growth medium was replaced with the culture medium containing high concentrations of NaCl or other osmolytes and drugs, and the cells were further incubated at 37 °C for different time periods in a humidified incubator.

Osmotic pressure of the hyperosmotic test medium was determined using a freezing point osmometer. The osmolarity of the media, containing either NaCl (100 mM), sucrose (125 mM), glycerol (200 mM), or none of these, was approximately 437, 408, 476, and 309 mOsmolar, respectively.

Veratridine and monensin (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in dimethylsulfoxide and ethanol, respectively. The solutions were stored in a freezer, and diluted with DMEM before use, and aliquots of these diluted solutions were then added to the cell cultures to yield the designated final concentrations. The concentrations of organic solvents in the cultures were less than 0.1%.

2.2. Semi-quantitative RT-PCR analysis

Total RNA was prepared from a single culture according to the method described previously (Chomczynski and Sacchi, 1987), and the steady-state levels of mRNAs were determined using a semi-quantitatively one-step RT-PCR technique as described previously with modifications (Morita et al., 1999). Total RNA (3 μg) was subjected to reverse transcription and successive amplification using an automated thermal cycler in 25 μl of the first strand buffer (75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.3) containing 500 μM dNTPs, 400 μM dithiothreitol, 5% dimethylsulfoxide, 25 pmol of the primers using 100 units of M-MLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and 2.5 units of recombinant Taq DNA