

Ouabain-Induced Signaling and Cell Survival in SK-N-SH Neuroblastoma Cells Differentiated by Retinoic Acid

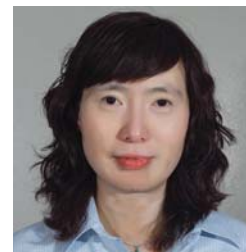
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Abstract: Ouabain stimulates activation of various signaling cascades such as protein kinase B (Akt) and Extracellular-signaling-regulated kinase 1/2 (ERK 1/2) in various cell lines. Retinoic acid (RA) is commonly used to induce neuroblastoma differentiation in cultures. Upon RA administration, human neuroblastoma cell line, SK-N-SH demonstrated neurite extensions, which is an indicator of neuronal cell differentiation. Here we report that ouabain-induced signaling is altered under the action of 1 μ M RA in human neuroblastoma SK-N-SH cells. RA increased the expression of p110 α subunit of phosphoinositide 3-kinase (PI3K), Akt and β 1 subunit of Na⁺/K⁺-ATPase. Ouabain activated Akt and ERK 1/2 in differentiated SK-N-SH cells; this effect was not observed in non-differentiated SK-N-SH cells. Long-term incubation of non-differentiated SK-N-SH with 1 μ M ouabain led to a decrease in the number of cells; this effect was reduced in differentiated SK-N-SH cells. Taken together, these results suggest that ouabain leads to cell death in neuroblastoma cells rather than neuronal cells due to the different response to ouabain manifested by activation of Akt and ERK 1/2.

Highlights

- RA increases the expression of p110 α subunit of PI3K, Akt and β 1 subunit of Na⁺/K⁺-ATPase
- Ouabain induces activation of Akt and ERK 1/2 in differentiated SK-N-SH cells but not in non-differentiated cells
- 1 μ M ouabain leads to a decrease in the number of cells in non-differentiated SK-N-SH
- Reduction of ouabain-induced cell death in differentiated SK-N-SH

Keywords: Na⁺/K⁺-ATPase, neuroblastoma, ouabain, retinoic acid, phosphoinositide 3-kinase, signaling cascades.

INTRODUCTION

Na⁺/K⁺-ATPase is the enzyme that transports two K⁺ ions into the cell and three Na⁺ ions out of the cell against their concentration gradients using the energy of one ATP molecule [1]. Specific inhibitors of Na⁺/K⁺-ATPase belong to the group of cardiotonic steroids (CTS), including marinobufagenin, telocinobufagin, digoxin and ouabain [2]. Besides its ion pumping function, Na⁺/K⁺-ATPase can function as a receptor to trigger intracellular signaling processes when bound to CTS [3, 4]. Ouabain and other CTS have been found in mammals [5-7] and can be endogenous regulators of cellular processes in areas such as the brain [8-10]. It has been shown that ouabain binding to Na⁺/K⁺-ATPase induces Src activation [11]. This in turn leads to Extracellular-signaling-regulated kinase 1/2 (ERK 1/2) activation through the epidermal growth factor receptor (EGFR)-Ras-Raf-MAPK/ERK kinase (MEK) pathway [4,

12]. Ouabain also activates phosphoinositide 3-kinase (PI3K) that triggers downstream of protein kinase B (Akt) activation [13-14]. Increased Akt activity enhances protein synthesis and prevents apoptosis [15]. In the other hand, there is extensive literature on ouabain-induced cell death by enhanced calcium oscillations, increase in intracellular reactive oxygen species (ROS), and Nuclear factor κ B (NF- κ B) activation [16-20]. In neuronal cells, Na⁺/K⁺-ATPase affects the functions of various proteins through direct protein-protein interaction [21-23]. Difference in the Na⁺/K⁺-ATPase isoforms expressed in neurons determines varying kinetic properties [24], different roles in restoring the gradients of Na⁺ and K⁺ ions [25], and cell signaling processes [26].

Neuroblastoma is an extracranial solid pediatric tumor arising from the developing neural crest along its migratory pathways [27] and contains several cell phenotypes, including neuroblastic (N), non-neuronal substrate-adherent (S), and intermediate (I) cell types [28]. Human neuroblastoma SK-N-SH cells are derived from a human bone marrow metastasis and can be used as a cell model system for studying cell signaling processes. The action of

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all-trans Retinoic acid (RA) arrests the cell cycle and causes differentiation along the neuronal lineage in N-type cells [29, 30]. In human neuroblastoma SH-SY5Y cell line, which is a thrice-cloned sub-line of SK-N-SH [31], ouabain-sensitive activity of Na⁺/K⁺-ATPase is regulated by cAMP [32]. Treatment of SH-SY5Y with ouabain (>1 μM) causes a 50% increase in ROS after 30 min. Four days incubation of SH-SY5Y with ouabain at different concentration decreases cell number (20% decrease for 10 nM ouabain, and 75% decrease for 1 μM ouabain) [33]. It has also been shown that in SH-SY5Y cells, ouabain stimulates ERK 1/2 activation through the α3, but not the α1 subunit of Na⁺/K⁺-ATPase [34].

CTS have been shown to act as proapoptotic [35] as well as antiapoptotic [36] agents depending on the cell type. Studies on various CTS-triggered cell responses can be useful in cancer therapy. Currently, CTS are being tested as potential agents to trigger apoptosis in tumor cells [35, 37]. The aim of this study was to assess ouabain-induced signaling in SK-N-SH neuroblastoma cells during the differentiation mediated by RA.

MATERIALS AND METHODS

Materials

Chemicals of the highest purity and culture media were purchased from Sigma and Invitrogen. Akt and phosphorylated (Ser473) Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ERK 1/2, phosphorylated ERK 1/2, α2 subunit of Na⁺/K⁺-ATPase, PI3K p110α, PI3K p110γ, PI3K p85α, Src, goat anti-mouse IgG-horseradish peroxidase, and goat anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against Na⁺/K⁺-ATPase α1 subunit (α6F) was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Na⁺/K⁺-ATPase α3 subunit antibodies (MA3-915) were purchased from Pierce Biotechnology (Rockford, IL).

Cell Cultures

Human neuroblastoma SK-N-SH cells from ATCC were grown in Eagle's Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine (Sigma), 10% Fetal Bovine Serum (Invitrogen), 1 mM sodium pyruvate, penicillin (100 units/ml) and streptomycin (100 mg/ml), and were maintained at 37°C in a saturated humid atmosphere with 5% CO₂. As they approached confluence, the cells were sub-cultured following treatment with 0.25% Trypsin-EDTA.

Differentiation by RA

All-trans RA was dissolved in DMSO and 10 mM stock solution was kept at -20°C. For each experiment, RA was diluted to the desired concentration directly in the culture medium. Cells cultured in medium containing 0.01% DMSO were used as control. Final concentration of 1 μM RA was used for differentiation. RA treatment started on the second day after plating. Cells were given fresh medium every 48 h.

Cells were incubated with and without RA for 10 days except as indicated elsewhere.

Western Blotting Analysis

Protein samples were subjected to 8 or 10% SDS-PAGE, transferred to a Polyvinylidene fluoride (PVDF) membrane, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were developed and detected using enhanced chemiluminescence. For quantitative comparisons, images were scanned with a densitometer. Different dilutions of samples were subjected to SDS-PAGE, and multiple exposures of the films were used to ensure the quantitation within the linear range of the assays.

Cell Proliferation Assay

Cell numbers were measured using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Inc.) as described [38]. Briefly, cells were incubated with or without ouabain for 3 days with same composition of culture media after 7 days of RA or vehicle induction. Before the measurement, the cells were rinsed with PBS twice to remove the floating cells and then detached and separated by 0.25% trypsin-EDTA. After stopping the enzyme action by addition of fetal bovine serum (FBS), the cells were resuspended in PBS. The cell size distribution was analyzed before the assay and the same range of diameter was set for counting in both differentiated and non-differentiated cells.

Statistical Analysis

Values are reported as the mean ± SE of the results of a minimum of three experiments. Student's t-test was used to compare two groups. The significance was accepted at p<0.05.

RESULTS

RA Induced Differential Changes in Neuroblastoma Cells

The results were consistent as reported in the literature [29-30, 39] that human neuroblastoma SK-N-SH cells are divided into several types (neuroblastic N-type cells, substrate-adherent S-type cells and intermediate I-type cells). Upon RA administration, neuroblastoma cells became stretching, exhibited neurite extensions and acquire a neuronal phenotype (Fig. 1A). The number of cells dramatically decreased to less than 50% under the action of 1 μM RA (Fig 1B).

Proteins of our interest were compared when cells were incubated for 12 days with or without 1 μM RA (Fig. 1C). The α subunit of Na⁺/K⁺-ATPase in non-differentiated and differentiated cells was represented by two isoforms: the ubiquitous α1 isoform and the nervous tissue specific α3 isoform. The amount of α1 and α3 subunits was not affected by RA-induced differentiation. The α2 subunit, a specific isoform for skeletal muscle, heart, and glial cells, was detected in minor amount, which is consistent with previous finding in SK-SY5Y cells [34]. A significant increase in the amount of β1 subunit of Na⁺/K⁺-ATPase was detected in

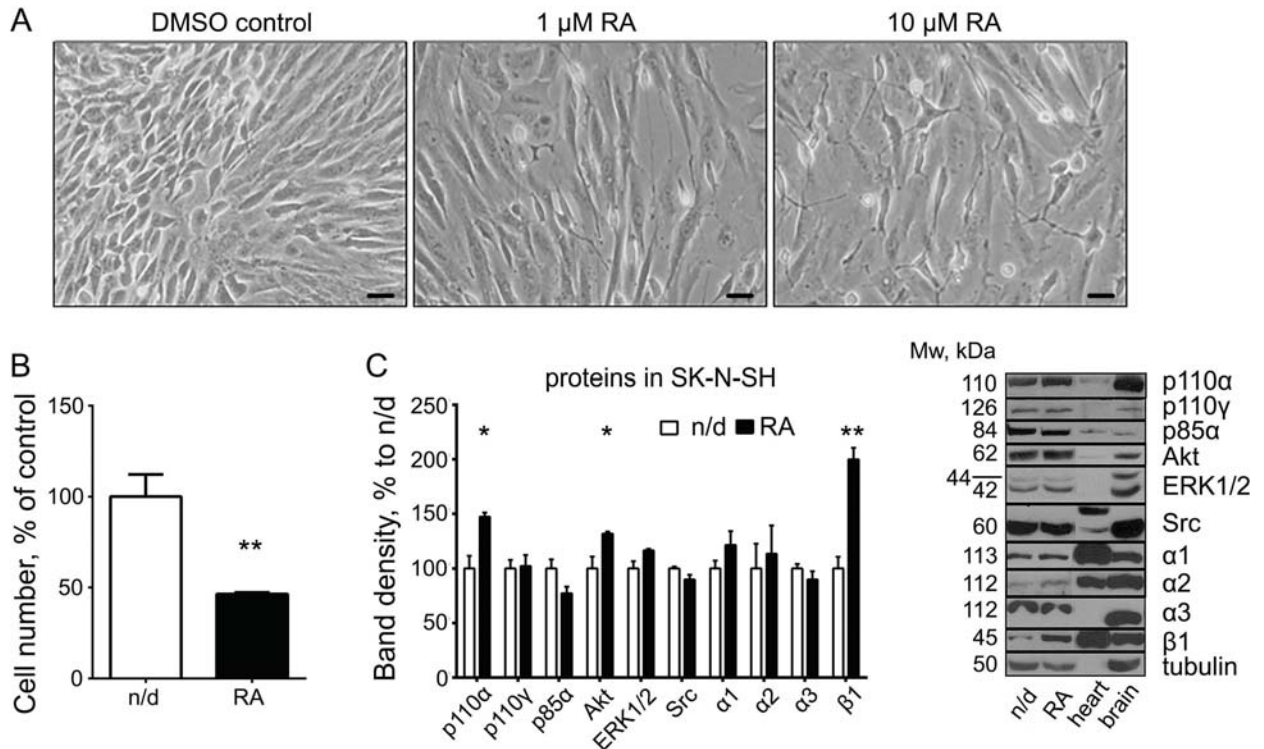


Fig. (1). RA induced changes in SK-N-SH cells. **A – Morphological changes after 10 days of RA incubation.** Phase contrast microscopy. Scale bar: 25μm. **B - Cell number of non-differentiated (n/d) and 1 μM RA-induced differentiated (RA) SK-N-SH.** Cells were rinsed with PBS and treated with trypsin-EDTA. Cell numbers were analyzed by Z2 cell counter. The number of non-differentiated cells was set to 100%. The graph represents data from four independent experiments. ** p < 0.01 vs n/d. **C – Changes in protein expression for non-differentiated (n/d, white bars) and 1 μM RA-induced differentiated (RA black bars) SK-N-SH.** Representative Western blots were shown the levels of proteins of interest in non-differentiated (n/d), differentiated (RA) SK-N-SH, cardiac tissue from rat (heart) and nervous tissue from rat (brain). Tubulin was used as a loading control. Lysates were analyzed by Western blotting (WB) with antibodies. The optical density of the n/d was set to 100%. Comparisons were normalized to the tubulin signal. The graph represents data from three independent experiments. * p < 0.05 vs n/d, ** p < 0.01 vs n/d.

differentiated cells compared to non-differentiated cells. There were no significant changes in the amount of Src and the mitogen-activated protein kinase, ERK 1/2 observed between the two groups. Protein expression of Akt and the p110α subunit of PI3K were augmented in differentiated SK-N-SH cells compared to non-differentiated cells.

Ouabain Induced Short-Term Activation of Signaling Cascades

Previously it has been shown that ouabain activates Akt and ERK 1/2 in cardiac myocytes and fibroblasts [7, 12-14]. It has also been shown that in non-differentiated SK-N-SH cells, ERK 1/2 is activated after 180 min of ouabain exposure via the α3 subunit of Na⁺/K⁺-ATPase [33]. Short-term activation of Akt and ERK 1/2 was detected in current study. The ratio of phosphorylated form to total Akt and ERK 1/2 was indicated as Akt and ERK 1/2 activation in differentiated and non-differentiated SK-N-SH cells by western blotting (Figs. 2, 3).

As shown in Fig. (2), in non-differentiated SK-N-SH cells, activation of Akt and ERK 1/2 by ouabain was not observed. Among the ouabain concentrations from 1 nM to 10 μM, the levels of phosphorylation of the two kinases

remained the same. Surprisingly, in differentiated SK-N-SH cells, both Akt and ERK 1/2 were significant activated by 10 nM ouabain. This activation by ouabain was dose-dependent (Fig. 3A). To determine the time course of ERK 1/2 and Akt activation, 10 nM ouabain (the minimal concentration caused an increase) was used (Figs. 2B, 3B). In differentiated SK-N-SH cells, ERK 1/2 activation was increased by ouabain and reached its peak after 10 min and remained the same up to 30 minutes of incubation (Fig. 3B). Akt activation reached its peak at 10 min and decreased after 30 minutes of incubation. The results obtained from the non-differentiated cells were quite different. The levels of phosphorylation of both ERK 1/2 and Akt were not increased by ouabain, even it had a trend reduce after 15 min of incubation (Fig. 2B). Thus, the data indicated that low doses of ouabain led to the activation of ERK 1/2 and Akt in differentiated SK-N-SH cells but not in non-differentiated SK-N-SH cells.

Long-Term Incubation of SK-N-SH with Ouabain

Previously, it has been shown that in non-differentiated SH-SY5Y cells, 10 nM ouabain causes 20% of cell death, while 1 μM ouabain causes 75% of cell death [34]. Long-

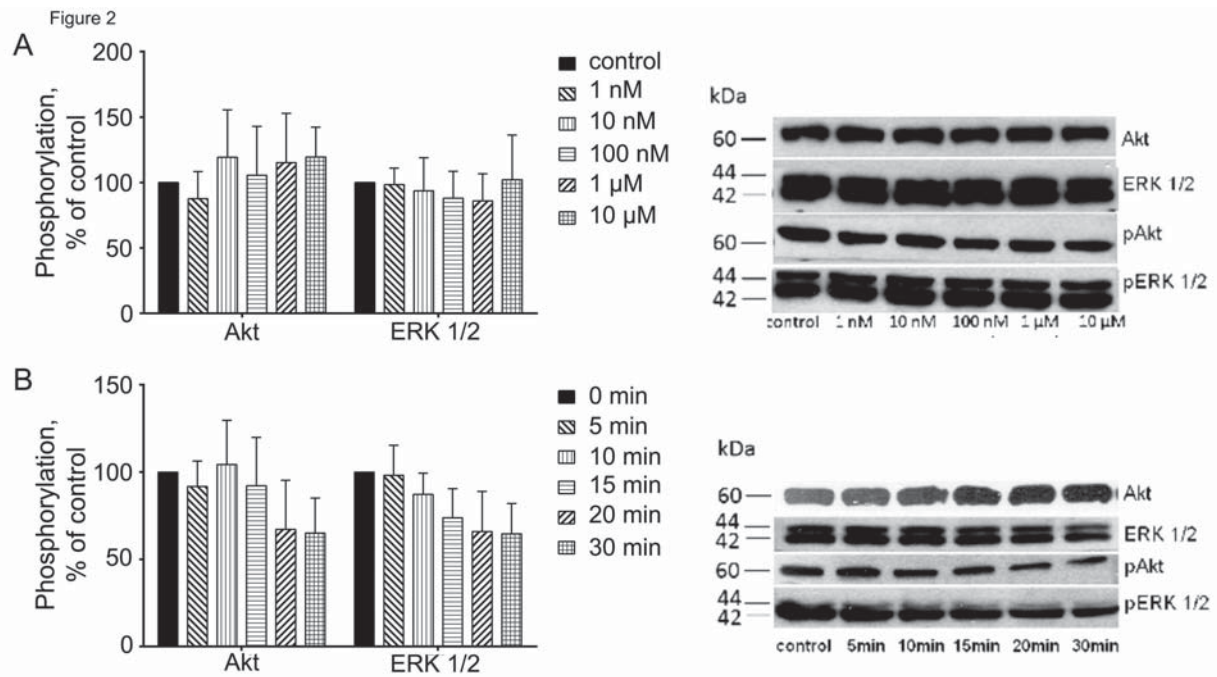


Fig. (2). Effects of ouabain on activation of Akt and ERK 1/2 in non-differentiated SK-N-SH. **A** – Levels of phosphorylation of ERK 1/2 and Akt after 10 min incubation with different concentrations of ouabain. **B** – Time course of 10 nM ouabain incubation on levels of phosphorylation of ERK 1/2 and Akt. The optical density of untreated cells was set as 100%. Data were presented as the ratio of p-Akt/Akt and p-ERK/ERK signal respectively. The graph represents data from three independent experiments. * $p < 0.05$ vs no ouabain.

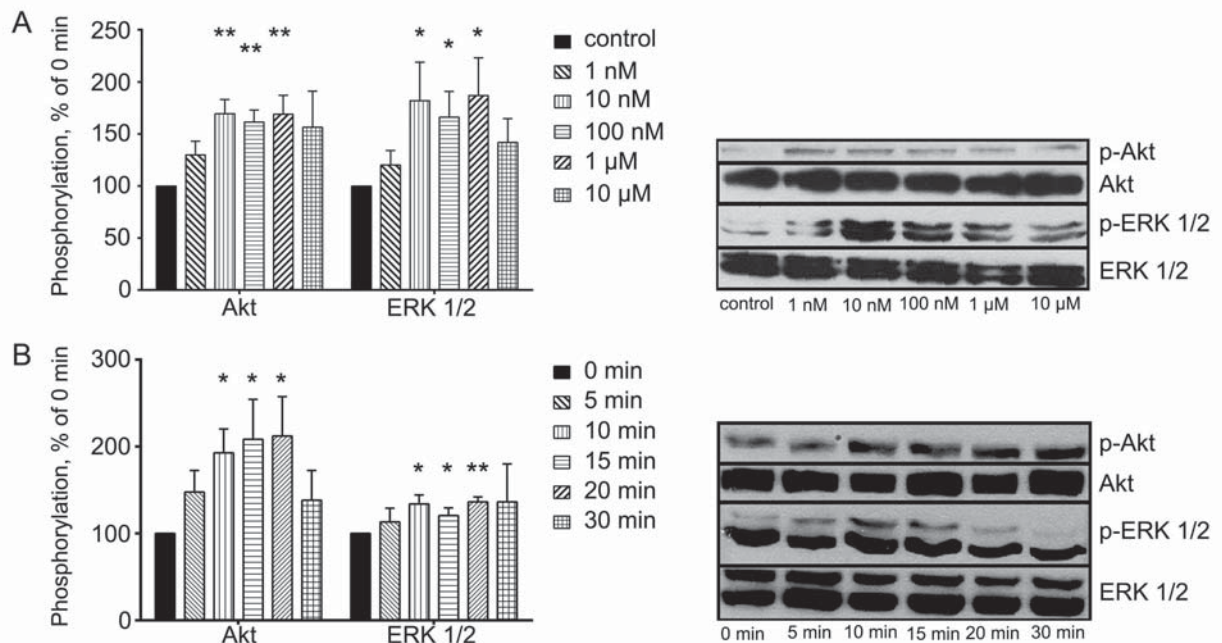


Fig. (3). Akt and ERK 1/2 activation by ouabain in differentiated SK-N-SH. **A** – Levels of phosphorylation of ERK 1/2 and Akt after 10 min incubation with different concentrations of ouabain. **B** – Time course of 10 nM ouabain incubation on levels of phosphorylation of ERK 1/2 and Akt. The optical density of untreated cells was set as 100%. Data were presented as the ratio of p-Akt/Akt and p-ERK/ERK signal respectively. The graph represents data from three independent experiments. * $p < 0.05$ vs to no ouabain, ** $p < 0.01$ vs no ouabain.

term effects of ouabain on cell proliferation were assessed in both differentiated and non-differentiated SK-N-SH cells (Fig. 4A).

10 nM ouabain did not affect cell proliferation in both groups, while 1 μM ouabain caused 60% of cell death in the non-differentiated SK-N-SH cells but only 15% of cell death in the differentiated cells. RA caused cell death during the

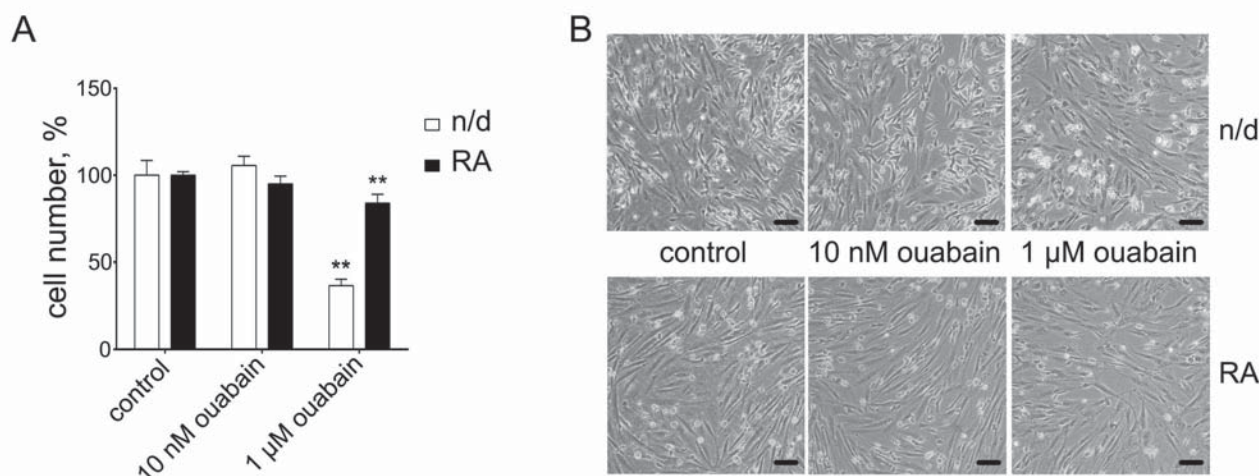


Fig. (4). Cell survival in non-differentiated (n/d) and RA-differentiated (RA) SK-N-SH after long-term ouabain incubation. Cells were incubated with or without RA for 7 days and then were exposure to 10 nM or 1 μ M ouabain for 3 days in non-differentiated and differentiated SK-N-SH. Cells were detached and separated with trypsin-EDTA and analyzed by Z2 cell counter. **A - Quantitative data on cell numbers.** The graph represents data from seven independent experiments. ** $p < 0.01$ vs no ouabain; # $P < 0.05$ n/d vs RA under the same treatment. **B- Morphological changes.** Scale bar: 50 μ m.

differentiation. The amount of cell death was similar that 1 μ M ouabain caused cell death in non-differentiated cells. We also noticed that ouabain did not cause morphological changes in differentiated SK-N-SH, but non-differentiated neuroblastoma cells acquired a neuron-like phenotype with longer cell bodies and more neurite processes (Fig. 4B).

DISCUSSION

The aim of this study was to exam ouabain-induced cell signaling in differentiated cells and non-differentiated SK-N-SH cells. Our study showed that both differentiated and non-differentiated SK-N-SH cells contain the $\alpha 1$ and $\alpha 3$ subunits of Na^+/K^+ -ATPase and small amount of $\alpha 2$ subunits of Na^+/K^+ -ATPase. While the amount of α subunits does not change with the differentiation, but a significant increase in the amount of fully glycosylated $\beta 1$ subunit of Na^+/K^+ -ATPase in differentiated SK-N-SH was observed. It has been reported that $\beta 3$ subunit of Na^+/K^+ -ATPase is involved during neuronal differentiation [40]. So far, there is no evidence that $\beta 1$ subunit of Na^+/K^+ -ATPase is attributed to ouabain-induced cell signaling. The β subunit is known to participate in the incorporation of Na^+/K^+ -ATPase α subunit into the membrane [41]. Higher ratio of β to α subunit also occurs in cardiomyocytes [44]. This might be a feature of terminally differentiated cells.

Class IA PI3K α is composed of catalytic subunit p110 α and regulatory subunit p85 [42]. Previously, it has been shown that the interaction between Na^+/K^+ -ATPase and p85 subunit, stimulates ouabain-induced activation of PI3K in cardiac myocytes [14]. It has been reported that PI3K/Akt is required for RA to induce neurite outgrowth and expression of neuronal markers [43]. We have shown that the amount of the PI3K p110 α subunit and Akt increases in differentiated cells, which might be contributed to RA-induced differentiation.

We have found that only in differentiated SK-N-SH cells, ouabain induced Akt and ERK 1/2 activation, the signaling

pathways have been demonstrated in many cell lines [7, 14]. It has been know that Akt activation promotes proliferation and increases cell survival. Interestingly, RA induced differentiation of SK-N-SH requires both ERK and Akt signaling [43]. These selective signaling pathways by ouabain might be beneficial to cancer therapy. Moreover, we also have shown that long-term incubation of 1 μ M ouabain caused less cell death in differentiated SK-N-SH cells compared to non-differentiated SK-N-SH cells. We have tested that IC_{50} of ouabain on human $\alpha 1$ subunit of Na^+/K^+ -ATPase is approximately 1.6 μ M (Liu L. unpublished data). More than IC_{50} dose of ouabain causes inhibition of Na^+/K^+ -ATPase and leads to cell death. It is reasonable to assume that the increase of cell death by ouabain in non-differentiated cells is due to significant inhibition of Na^+/K^+ -ATPase. It has been reported that ERK1/2 and Akt are critically involved in the regulation of apoptosis [43]. In differentiated cells, ouabain-induced activation of Akt and ERK 1/2 might prevent partly ouabain-induced cell death. Increased PI3K α and Akt by RA may augment ouabain-induced signaling. We also noted long-term exposure of non-differentiated cells to 1 μ M ouabain, the remaining cells acquired a neuron-like phenotype (Fig. 4B). Thus, it seems that ouabain has the potential to induce cell differentiation. Ouabain caused most non-differentiated cell death, but induced differentiation in the survivors. However, this speculation requires further investigation.

Previously it has been suggested that ouabain can be an endogenous hormone in mammals. For example, low concentrations of ouabain have been found in the cerebrospinal fluid [10]. Yet, the effects of CTS in neurons and neuron-like cells have been poorly studied so far. It has been suggested that the minor isoforms of the α subunit of Na^+/K^+ -ATPase ($\alpha 2$, $\alpha 3$) act as CTS signaling receptors in excitable tissues, while the $\alpha 1$ subunit mainly executes the ion pump function [26]. In non-differentiated SK-N-SH cells, it has been previously reported that ouabain causes ERK 1/2 activation after 3-hr incubation through $\alpha 3$ subunit

of Na⁺/K⁺-ATPase [34]. In the present study, we do not know which isoforms are responsible to ouabain-induced signaling after RA treatment. The data obtained in this study provide new insight into ouabain-induced signaling in human neuroblastoma and can be useful for exploring novel CTS-based cancer therapies.

LIST OF ABBREVIATIONS

CTS	=	CardioTonic Steroids
ERK	=	Extracellular-signaling-Regulated Kinase
PI3K	=	PhosphoInositide 3-Kinase
RA	=	Retinoic Acid

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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