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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

polymerase (MBI Fermentas, St. Leon-Rot, Germany). The mixture was incubated at 48 °C for 45 min to generate the first strand cDNA, and heated at 95 °C for 2 min to inactivate reverse transcriptase, and the cDNA was then amplified by subjecting the mixture to 35 cycles of DNA polymerase chain reaction (PCR) with each cycle including denaturation at 95 °C for 1 min, primer annealing at 58 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were separated on a 1.8% agarose gel containing 0.5 μg/ml of ethidium bromide in 1× TAE buffer (40 mM Tris–acetate, pH 8.0, and 2 mM EDTA), and signal intensities of the bands were determined by a computerized densitometric analysis using the Scion Image for Windows 4.02 software. The expression of β-actin mRNA was also determined as an internal standard, and the target mRNA expression was then normalized by calculating the ratio of target mRNA levels to the internal standard mRNA.

Specific PCR primers for rat Egr-1 and β-actin genes were designed based on the reported sequences, and commercially synthesized by Sigma Genosys Japan (Ishikari, Hokkaido, Japan). The sequences of these oligonucleotides are 5′-CAGCAGTCCCATTTACT CAG-3′ (forward) and 5′-TGGGACTGGTAGGTGT TATT-3′ (reverse) for Egr-1 (Milbrandt, 1987); 5′-TGG GTC AGAAGGACTCCTAC-3′ (forward) and 5′-CTTCAGAGGTAGTCTGTCAGGT-3′ (reverse) for β-actin (Bova et al., 1998). The expected size of PCR products for Egr-1 and β-actin is 348 bp and 520 bp, respectively.

### 2.3. Immunoblotting analysis

Cells were harvested in 500 μl of lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 2 μg/ml of aprotinin and 1 μg/ml of leupeptin), and the cell lysates were prepared for the immunoblotting analysis of Egr-1 protein. The lysates (20 μg of protein per lane) were analyzed by SDS-PAGE on a 10% polyacrylamide gel, and proteins were electroblotted onto a PVDF membrane. The membrane was incubated with a polyclonal rabbit anti-Egr-1 antibody C-19 (1:2000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the immunocomplex of Egr-1 protein was then visualized using ECL Western-blotting kit and Hyperfilm ECL (Amersham Biosciences Corp., Piscataway, NJ, USA) following the manufacturer's instructions. The signal intensities of corresponding bands were determined by a computerized densitometric analysis using the Scion Image for Windows 4.02 software. The amounts of total protein in the cell lysates were determined by a dye-binding method (Bradford, 1976) using bovine immunoglobulin G as a standard.

### 2.4. Imaging analysis

Cells were preloaded with membrane-permeable $Na^+$-specific fluorescence probe SBFI/AM (Molecular Probe, Inc., Eugene, OR, USA) or $Ca^{2+}$-specific fluorescence indicator Fura-2/AM (Molecular Probe, Inc., Eugene, OR, USA), and the intracellular $Na^+$ and $Ca^{2+}$ concentrations were then determined by measuring fluorescence intensities in a single cell using an image analyzer ARGUS-50 (Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan). Briefly, the cells growing on glass coverslips were incubated with 10 μM SBFI/AM at 37 °C for 40 min or 8 μM Fura-2/AM at 37 °C for 20 min in standard solution (140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.56 mM glucose, pH 7.4) as reported previously (Hiyama et al., 2002; Shirakawa and Miyazaki, 1995), and the fluorescence intensities at 340 nm and 380 nm were determined at 10 s intervals for 10 min after exposing the cells to high salt solutions, which was the standard solution containing different concentrations of additional NaCl. The ratio of fluorescence intensities ($F_{340}/F_{380}$) was calculated.

### 2.5. Data analysis

Results were presented as the mean ± SEM, and analyzed using an analysis of variance (ANOVA) followed by Tukey's post hoc test. A $p$ value of $<0.05$ was accepted as a statistically significant difference.

## 3. Results

### 3.1. Influence of high salt culture medium on egr-1 gene expression

Rat C6 glioma cells were exposed to the medium containing high concentrations of NaCl for relatively short periods, and Egr-1 mRNA levels in these glioma cells were then determined to investigate the influence of high salt culture conditions on *egr-1* gene expression. As shown in Fig. 1, the exposure to high salt medium reduced Egr-1 mRNA levels according to the time of exposure, and the significant reduction of Egr-1 mRNA was observed at 4 h after the exposure to high salt medium. On the other hand, the effect of high salt medium on Egr-1 protein contents in these glioma cells was examined, and the protein contents were also reduced by the exposure to high salt medium in parallel with the reduction of Egr-1 mRNA levels. In addition, the inhibitory action of high salt medium on Egr-1 mRNA expression was observed in a manner dependent on the concentration of additional NaCl in the medium (Fig. 2). These results seem to indicate that the exposure to high salt medium could induce the down-regulation

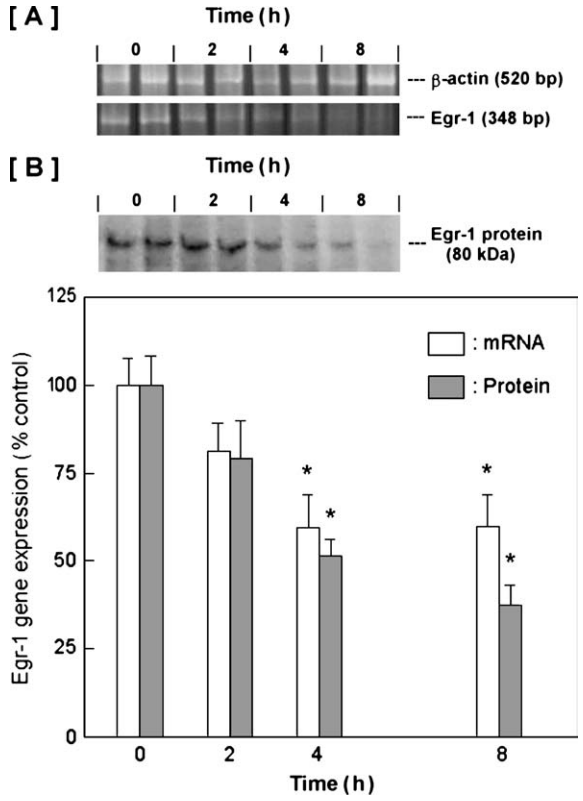


Fig. 1. Influence of high salt culture conditions on Egr-1 mRNA levels and its protein contents in rat C6 glioma cells. Cells were exposed to the medium containing NaCl (100 mM) for different time periods, and Egr-1 mRNA levels (A) and its protein contents (B) were then determined as described in the text. Results were expressed as the percent of control. Values are the mean ± SEM. $*p < 0.05$ ($n = 6$).

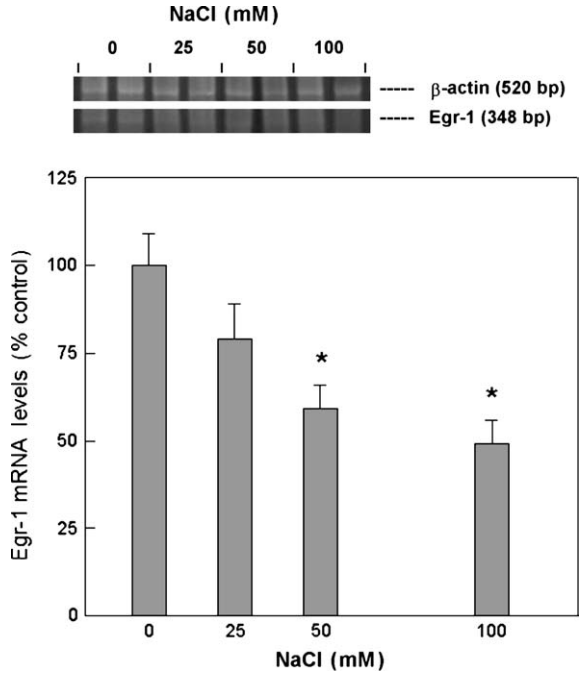


Fig. 2. Influence of NaCl concentrations in high salt culture medium on Egr-1 mRNA levels in rat C6 glioma cells. Cells were exposed to the medium containing different concentrations of NaCl for 4 h, and Egr-1 mRNA levels were then determined as described in the text. Results were expressed as the percent of control. Values are the mean ± SEM. $*p < 0.05$ ($n = 6$).

of *egr-1* gene expression in rat C6 glioma cells. However, since high salt medium used in these experiments was obviously hyperosmotic, it was still unidentified which of the salt concentration or the osmotic pressure of high salt medium might be responsible for the down-regulation of *egr-1* gene expression observed here.

### 3.2. Influence of hyperosmotic culture medium on egr-1 gene expression

To determine whether the hyperosmotic pressure of high salt medium might contribute to its inhibitory action on Egr-1 mRNA expression, the effect of culture medium containing 125 mM sucrose or 200 mM glycerol, which was osmotically almost equivalent to the medium containing 100 mM NaCl, on Egr-1 mRNA levels was examined. As shown in Fig. 3, neither the medium containing sucrose nor that containing glycerol induced any significant change in Egr-1 mRNA levels in the cells under the conditions in which the reduction of Egr-1 mRNA levels induced by high salt medium was consistently observed. Furthermore, the effects of a voltage-dependent $Na^+$ channel opener veratridine (50 μM) and a $Na^+$ ionophore monensin (10 μM) on Egr-1 mRNA expression were examined, and these drugs induced the reduction of Egr-1 mRNA levels, almost similar in extent to that induced by high salt medium (Fig. 4). These results seemed to indicate that the reduction of Egr-1 mRNA expression induced by high salt medium might not reflect the influence of hyperosmotic stress on the cells, and suggest that high salt medium could induce the reduction of Egr-1 mRNA levels probably through the enhancement of $Na^+$ influx, and hence a rise in intracellular $Na^+$ in glioma cells.

### 3.3. Influence of high salt culture medium on intracellular $Na^+$ and $Ca^{2+}$ concentrations

To further confirm that the elevation of intracellular $Na^+$ concentration actually occurred in the glioma cells exposed to high salt culture medium, the concentration of $Na^+$ within the cells was monitored under the high salt conditions using a fluorescence imaging technique. As shown in Fig. 5, the exposure to high salt solution elevated the intracellular concentration of $Na^+$ in a manner dependent on the concentration of additional NaCl, corresponding to the concentration required for the reduction of Egr-1 mRNA levels (Fig. 2), while the exposure to hyperosmotic solution containing 125 mM sucrose failed to induce any significant change in the

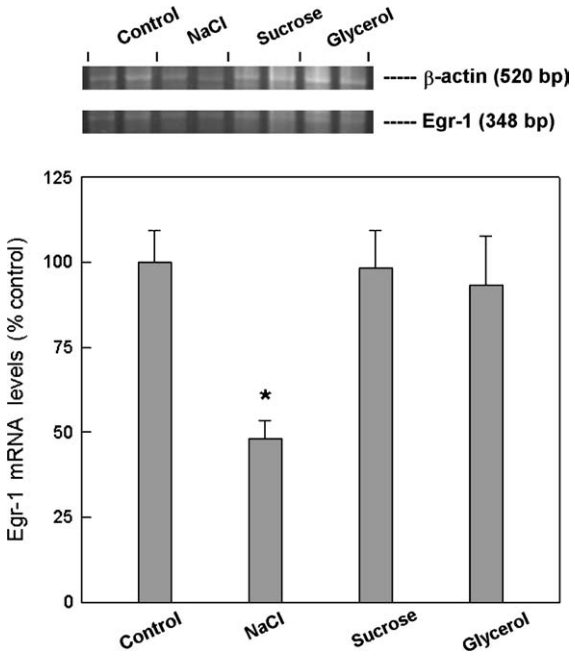


Fig. 3. Influence of osmolytes in culture medium on Egr-1 mRNA levels in rat C6 glioma cells. Cells were exposed to the medium containing NaCl (100 mM), sucrose (125 mM), or glycerol (200 mM) for 4 h, and Egr-1 mRNA levels were then determined as described in the text. Results were expressed as the percent of control. Values are the mean±SEM. $*p<0.05$ ($n = 6$).

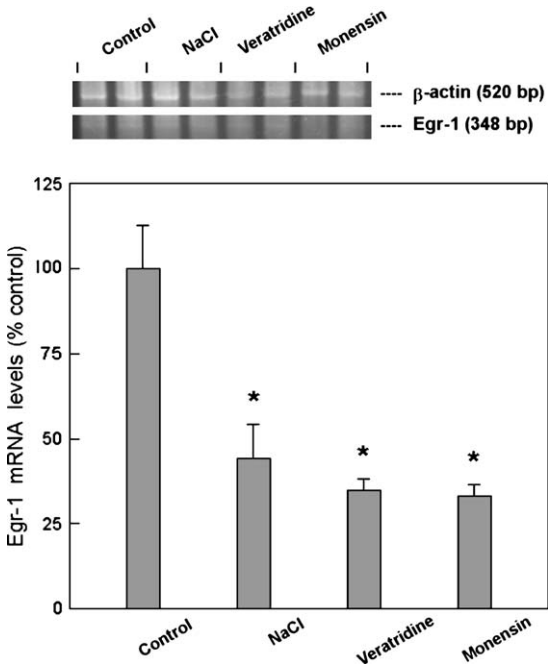


Fig. 4. Influence of $Na^+$ influx on Egr-1 mRNA levels in rat C6 glioma cells. Cells were exposed to the medium containing NaCl (100 mM), veratridine (50 μM) or monensin (10 μM) for 4 h, and Egr-1 mRNA levels were then determined as described in the text. Results were expressed as the percent of control. Values are the mean±SEM. $*p<0.05$ ($n = 6$).

concentration of $Na^+$ within the cells (data not shown). On the other hand, since the elevation of intracellular $Na^+$ concentration is well known to induce the mobilization of $Ca^{2+}$ within the cells, it seemed quite possible that the exposure to high salt medium might increase the intracellular $Ca^{2+}$ levels through the elevation of $Na^+$ concentration within the cells, thus resulting in the suppression of *egr-1* gene expression in the glioma cells. Then, the influence of high salt conditions on the intracellular $Ca^{2+}$ levels was examined, and the exposure to high salt solution failed to induce any notable change in the concentration of $Ca^{2+}$ within the cells under the conditions in which the elevation of intracellular $Na^+$ concentration was observed (Fig. 6). These results indicated that the exposure to high salt culture medium induced the suppression of *egr-1* gene expression through the elevation of intracellular $Na^+$ concentration, thus suggesting a possible connection between the extracellular $Na^+$ concentration and the expression of immediate early genes in glial cells.

## 4. Discussion

Effects of $Na^+$ flux modulators on cell proliferation has been extensively investigated in a variety of cell lines, most of which are derived from peripheral tissues, and considerable evidence for a possible implication of $Na^+$ influx in the regulation of DNA synthesis and cell growth has been presented. However, little is known about the influence of $Na^+$ flux on the cells derived from the central nervous system, such as neuronal and glial cells. Previous studies have shown that artificial cerebrospinal fluid containing high concentration of NaCl can increase both c-fos mRNA and its protein levels in glial cells in rat supraoptic nucleus (Ludwig et al., 1997), but the influence of high concentration of $Na^+$ itself in this artificial cerebrospinal fluid on glial cells still remains to be further investigated.

In the present studies, the influence of high salt culture conditions on the expression of *egr-1* gene, one of the immediate early genes implicated in the proliferation and differentiation of various cells, was examined by measuring Egr-1 mRNA levels and its protein contents in rat C6 glioma cells, and both Egr-1 mRNA and protein levels were shown to be gradually decreasing according to the time of culture (Fig. 1) in a manner dependent on the concentration of NaCl in the culture medium (Fig. 2). In previous studies, the medium containing high concentrations of NaCl has been reported to induce the activation of *egr-1* gene expression through its action on the cells as hyperosmotic stress (Cohen et al., 1991; Wollnik et al., 1993).

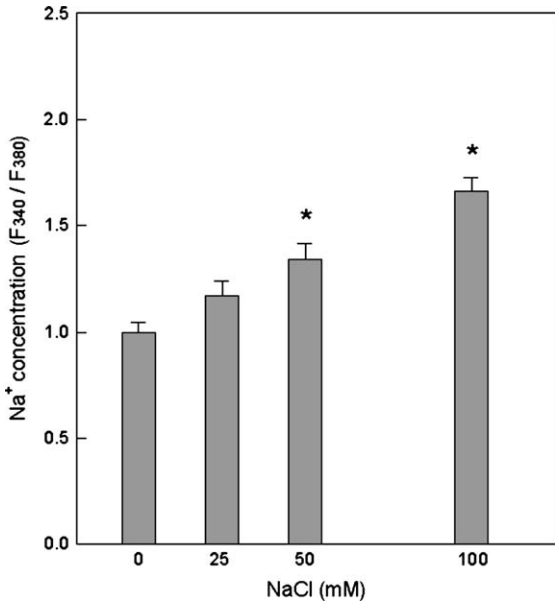


Fig. 5. Influence of high salt culture conditions on intracellular concentration of $Na^+$ in rat C6 glioma cells. Cells were exposed to the solution containing different concentrations of NaCl for 10 min, and the fluorescence intensities at 340 nm ($F_{340}$) and 380 nm ($F_{380}$) were determined as described in the text. Results were expressed as the ratio of fluorescence intensities ($F_{340}/F_{380}$). Values are the mean±SEM. $^*p<0.05$ ($n = 14$).

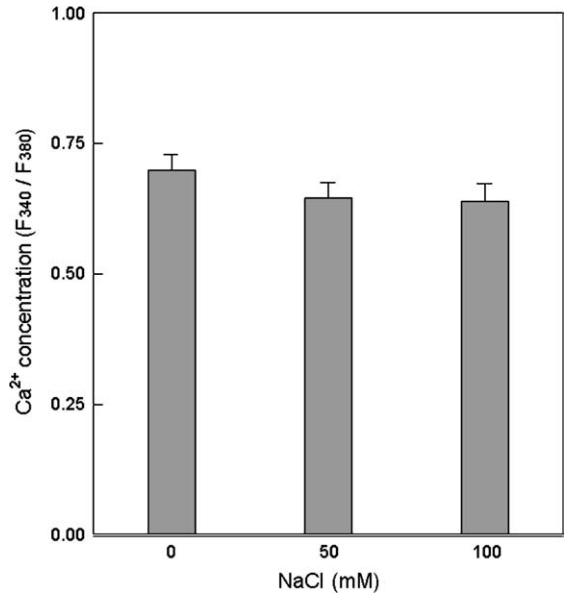


Fig. 6. Influence of high salt culture conditions on intracellular concentration of $Ca^{2+}$ in rat C6 glioma cells. Cells were exposed to the solution containing different concentrations of NaCl for 10 min, and the fluorescence intensities at 340 nm ($F_{340}$) and 380 nm ($F_{380}$) were determined as described in the text. Results were expressed as the ratio of fluorescence intensities ($F_{340}/F_{380}$). Values are the mean±SEM. $^*p<0.05$ ($n = 19$).

On the contrary, the results presented here clearly indicate that high salt culture medium can exert an inhibitory rather than a stimulatory action on *egr-1* gene expression in rat C6 glioma cells, and hence it seems possible that the down-regulation of *egr-1* gene expression observed here may be attributed to any other factor(s) than hyperosmotic pressure.

The influence of hyperosmotic culture medium containing either sucrose or glycerol, which was osmotically almost equivalent to high salt medium, on *egr-1* gene expression was also examined, and neither of them was shown to induce any notable change in Egr-1 mRNA levels in the cells under the conditions in which the inhibitory action of high salt medium was observed (Fig. 3). Therefore, it seems conceivable that the reduction of Egr-1 mRNA levels induced by high salt culture conditions may be due to the high concentration of NaCl itself in the medium rather than its hyperosmotic pressure. Furthermore, both a voltage-gated $Na^+$ channel opener, veratridine, and a $Na^+$ ionophore, monensin, were shown to induce the reduction of Egr-1 mRNA levels, similar in extent to that induced by high salt medium (Fig. 4). Since these compounds are generally known to induce $Na^+$ influx into the cells through different pathways, which need to be further characterized in these glioma cells, it seems possible that the enhancement of $Na^+$ influx into the cells, and hence a rise in the intracellular $Na^+$ concentration, may contribute to the down-regulation of *egr-1* gene expression observed in the glioma cells under high salt culture conditions.

Furthermore, the influence of high salt conditions on the intracellular $Na^+$ and $Ca^{2+}$ levels in the glioma cells was examined by measuring the concentrations of $Na^+$ and $Ca^{2+}$ within the cells using fluorescence imaging analysis, and the exposure to high salt solution was clearly shown to induce a significant elevation of $Na^+$ concentration within the cells (Fig. 5). Thus, it seems possible that the intracellular concentration of $Na^+$ itself may be related to the reduction of *egr-1* gene expression observed under the high salt conditions. On the other hand, the elevation of $Na^+$ concentration in the cell cytoplasm is generally known to induce a rise in the intracellular $Ca^{2+}$ levels as a consequence of mobilizing $Ca^{2+}$ from intracellular store sites and/or facilitating $Ca^{2+}$ flux through the ion exchange system, and intracellular $Ca^{2+}$ is also well characterized as an important factor mediating a variety of cellular metabolism and function.

Therefore, it seems possible that the effect of high salt medium on *egr-1* gene expression may be mediated by a rise in the intracellular $Ca^{2+}$ levels resulting from the elevation of $Na^+$ concentration within the cells. However, because the exposure to high salt solution failed to show any notable effect on the intracellular $Ca^{2+}$ levels, it seems unlikely that the intracellular concentration of $Ca^{2+}$ may be connected with the reduction of *egr-1* gene expression observed under the high

salt culture conditions. Recently, inhibition of the $Na^+$-$K^+$ pump has been shown to induce the expression of immediate early gene *c-fos* in rat vascular smooth muscle cells probably through an intracellular $Na^+$-mediated $Ca^{2+}$-independent mechanism (Taurin et al., 2002). Thus, it is reasonable to consider that the concentration of $Na^+$ itself within the cells may be critical in the regulation of immediate early gene expression.

The present studies indicate that the exposure of rat C6 glioma cells to high salt culture medium results in down-regulation of *egr-1* gene expression under the experimental conditions used in this work, and suggest that the intracellular $Na^+$ concentration may be involved in the cellular mechanism of the down-regulation of *egr-1* gene expression observed under the high salt culture conditions. However, it seems still necessary to address a critical question for understanding the influence of high salt culture conditions on *egr-1* gene expression. In previous studies, the high salt culture conditions have been shown to induce the activation of *egr-1* gene expression in rat cardiomyocytes and canine kidney cells through its hyperosmotic action (Cohen et al., 1991; Wollnik et al., 1993).

On the contrary, the findings presented here suggest that high salt medium may induce suppression rather than stimulation of *egr-1* gene expression through a mechanism unrelated to its hyperosmotic action on the cells. Although it seems quite difficult to give a reasonable explanation for the difference in these responses of *egr-1* gene expression to high salt culture conditions, the difference in the basal activity of *egr-1* gene expression in different cell types is presumed to be one of the possible factors inducing their different responsiveness to given stimuli. In fact, a protein phosphatase inhibitor, okadaic acid, has previously been shown to induce the substantial expression of *egr-1* gene in human and mouse fibroblasts (Cao et al., 1992).

In contrast, the effect of okadaic acid on *egr-1* gene expression in human oral squamous carcinoma cell lines and osteoblastic cell lines has recently been examined, and this inhibitor has been reported to show its suppressive action on *egr-1* gene expression only in the cells highly expressing *egr-1* gene, and induce the transient elevation of *egr-1* gene expression in the cells in which the basal expression is relatively lower (Okamura et al., 2002). Therefore, it seems necessary to examine whether the influence of high salt culture conditions observed here is specific for the type and origin of the cells. In addition, since the immediate early gene *egr-1* is known to encode a zinc-finger protein EGR-1, which is one of the transcription factors implicated in the proliferation of various cells, it also seems interesting to investigate the influence of high salt culture conditions on the proliferation and differentiation of glial cells. Further studies on the proliferation of rat C6 glioma cells under the high salt culture conditions are in progress to define the biological significance of this phenomenon.

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