Abstract: Accurate evaluation of the anti-cancer effects of ouabain, a cardiac glycoside, requires an understanding of its signaling pathway. This study examined the effects of ouabain stimulation on spontaneous interleukin (IL)-8 and IL-1α secretion in the HSC3 oral squamous cell carcinoma cell line. IL-8 secretion was reduced and IL-1α secretion was increased in the cells. Real-time polymerase chain reaction confirmed that these changes were regulated at the transcriptional level. Further analysis revealed that ouabain stimulation induced phosphorylation of activator protein (AP)-1 components (c-Jun and c-Fos) but not nuclear factor kappa B (NF-κB) components (p65 and p50). A luciferase assay demonstrated that the NF-κB-binding site located at 1 kb upstream of the TATA box in the IL-8 gene contributed to the reduction in IL-8 secretion. Pre-incubation of the cells with BAPTA-AM and L-glutathione increased IL-8 secretion, which indicates that Ca2+ ions and reactive oxygen species are associated with the ouabain-mediated reduction in IL-8 levels. The inhibitory effect of ouabain was attributed to reduced nuclear translocation of the NF-κB p65 subunit. Taken together, these findings indicate that ouabain exerts opposing effects on transcription factors NF-κB and AP-1.

Keywords: ouabain; oral squamous cell carcinoma; NF-κB; AP-1.

Introduction

Most oral cancers are histologically diagnosed as oral squamous cell carcinoma (OSCC) (1). Consistent activation of several transcription factors in OSCC cell lines results in spontaneous secretion of cytokines that contribute to tumor progression (1). Thus, inhibition of these cytokines should be a promising approach to prevent OSCC growth and invasion.

Cardiac glycosides are naturally derived compounds that share a common structural steroidal framework (2). They bind to the sodium/potassium pump (Na+/K+-ATPase) on the cell surface and exert various biological effects. Ouabain is a cardiac glycoside with cardiotonic properties; it binds to Na+/K+-ATPase and transcriptionally induces expression of Na+/K+-ATPase messenger ribonucleic acid (mRNA) (3).

Furthermore, the binding of ouabain to Na+/K+-ATPase partially suppresses the activities of the ion pump and initiates two distinct events: intracellular ionic changes (3-7) and interaction of Na+/K+-ATPase with other cell surface proteins (8,9). Two years after Na+/K+-ATPase was identified as a target of ouabain, the anti-proliferative effect of cardiac glycosides on malignant cells was reported (10). By increasing the concentration of Ca2+ ions in the cell, cardiac glycosides exert anti-cancer activity via endoplasmic reticulum stress, apoptosis, and disruption of mitochondrial integrity (2,11,12). Research on the susceptibilities of various cancer cells to cardiac glycosides began soon after the anti-cancer effects of these compound were discovered (13). However, the
effects of these compounds on OSCC have not been thoroughly investigated. In the present study, the ouabain signaling pathway was examined in order to clarify its anti-cancer effect in OSCC.

**Materials and Methods**

**Reagents**

Ouabain (C_{29}H_{44}O_{12}•8H_2O; Sigma, Tokyo, Japan) was chosen as a stimulator, BAPTA-AM (Dojindo Molecular Technologies, Tokyo, Japan) was selected as an intracellular calcium chelator, and L-glutathione was chosen as a reactive oxygen species inhibitor (Sigma).

**Cells**

The human OSCC cell line HSC3 (obtained from the Japanese Collection of Research Bioresources [JCRB] Cell Bank, Ibaraki, Japan) was maintained as described previously (14).

**Enzyme-linked immunosorbent assay (ELISA)**

The HSC3 cells were seeded in a 24-well culture plate at a density of 2 × 10^5 and cultured for 18 h in a 5% CO_2 incubator. The cells were then stimulated with 0, 30, 60, and 120 μM of ouabain for 1 h, washed with fresh medium, and incubated for another 6 h. After incubation, the culture supernatants were collected and centrifuged for 3 min at 14,000 × g. The supernatants were collected in new tubes and subjected to ELISA measurements for interleukin (IL)-1α and IL-8 (DuoSet ELISA Development System, R&D system, Tokyo, Japan) (IL-8, n = 10; IL-1α, n = 8). The cells then were pre-incubated with 10 μM of BAPTA-AM and 10 μM of L-glutathione for 1 h before addition of ouabain, to examine the effects of Ca^{2+} ions and reactive oxygen species (ROS) on IL-8 secretion.

**Deoxyribonucleic acid (DNA) construction**

The luciferase reporter vector encompassing the IL-8 untranslated region (UTR) was described previously (14). This vector contains two NF-κB-binding sites, and the shorter fragment, which contains only the lower binding site, was amplified and subcloned to the pGL4 basic vector (NF-κB-short vector) to eliminate the upper binding site.

**Luciferase assay**

HSC3 cells (1 × 10^5/48-well plate) were rinsed twice with OPTI-MEM medium (GE Healthcare, Tokyo, Japan). The luciferase reporters (1 μg) and internal control vector pRL-CMV (20 pg) were co-transfected into the cells by using Lipofectamine Plus reagent (3 μL each/sample; Life Technologies) for 5 h. After transfection, the cells were washed with 10% FCS-RPMI and cultured for another 18 h. The transfectants were stimulated with or without 30 μM of ouabain for 1 h. After washing the cells and culturing them for another 6 h, the cell lysates were harvested and subjected to luciferase activity measurements (Dual-Luciferase Reporter Assay System, Promega; n = 8).

**Immunofluorescence staining**

HSC3 cells were plated on 10-mm cover slips (Matsunami, Kishiwada, Japan) and cultured for 18 h. The cells were cultured with or without 30 μM of ouabain for 1 h. After washing with phosphate-buffered salts (PBS), the cells were cultured for another 6 h, washed with PBS, and fixed with 2% paraformaldehyde for 10 min. The cells were washed with PBS and incubated with 1% Triton X-100 for 10 min. Nonspecific binding was blocked by incubating the cells with 1% bovine serum albumin (BSA)-PBS for 30 min at room temperature. Then, the cells were incubated with rabbit anti-p65 antibody (×100 dilution with 1% BSA-PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Finally, the cells were washed and mounted on glass slides. The images were captured with a digital microscope (Keyence, Tokyo, Japan) (n = 9).

**Statistical analysis**

The data were primarily analyzed with the Kolmogorov-Smirnov test and the Levene test. In addition, the data were statistically analyzed by one-way analysis of variance (ANOVA) and the Tukey-Kramer test. Results are presented as mean ± SD by using the SPSS software package (v22; IBM Corp., Chicago, IL, USA). A P value of <0.05 was considered to indicate statistical significance. Single and double asterisks indicate P < 0.05 and P < 0.01, respectively.

**Results**

**Effect of ouabain on cytokine secretion**

HSC3 spontaneously secreted IL-8 (585 ± 33.3 pg/mL) and IL-1α (93 ± 7.2 pg/mL) (Fig. 1a). IL-8 levels decreased to 299 ± 45.8 pg/mL in an ouabain concentration-dependent manner (Fig. 1a), whereas IL-1α secretion increased to 122 ± 4.59 pg/mL (Fig. 1b). To examine whether these changes were regulated at the transcriptional level, total RNA was purified and subjected to real-time PCR. When the mRNA expression level in the absence of ouabain was set as 1, IL-8 expression decreased to 0.153 ± 0.05 in the cells (Fig. 2a). In contrast, IL-1α expression increased to 1.14 ± 0.04 after
3 h of stimulation with ouabain (Fig. 2b).

**Effect of ouabain on transcription factors**

mRNA expression levels of IL-8 and IL-1α are mainly regulated by the transcription factors NF-κB and AP-1, respectively. The effect of ouabain on these transcription factors was evaluated by a luciferase assay. The structures of the luciferase reporter vectors are shown in Fig. 3. The luciferase activities of ouabain-stimulated HSC3 cells transfected with NF-κB-Luc and AP-1-Luc were significantly ($P < 0.01$) lower (47%) and higher (333%), respectively, than those of the controls (Fig. 4a, b). To identify the NF-κB-binding site that may have contributed to the reduction in luciferase activity, the assay was
conducted with both the NF-κB-long and -short vectors. Luciferase activity in cells transfected with the NF-κB-short vector showed no change in relation to ouabain stimulation (Fig. 4c). However, luciferase activity was significantly lower (43%; \( P < 0.01 \)) in cells transfected with the long vector than in the controls (Fig. 4d).

**Effects of Ca\(^{2+}\) ions and ROS on IL-8 secretion**

To examine the effects of Ca\(^{2+}\) ions and ROS on ouabain-mediated IL-8 secretion, HSC3 cells were pre-incubated with or without BAPTA-AM or L-glutathione. Spontaneous secretion of IL-8 was not affected by BAPTA-AM or L-glutathione (Fig. 5). However, the addition of each reagent significantly (\( P < 0.01 \)) increased (roughly doubled) IL-8 secretion (Fig. 5). In contrast, IL-8 secretion was similar to baseline levels in HSC3 cells cultured with both reagents (Fig. 5).

**Signaling pathway**

HSC3 cells were stimulated with or without ouabain, and the cell lysates were harvested for Western blotting. The amounts of total or phosphorylated p65 and p50 were unchanged after stimulation with ouabain (Fig. 6a, b). Conversely, phosphorylated c-Jun and c-Fos levels were significantly higher after 15 and 30 min of ouabain stimulation (Fig. 6c, d).

**Immunofluorescence staining**

To examine the intracellular distribution of NF-κB p65 subunit after ouabain stimulation, HSC3 cells were stimulated with or without ouabain and subjected to immunofluorescence staining using goat anti-p65 antibody followed by FITC-conjugated rabbit anti-goat IgG. Representative data from at least nine independent experiments are shown (\( n = 9, **P < 0.01 \)). Fluorescence intensity was evaluated with a Keyence microscope. Scale bar: 20 μm
ratios of the fluorescence intensities in the nucleus and cytoplasm (Fig. 7b) indicate that a significant ($P < 0.01$) amount of p65 subunit had moved to the cytoplasm after ouabain stimulation (Fig. 7b).

**Discussion**

Ouabain binding to Na$^+/K^+$-ATPase induces both NF-$\kappa$B and AP-1 by increasing intracellular Ca$^{2+}$ ion levels (15). The present findings show that ouabain stimulation had opposing effects on IL-8 and IL-1$\alpha$ secretion in HSC3 cells, namely, IL-8 secretion was reduced and IL-1$\alpha$ secretion was increased in the cells. Real-time PCR revealed that these changes were regulated at the transcriptional level, as well. In general, IL-8 mRNA induction is regulated mainly by NF-$\kappa$B, the binding site of which is located within 150 bp upstream of the TATA box in the IL-8 5'-UTR (16). The contribution of NF-$\kappa$B was evaluated with a luciferase assay using the 1.3-kb 5'-UTR, which contains two NF-$\kappa$B binding sites. Luciferase activity was significantly down-regulated by ouabain when the NF-$\kappa$B-long vector was used, indicating that the upper binding site was more prominently involved in ouabain-mediated down-regulation of IL-8 mRNA expression. Cardiac glycoside-mediated down-regulation of some genes has been reported in prostate cancer (17). Another cardiac glycoside, reevesioside A, down-regulated expression of c-myc and its targets (17). Although previous studies showed that apoptosis is induced in osteosarcoma (18) and Burkitt’s lymphoma cell lines (19), and that detachment from the extracellular matrix is induced in lung cancer cells (20), the underlying signaling is not clearly understood. Future studies should carefully examine the mechanisms involved in ouabain-mediated IL-8 mRNA inhibition.

Intriguingly, the 5'-UTR region also contains an AP-1 binding site. The contributory effect of AP-1 on IL-8 expression is less significant and depends on the cell type (15). The dominant negative mutant of c-Jun did not affect IL-8 secretion in fibroblasts (21) but had an inhibitory effect in endothelial cells (22). This is consistent with the present findings showing that ouabain stimulation increased luciferase activity of the AP-1 reporter transfectant without affecting IL-8 secretion.

To further confirm the contradictory effects of ouabain on NF-$\kappa$B and AP-1, the phosphorylation status of their components was examined. Ouabain stimulation induced phosphorylation of c-Jun and c-Fos (AP-1 components) but not of NF-$\kappa$B subunits. Phosphorylation of the AP-1 components was observed at an early stage (15-30 min after stimulation). Nuclear translocation of the p65 subunit was weakly inhibited by ouabain stimulation. AP-1 activation is controlled by mitogen-activated protein kinases (MAPK), which are activated by ROS via unknown mechanisms (23). ROS are produced after ouabain binding to Na$^+/K^+$-ATPase and can phosphorylate the AP-1 component through c-Jun N-terminal kinases.

To clarify the effects of ROS and Ca$^{2+}$ ions on IL-8 secretion, HSC3 cells were pre-incubated with a ROS inhibitor and a Ca$^{2+}$ chelator. Surprisingly, pre-incubation with either reagent significantly increased IL-8 secretion. Furthermore, treatment with both inhibitors reduced IL-8 secretion to the baseline level. The binding of ouabain to Na$^+/K^+$-ATPase leads to interaction with epidermal growth factor receptor (EGFR) and Src, which culminates in ROS production and an increase in cellular Ca$^{2+}$ levels. These two important events are together thought to activate NF-$\kappa$B and AP-1 (24). Pre-treatment with both reagents abolished the ouabain-induced reduction in IL-8 secretion, which indicates that ROS and Ca$^{2+}$ ions both have roles in inactivating NF-$\kappa$B. However, the reasons why these reagents increased IL-8 secretion remain to be determined.

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**Conflict of interest**

The authors have no conflict of interest to declare.

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