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Odoroside A and ouabain inhibit Na^+/K^+-ATPase and prevent NF-κB-inducible protein expression by blocking Na^+-dependent amino acid transport

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ABSTRACT

Inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin-1 (IL-1), trigger the activation of transcription factor NF-κB that induces the expression of a variety of genes, including intercellular adhesion molecule (ICAM)-1. Odoroside A [3β-O-(β-D-diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide] was found to inhibit the cell-surface expression of ICAM-1 induced by TNF-α and IL-1 at comparable concentrations in human lung carcinoma A549 cells. In this study, the molecular mechanism underlying the inhibition of TNF-α-induced cell-surface ICAM-1 expression by odoroside A together with the specific Na^+/K^+-ATPase inhibitor ouabain was further investigated. Odoroside A and ouabain neither prevented IκBα degradation nor NF-κB translocation to the nucleus upon TNF-α stimulation. While odoroside A and ouabain had no inhibitory effect on the induction of ICAM-1 mRNA, they inhibited the TNF-α-induced ICAM-1 expression at the protein level. Consistent with these results, odoroside A and ouabain potently reduced *de novo* protein synthesis, largely due to its ability to block Na^+-dependent transport of amino acids across the plasma membrane, but not to interfering with the translation machinery. As a direct molecular target, odoroside A was found to inhibit the ATP-hydrolyzing activity of Na^+/K^+-ATPase as potently as ouabain. These results clearly demonstrate that odoroside A and ouabain prevent NF-κB-inducible protein expression by blocking the Na^+-dependent amino acid transport.

1. Introduction

Inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin-1 (IL-1) play an essential role in inflammation and induce the expression of a variety of genes responsible for inflammatory responses, such as intercellular adhesion molecule-1 (ICAM-1; CD54) [1]. ICAM-1 is a cell-surface glycoprotein that belongs to the immunoglobulin superfamily and serves as a ligand for lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and for Mac-1 (CD11b/CD18) [2]. ICAM-1 is expressed at low levels on many types of cells and its expression is predominantly upregulated at the transcriptional level [1]. During inflammation, the inducible expression of ICAM-1 on vascular endothelium is regulated mainly by inflammatory cytokines, and ICAM-1 expression facilitates the adhesion and subsequent transmigration of leukocytes expressing LFA-1 or Mac-1 into inflamed sites [3,4]. ICAM-1 expression is induced primarily by the transcription factor nuclear factor κB (NF-κB) [1].

The NF-κB signaling pathway is activated by various stimuli, including inflammatory cytokines. Upon TNF-α stimulation, TNF receptor 1 (TNF-R1) recruits adaptor proteins to form a membrane-bound complex that triggers the activation of IκB (inhibitor of κB) kinase [5]. Interleukin-1 receptor (IL-1R) recruits different adaptor proteins to its cytoplasmic domain, and this complex also initiates the activation of IκB kinase [6,7]. IκB exists basically as a complex with the NF-κB heterodimer in the cytosol and phosphorylation of IκB causes its ubiquitinylation and subsequent hydrolysis by the

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proteasome [6,7]. The NF-κB heterodimer becomes free and translocates to the nucleus where it activates a variety of genes responsible for inflammation as well as cancer development and progression [8].

We have purified various natural products from the extracts of *Nerium oleander* and investigated their structure–activity relationships with focus on anti-inflammatory and anticancer activities [9–12]. In this study, as one of the most active compounds isolated from *N. oleander*, we report that odoroside A [3β-O-(β-D-diginosyl)-14-hydroxy-5β,14β-card-20(22)-enolide] [12–15] (Fig. 1A) isolated from the extracts of *N. oleander* inhibits the cell-surface expression of ICAM-1 induced by TNF-α and IL-1 in human lung carcinoma A549 cells. Odoroside A is classified into a group of cardenolide glycosides, some of which are defined as inhibitors of Na^+/K^+-ATPase and are used as drugs for treating cardiac failure and as anticancer drugs in clinical trials [16]. It has been shown that such cardenolide glycosides as oleandrin block TNF-α-induced NF-κB activation [17,18]. Moreover, it has been reported that the Na^+/K^+-ATPase inhibitor ouabain is able to activate the NF-κB pathway [19,20]. However, compared with other cardenolide glycosides [16], the biological activities of odoroside A have been poorly characterized. Therefore, in this study, we investigated the molecular mechanism underlying the inhibition of TNF-α-induced cell-surface ICAM-1 expression by odoroside A together with its structurally related cardenolide glycoside ouabain. In contrast to previous studies [17–20], our present results demonstrate that odoroside A and ouabain do not affect the early NF-κB signaling pathway but rather prevents NF-κB-inducible protein expression by blocking Na^+-dependent amino acid transport.

2. Materials and methods

2.1. Cell culture

Human lung carcinoma A549 cells were provided by the Health Science Research Resources Bank (Tokyo, Japan). A549 cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS, USA), 100 U/ml penicillin G (Sigma–Aldrich, Co., St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma–Aldrich).

2.2. Reagents

Recombinant human TNF-α and human IL-1 were kindly provided by Dainippon Pharmaceutical (Osaka, Japan). Bovine serum albumin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich. *o*-Phenylenediamine dihydrochloride, trichloroacetic acid, sodium orthovanadate, and choline chloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ATP, L-glutamine, glycine, L-leucine, L-lysine, L-phenylalanine, and L-serine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The proteosome inhibitor MG-132 was purchased from Peptide Institute, Inc. (Osaka, Japan). The protein synthesis inhibitor puromycin and the Na^+/K^+-ATPase inhibitor ouabain were obtained from Sigma–Aldrich, respectively. MG-132 and ouabain were dissolved in dimethyl sulfoxide as a 10 mM stock solution and puromycin was dissolved in methanol as a 10 mM stock solution. These inhibitors were further diluted with media or buffers to the indicted concentrations. Odoroside A was isolated from the stems and twigs of *N. oleander* as described in Supplementary data [12]. Odoroside A was dissolved in dimethyl sulfoxide as a 10 mM stock solution and further diluted with media or buffers to the indicated concentrations.

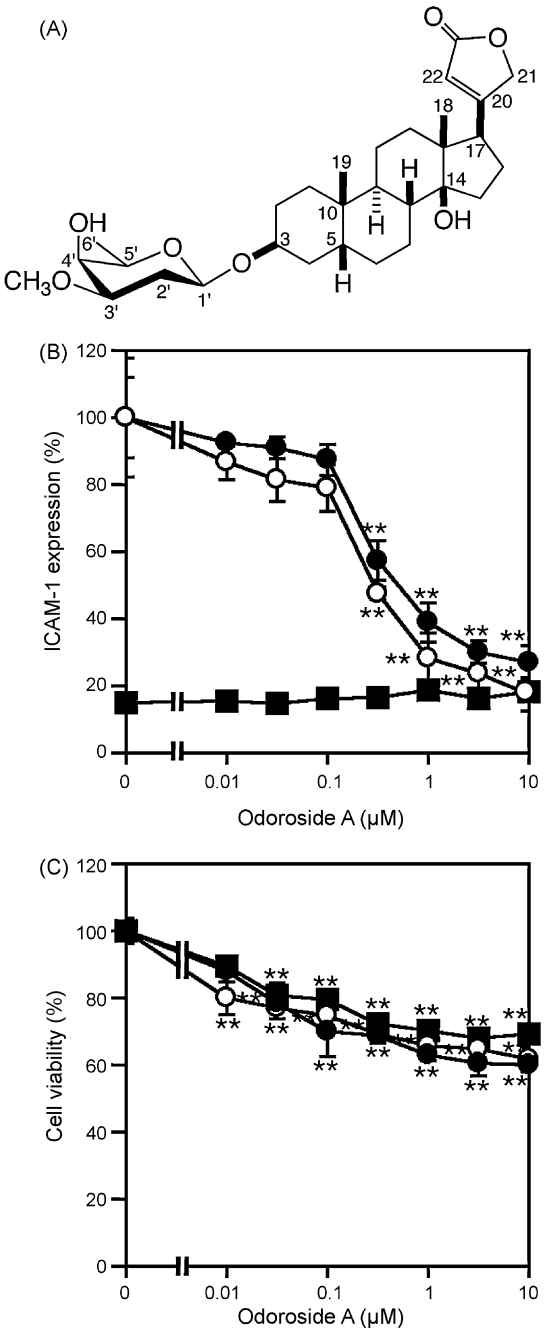

Fig. 1. Odoroside A inhibits expression of cell-surface ICAM-1 induced by TNF-α or IL-1. (A) Structure of odoroside A. (B) A549 cells were pretreated with various concentrations of odoroside A for 1 h and then incubated with TNF-α (2.5 ng/ml; open circles) or IL-1 (0.25 ng/ml; filled circles) for 6 h in the presence of odoroside A. Absolute values for TNF-α-stimulated and IL-1-stimulated cells were 0.520 ± 0.050 and 0.459 ± 0.064, respectively. A549 cells were pretreated with various concentrations of odoroside A for 1 h and then incubated without cytokines (filled squares) for 6 h in the presence of odoroside A. Absolute value for non-stimulated cells was 0.111 ± 0.005, compared with 0.481 ± 0.024 for TNF-α-stimulated cells as positive control. ICAM-1 expression (%) is shown as means ± S.D. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with non-stimulated, TNF-α-stimulated or IL-1-stimulated cells. (C) A549 cells were pretreated with various concentrations of odoroside A for 1 h and then incubated with TNF-α (2.5 ng/ml; open circles) or IL-1 (0.25 ng/ml; filled circles) or without cytokines (filled squares) for 6 h in the presence of odoroside A. Cell viability (%) was measured by MTT assay. Data points represent means ± S.D. ($n = 3$). Absolute values for non-stimulated, TNF-α-stimulated and IL-1-stimulated cells were 0.809 ± 0.018, 0.844 ± 0.030 and 0.871 ± 0.027, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with non-stimulated, TNF-α-stimulated or IL-1-stimulated cells.

2.3. Antibodies

Antibodies to β-actin (AC-15; Sigma–Aldrich), FLAG (M2; Sigma–Aldrich), c-FLIP (Dave-II; Alexis, Lausen, Switzerland), ICAM-1 (clone 15.2; Leinco Technologies, Inc., St. Louis, MO, USA), ICAM-1 (clone 28; BD Biosciences, Franklin Lakes, NJ, USA), IκBα (clone 25; BD Biosciences), NF-κB p65 (AHP288; Serotec, Inc., Raleigh, NC, USA), and phospho-IκBα (Ser32/36) (5A5; Cell Signaling Technology, Inc., Danvers, MA, USA) were commercially obtained.

2.4. Assay for cell-surface expression of ICAM-1

Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde–PBS for 15 min. After being washed twice with PBS, the cells were incubated with 1% bovine serum albumin (Sigma–Aldrich)–PBS overnight. Fixed cells were treated with mouse anti-human ICAM-1 IgG antibody (15.2) for 60 min and then washed three times with 0.02% Tween 20–PBS. The cells were further treated with horseradish-peroxidase-linked anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 60 min and then washed three times with 0.02% Tween 20–PBS. To develop the colorimetric reaction, the cells were incubated with the substrate solution (0.2 M sodium citrate (pH 5.3), 0.1% *o*-phenylenediamine dihydrochloride, 0.02% H_2O_2) for 20 min at 37 °C. Absorbance at 415 nm was measured with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). ICAM-1 expression (%) was calculated as [(experimental absorbance in the presence of cytokines and test compounds − background absorbance)/(control absorbance in the presence of cytokines − background absorbance)] × 100.

2.5. Assay for cell viability

Cells were pulsed with MTT (500 μg/ml) for 4 h and resultant MTT formazan was solubilized with 10% sodium dodecyl sulfate (SDS) overnight. Absorbance at 595 nm was measured with the Model 680 microplate reader. Cell viability (%) was calculated as [(experimental absorbance − background absorbance)/(control absorbance − background absorbance)] × 100.

2.6. Preparation of cell lysates and Western blotting

Cells were washed once with PBS and lysed in Triton X-100 lysis buffer consisting of 50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 2 mM DTT, 2 mM sodium orthovanadate, and the protease inhibitor mixture Complete™ (Roche Diagnostics, Mannheim, Germany). Postnuclear lysates were collected as supernatants by centrifugation (800 × *g*, 7 min). Pellets were washed twice with Hepes buffer (20 mM Hepes–NaOH (pH 8.0), 20% glycerol, 100 mM KCl, 1 mM EDTA, 0.5 mM DTT, leupeptin (10 μg/ml), 0.5 mM PMSF) and then lyzed in the Hepes buffer by sonication, followed by centrifugation (9000 × *g*, 2 min). Protein samples (20–30 μg/lane) were separated by SDS-PAGE and transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were incubated with primary antibodies and then horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch), followed by analysis using ECL Western blotting detection reagents (GE Healthcare). The experiments including preparation of cell lysates and Western blotting were repeated at least twice to confirm reproducibility.

2.7. PCR

Total RNA was extracted from A549 cells using Sepasol-RNA (Nacalai Tesque) and reverse-transcribed to cDNA using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. cDNA was amplified with a Bio-Rad iCycler (Bio-Rad Laboratories) using KOD DNA polymerase (TOYOBO, Co. Ltd., Osaka, Japan) and the following primers:

5′-GGGAGGCTCCGTGCTGGTGA-3′ (sense) and
5′-TCAGTGCGGCACGAGAAATTG-3′ (antisense) for ICAM-1 (447 bp) [21], and
5′-GGCATCGTGATGGACTCCG-3′ (sense) and
5′-GCTGGAAGGTGGACAGCGA-3′ (antisense) for β-actin (613 bp).

PCR conditions for ICAM-1 and β-actin were 94 °C for 3 min, followed by 25 and 20 cycles of 94 °C for 15 s, 58 °C for 30 s, 68 °C for 1 min, and 68 °C for 10 min, respectively. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and stained with 1 μg/ml ethidium bromide (Invitrogen).

2.8. Assay for protein synthesis

Cells were pulsed with [4,5-^{3}H]L-leucine (5.92 TBq/mmol; Moravek Biochemicals, Inc., Brea, CA, USA) for 2 h. The labeled cells were washed twice with PBS and then lyzed with 250 mM NaOH for 15 min, followed by 1 h incubation on ice in the presence of 5% trichloroacetic acid. The precipitates were collected by centrifugation (10 000 × *g*, 5 min), then washed with 10% trichloroacetic acid, and measured for radioactivity.

2.9. Assay for cell-free protein synthesis

FLAG-tagged human β-actin cDNA in the pCR3 expression vector (Invitrogen) was subjected to cell-free reaction (30 °C, 90 min) for transcription and translation by TnT$^{(R)}$ Coupled Reticulocyte Lysate Systems (Promega Co., Madison, WI, USA). After centrifugation (7700 × *g*, 10 min), the resultant products were separated by SDS-PAGE and analyzed by Western blotting using antibodies reactive to FLAG and β-actin.

2.10. Assay for amino acid transport

Cells were pulsed with [2,3,4-^{3}H]L-glutamine (1.628 TBq/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA), [2-^{3}H]glycine (2.479 TBq/mmol, Moravek), [4,5-^{3}H]L-leucine (2.664 TBq/mmol, Moravek), [4,5-^{3}H(N)]L-lysine (1.665 TBq/mmol, Moravek), [ring-2,6-^{3}H]L-phenylalanine (2.035 TBq/mmol, Moravek), or [2,3-^{3}H]L-serine (0.74 TBq/mmol, Moravek) for 5 min in control incubation buffer (25 mM Hepes, 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, pH 7.4) or Na^+-free incubation buffer (25 mM Hepes, 125 mM choline chloride, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, pH 7.4). The cells were immediately washed three times with ice-cold PBS and lysed in 100 mM NaOH, followed by measurement of radioactivity.

2.11. Assay for Na^+/K^+-ATPase activity

Highly purified Na^+/K^+-ATPase from porcine kidney (15.6 μmol Pi/min/mg protein at 37 °C) was the gift of Drs. Yoshikazu Tahara and Yutaro Hayashi (Department of Biochemistry, Kyorin University School of Medicine, Tokyo, Japan) [22,23]. Assay conditions for the ATP-hydrolyzing activity of Na^+/K^+-ATPase were described previously [22,23]. Na^+/K^+-ATPase (0.5 μg) was incubated in 23 mM Hepes, 15 mM imidazole, 100 mM NaCl, 25 mM KCl, 3.9 mM $MgCl_2$, 1 mM EDTA, pH 7.0 in the presence of 0.5 mM

ATP at 37 °C for 15 min. The colorimetric assay for inorganic phosphates was performed as described previously [24,25].

2.12. Statistical analysis

Statistical significance was assessed by one-way ANOVA followed by Tukey test for multiple comparisons.

3. Results

3.1. Odoroside A inhibits cell-surface expression of ICAM-1 induced by TNF-α and IL-1

Inflammatory cytokines, such as TNF-α or IL-1, induce the expression of various cell-surface molecules that are involved in cell–cell interaction during inflammatory responses [1]. Upon stimulation with TNF-α or IL-1, A549 cells were induced to express ICAM-1 on their surfaces. Odoroside A was found to inhibit the cell-surface expression of ICAM-1 induced by TNF-α and IL-1 in a dose-dependent manner and at comparable concentrations (IC_{50} values of 0.27 μM and 0.42 μM, respectively), while odoroside A itself did not induce the cell-surface ICAM-1 expression in the absence of cytokines (Fig. 1B). Under these conditions, odoroside A decreased cell viability to some extent irrespective of TNF-α or IL-1 as judged by the MTT assay (Fig. 1C). These data indicate that the inhibition of ICAM-1 expression by odoroside A is not due to nonspecific cytotoxicity.

3.2. Odoroside A does not inhibit TNF-α-induced NF-κB translocation to nucleus

In A549 cells, ICAM-1 expression has been shown to be highly NF-κB-dependent upon stimulation with TNF-α and IL-1 [26]. TNF-α induces the rapid phosphorylation of IκBα by IκB kinase, and phosphorylated IκBα immediately undergoes proteasomal degradation [6,7]. IκBα was constitutively expressed as an unphosphorylated form in A549 cells and became barely detectable within 15 min upon TNF-α stimulation (Fig. 2A). Phosphorylated IκBα accumulated in TNF-α-stimulated A549 cells when treated with the proteasome inhibitor MG-132 (Fig. 2A). In contrast to previous reports [17,18], neither the unphosphorylated nor the phosphorylated form of IκBα was detectable in TNF-α-stimulated A549 cells in the presence of odoroside A (Fig. 2A). Moreover, odoroside A did not reduce the phosphorylation of IκBα in the presence of TNF-α and MG-132 (Fig. 2A). These results indicate that odoroside A does not affect the NF-κB signaling pathway leading to IκBα degradation.

The NF-κB heterodimer forms a complex with IκBα in the cytosol, and IκBα degradation leads to the nuclear translocation of the NF-κB heterodimer [6,7]. Upon TNF-α stimulation, the NF-κB subunit p65 was translocated from the cytosol to the nucleus (Fig. 2B). Unlike MG-132, odoroside A did not inhibit the p65 translocation to the nucleus (Fig. 2B). These results suggest that odoroside A does not inhibit TNF-α-induced nuclear translocation of the NF-κB heterodimer.

3.3. Odoroside A does not affect TNF-α-induced mRNA expression but inhibits TNF-α-induced protein expression

ICAM-1 expression is upregulated at the mRNA level in an NF-κB-dependent manner [1,26]. A549 cells were pretreated with odoroside A or MG-132 for 1 h and then incubated with TNF-α for 4 h in the presence of odoroside A or MG-132. TNF-α was able to induce ICAM-1 mRNA expression, while MG-132 strongly prevented the induction of ICAM-1 mRNA expression to almost unstimulated levels (Fig. 3A). By contrast, odoroside A did not inhibit the TNF-α-induced ICAM-1 mRNA expression even at 10 μM (Fig. 3A). To determine if odoroside A inhibits the induction of ICAM-1 protein during the translation process, the expression of ICAM-1 as a protein product was analyzed by Western blotting. Consistent with ICAM-1 mRNA expression, TNF-α induced a marked increase in ICAM-1 protein expression in A549 cells (Fig. 3B). Indeed, odoroside A inhibited the induction of ICAM-1

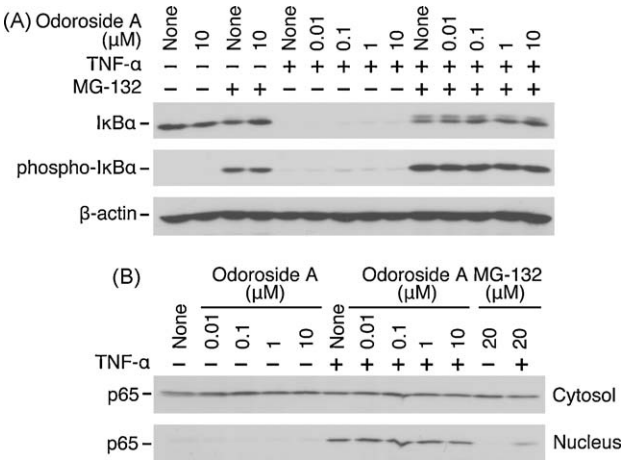

Fig. 2. Odoroside A does not affect TNF-α-induced NF-κB signaling pathway. (A) A549 cells were pretreated with various concentrations of odoroside A in the presence (+) or absence (−) of MG-132 (20 μM) for 1 h and then treated with (+) or without (−) TNF-α (2.5 ng/ml) for 15 min in the presence (+) or absence (−) of odoroside A or MG-132. Cell lysates were analyzed by Western blotting. (B) A549 cells were pretreated with various concentrations of odoroside A or MG-132 (20 μM) for 1 h and then treated with (+) or without (−) TNF-α (2.5 ng/ml) for 30 min in the presence or absence of odoroside A or MG-132. Cytosolic and nuclear fractions were analyzed by Western blotting.

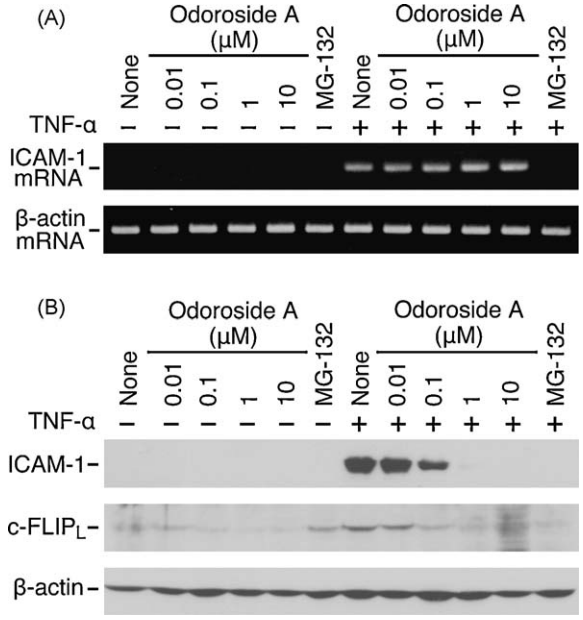

Fig. 3. Odoroside A does not affect TNF-α-induced mRNA expression, but inhibits TNF-α-induced protein expression. (A) A549 cells were pretreated with various concentrations of odoroside A or MG-132 (20 μM) for 1 h and then incubated with (+) or without (−) TNF-α (2.5 ng/ml) for 4 h in the presence or absence of odoroside A or MG-132. Expression of ICAM-1 mRNA and β-actin mRNA was measured by semi-quantitative PCR. (B) A549 cells were pretreated with various concentrations of odoroside A or MG-132 (20 μM) for 1 h and then incubated with (+) or without (−) TNF-α (2.5 ng/ml) for 6 h in the presence or absence of odoroside A or MG-132. Cell lysates were analyzed by Western blotting using antibodies reactive to ICAM-1, c-FLIP, and β-actin.

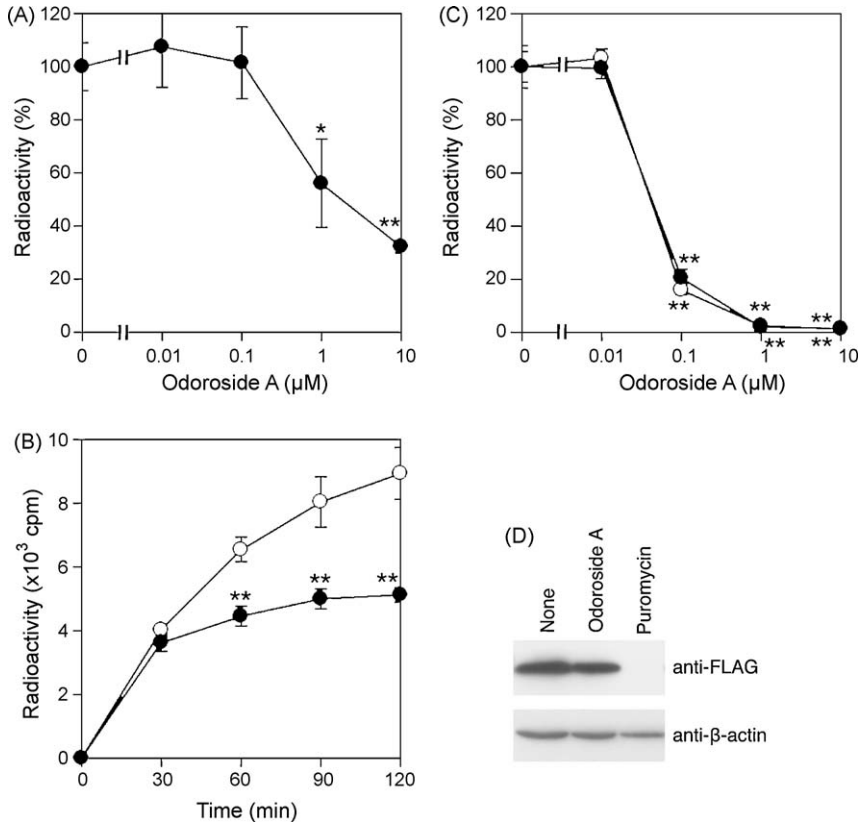

Fig. 4. Odoroside A inhibits *de novo* protein synthesis. (A) A549 cells were pretreated with various concentrations of odoroside A for 1 h and pulsed with [^{3}H]L-leucine for 2 h in the presence of odoroside A. Radioactivity incorporated into acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Control absolute value was 2393 ± 217 cpm. $^*P < 0.05$ and $^{**}P < 0.01$, compared with control. (B) A549 cells were pretreated with odoroside A (10 μM) (filled circles) or not pretreated (open circles) for 1 h and then pulsed with [^{3}H]L-leucine for the indicated times in the presence (filled circles) or absence (open circles) of odoroside A. Radioactivity incorporated into the cells was measured and is shown as means ± S.D. (n = 3). $^*P < 0.05$ and $^{**}P < 0.01$, compared with odoroside-A-treated cells for the same incubation times. (C) A549 cells were pretreated with various concentrations of odoroside A for 1 h, then incubated with (filled circles) or without (open circles) TNF-α (2.5 ng/ml) for 4 h, and pulsed with [^{3}H]L-leucine in the presence of odoroside A. Radioactivity incorporated into acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Absolute values for non-stimulated cells and TNF-α-stimulated cells were 1884 ± 109 and 2060 ± 163 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells. (D) FLAG-tagged human β-actin cDNA was transcribed and translated by rabbit reticulocyte lysates for 90 min in the presence or absence of odoroside A (10 μM) or puromycin (20 μM). The translated products were analyzed by Western blotting using antibodies reactive to FLAG and β-actin.

protein expression in a dose-dependent manner, and the inhibition was almost complete at concentrations higher than 1 μM (Fig. 3B). To generalize the inhibitory effect of odoroside A on the TNF-α-induced protein expression, the expression level of another NF-κB-inducible protein was investigated. The caspase-8 modulator c-FLIP is known to be an NF-κB-inducible protein that renders resistance to TNF-α-induced apoptosis [27,28]. As shown in our earlier paper [29], c-FLIP$_L$ was constitutively expressed in A549 cells and was substantially upregulated upon TNF-α treatment (Fig. 3B). c-FLIP$_L$ expression was also decreased to background levels by odoroside A at concentrations higher than 1 μM (Fig. 3B).

3.4. Odoroside A inhibits de novo protein synthesis

To investigate whether odoroside A affects *de novo* protein synthesis, A549 cells were pretreated with odoroside A for 1 h and then pulsed with [^{3}H]L-leucine for 2 h, followed by measurement of radioactivity incorporated into proteins. Odoroside A inhibited the incorporation of [^{3}H]L-leucine at concentrations of 1–10 μM (Fig. 4A). Similar results were observed with two other human cell lines, cervix adenocarcinoma HeLa cells and fibrosarcoma HT-1080 cells (data not shown), indicating that odoroside A generally inhibits *de novo* protein synthesis at the cell level. The time-course experiment revealed that odoroside A does not affect the initial incorporation of [^{3}H]L-leucine into the cell during 30 min incubation, but potently shut down the successive incorporation of [^{3}H]L-leucine after 60 min incubation (Fig. 4B). These results suggested that odoroside A may indirectly reduce the incorporation of L-leucine into proteins. TNF-α stimulation did not affect the inhibitory effect of odoroside A on protein synthesis (Fig. 4C). To further address if odoroside A directly targets translation machinery, FLAG-tagged β-actin cDNA was used as a template to be transcribed and translated into protein products in the cell-free system based on rabbit reticulocyte lysates. The β-actin band reactive to anti-FLAG antibody was completely reduced by the protein synthesis inhibitor puromycin, while odoroside A was totally ineffective (Fig. 4D).

3.5. Odoroside A inhibits Na$^+$-dependent amino acid transport

The above observation that odoroside A inhibits protein synthesis without affecting the translation machinery suggested the possibility that odoroside A prevents the uptake of amino acids across the plasma membrane. To investigate the effect of odoroside A on amino acid uptake, A549 cells were pretreated with odoroside A for 30 min and then pulsed with several [^{3}H]amino acids for a short period (5 min), followed by measurement of cell-associated radioactivity. The uptake of glycine, L-glutamine, and L-serine into the cell was greatly decreased in the absence of Na$^+$, whereas L-leucine, L-phenylalanine, and L-lysine were incorporated into the cell irrespective of Na$^+$ (Fig. 5A). Under normal ionic conditions, while odoroside A at 100 μM barely affected the uptake of L-

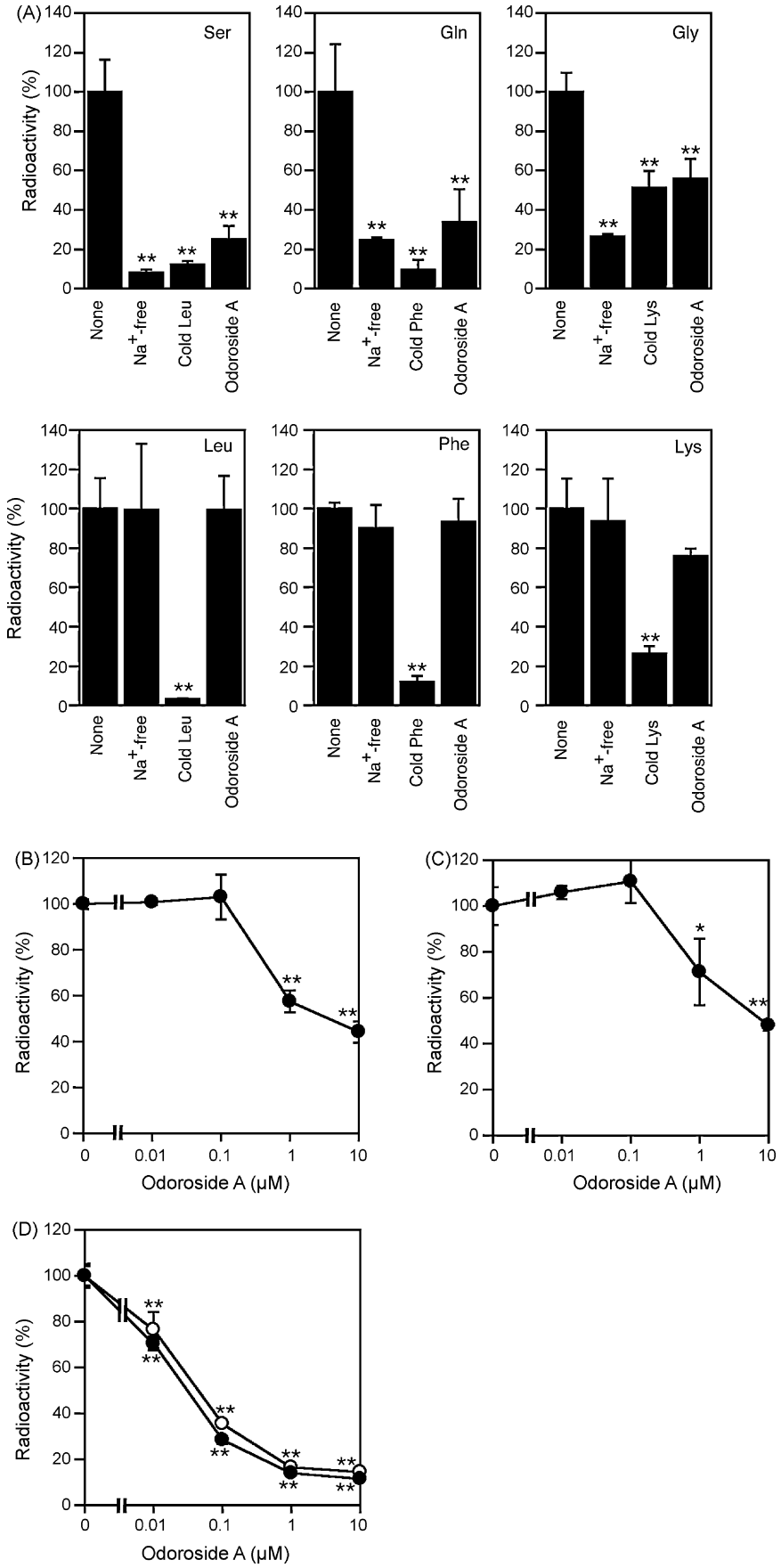

Fig. 5. Odoroside A inhibits Na^+-dependent transport of amino acids. (A) A549 cells were pretreated with or without odoroside A (100 μM) or unlabeled amino acids (100 μM) for 30 min and then pulsed with [^{3}H]labeled amino acids for 5 min in the control incubation buffer or the Na^+-free incubation buffer (Na^+-free). Radioactivity incorporated into the cells was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Control absolute values for L-serine, L-glutamine, glycine, L-leucine, L-phenylalanine, and L-lysine

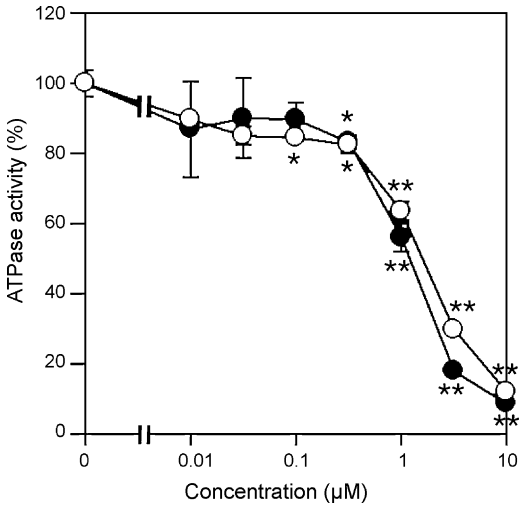

Fig. 6. Odoroside A inhibits Na^+/K^+-ATPase activity. Na^+/K^+-ATPase purified from porcine kidney was preincubated with various concentrations of odoroside A (filled circles) or ouabain (open circles) for 15 min and then incubated with ATP for 15 min in the presence of odoroside A or ouabain. The inorganic phosphates produced by ATP hydrolysis were measured. Data points represent means ± S.D. ($n = 3$). Control absolute value was 5933 ± 217 (nmol/mg/min). $^*P < 0.05$ and $^{**}P < 0.01$, compared with control.

leucine, L-phenylalanine, and L-lysine, odoroside A at the same concentration potently prevented the uptake of glycine, L-glutamine, and L-serine to levels at which A549 cells were treated with excess unlabeled amino acids as competitors (Fig. 5A). Odoroside A also inhibited the uptake of L-serine and L-glutamine in a dose-dependent manner and at concentrations of 1–10 μM (Fig. 5B and C). TNF-α stimulation did not affect the inhibitory effect of odoroside A on the uptake of L-serine (Fig. 5D). Collectively, these data indicate that odoroside A primarily inhibits the Na^+-dependent uptake of amino acids across the plasma membrane.

3.6. Odoroside A is an inhibitor of Na^+/K^+-ATPase

It is known that Na^+/K^+-ATPase pumps Na^+ out and K^+ in via hydrolysis of ATP and plays a major role in maintaining Na^+ and K^+ balance across the plasma membrane [30]. To address if Na^+/K^+-ATPase is a direct target of odoroside A, Na^+/K^+-ATPase purified from porcine kidney was pretreated with odoroside A and incubated in the presence of ATP, followed by measurement of inorganic phosphates as products of hydrolysis. Odoroside A inhibited ATPase activity in a dose-dependent manner at the IC_{50} value of 1.2 μM (Fig. 6). Under the same experimental condition, the specific Na^+/K^+-ATPase inhibitor ouabain inhibited ATPase activity at the IC_{50} value of 1.6 μM (Fig. 6). Thus, these results indicate that odoroside A is able to inhibit Na^+/K^+-ATPase as potently as ouabain.

3.7. Ouabain does not affect TNF-α-induced mRNA expression but inhibits TNF-α-induced protein expression

It has been shown that there is species/tissue variability in response to ouabain, due to different Na^+/K^+-ATPase isoforms [31]. A ^{86}Rb uptake test may be more appropriate to measure Na^+/K^+-ATPase activity directly in intact cells. However, ^{86}Rb is not allowed to use in the Radioisotope Laboratory in our Institute. As an alternative way, ouabain was further employed to address if its inhibitory effects on intact cells resemble those of odoroside A.

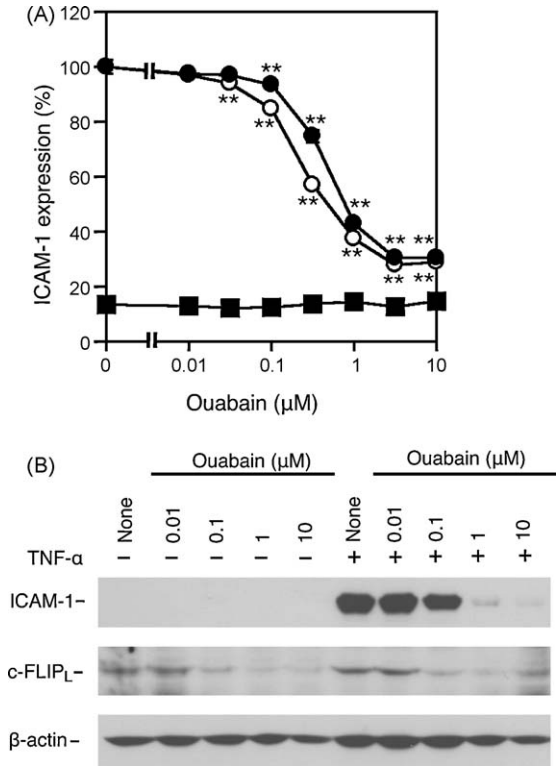

Fig. 7. Ouabain inhibits ICAM-1 and c-FLIP expression induced by TNF-α or IL-1. (A) A549 cells were pretreated with various concentrations of ouabain for 1 h and then incubated with TNF-α (2.5 ng/ml; open circles) or IL-1 (0.25 ng/ml; filled circles) or without cytokines (filled squares) for 6 h in the presence of ouabain. ICAM-1 expression (%) is shown as means ± S.D. ($n = 3$). Absolute values for non-stimulated, TNF-α-stimulated and IL-1-stimulated cells were 0.103 ± 0.009, 0.505 ± 0.011 and 0.548 ± 0.004, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-stimulated, TNF-α-stimulated or IL-1-stimulated cells. (B) A549 cells were pretreated with various concentrations of ouabain for 1 h and then incubated with (+) or without (−) TNF-α (2.5 ng/ml) for 6 h in the presence or absence of ouabain. Cell lysates were analyzed by Western blotting using antibodies reactive to ICAM-1, c-FLIP, and β-actin.

A549 cells were pretreated with ouabain for 1 h and then treated with TNF-α or IL-1 for 6 h. Ouabain inhibited the TNF-α- and IL-1-induced ICAM-1 expression in a dose-dependent manner and at similar concentrations as odoroside A (IC_{50} values of 0.48 μM and 0.78 μM, respectively) (Fig. 7A). Under these conditions, ouabain exerted an inhibitory effect on the cell viability to some extent regardless of TNF-α or IL-1 (supplementary Fig. 1). These results indicate that the inhibition of ICAM-1 expression by ouabain is not caused by nonspecific cytotoxicity as in the case of odoroside A.

Ouabain neither affected the TNF-α-induced phosphorylation of IκBα and its subsequent degradation (supplementary Fig. 2) nor the TNF-α-induced p65 translocation to the nucleus (supplementary Fig. 3). The ICAM-1 mRNA induced by TNF-α stimulation was not reduced by ouabain even at 10 μM (supplementary Fig. 4). However, similar to odoroside A, ouabain inhibited the TNF-α-induced expression of ICAM-1 as well as the constitutive and

were 24 272 ± 3968, 30 875 ± 7513, 2317 ± 227, 47 300 ± 7281, 25 585 ± 794, and 4091 ± 1264 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells. (B and C) A549 cells were pretreated with various concentrations of odoroside A for 30 min and then pulsed with [^{3}H] L-serine (B) or [^{3}H] L-glutamine (C) for 5 min in the control incubation buffer. Radioactivity incorporated into the cells was measured. Radioactivity (%) is shown as means ± S.D. ($n = 3$). Control absolute values for L-serine and L-glutamine were 12 152 ± 142 and 7690 ± 424 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells. (D) A549 cells were pretreated with various concentrations of odoroside A for 1 h and then incubated with (filled circles) or without (open circles) TNF-α (2.5 ng/ml) for 4 h in the presence of odoroside A. The cells were pulsed with [^{3}H] L-serine for 5 min in the control incubation buffer. Radioactivity (%) is shown as means ± S.D. ($n = 3$). Control absolute values for non-stimulated cells and TNF-α-stimulated cells were 9842 ± 522 and 10 828 ± 451 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells.

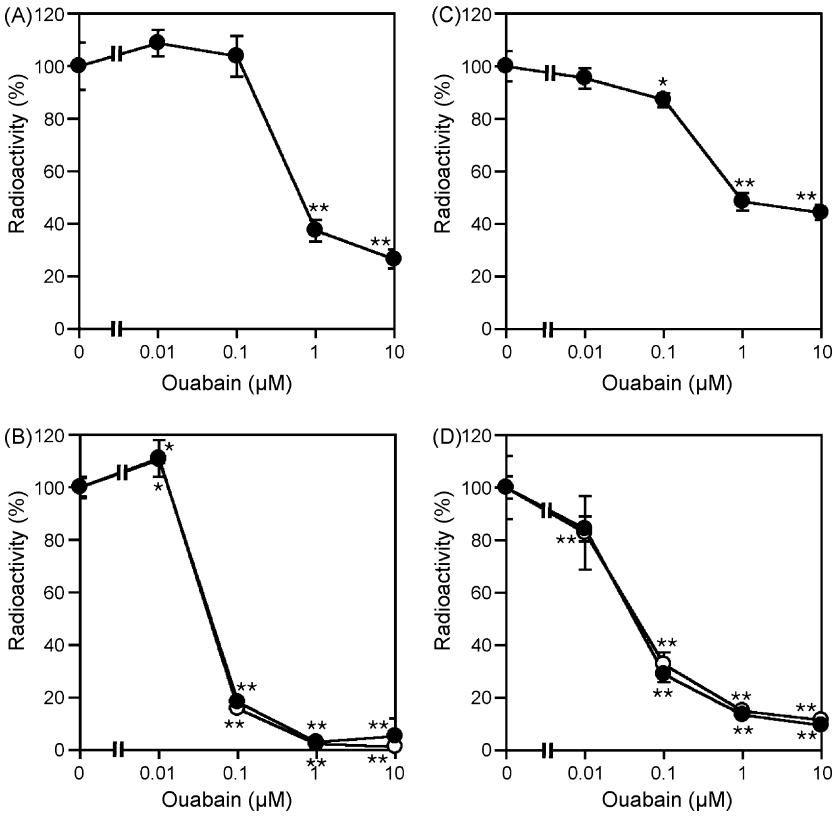

Fig. 8. Ouabain inhibits *de novo* protein synthesis and Na^+-dependent amino acid transport. (A) A549 cells were pretreated with various concentrations of ouabain for 1 h and pulsed with [^{3}H]L-leucine for 2 h in the presence of ouabain. Radioactivity incorporated into acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Control absolute value was 2393 ± 217 cpm. $^*P < 0.05$ and $^{**}P < 0.01$, compared with control. (B) A549 cells were pretreated with various concentrations of ouabain for 1 h and then incubated with (filled circles) or without (open circles) TNF-α (2.5 ng/ml) for 6 h in the presence of ouabain. The cells were pulsed with [^{3}H]L-leucine for the last 2 h incubation. Radioactivity incorporated into acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Absolute values for non-stimulated cells and TNF-α-stimulated cells were 2016 ± 73 and 1968 ± 82 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells. (C) A549 cells were pretreated with various concentrations of ouabain for 1 h and then pulsed with [^{3}H] L-serine for 5 min in the control incubation buffer. Radioactivity incorporated into the cells was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). Control absolute value for L-serine was 36 719 ± 2089 cpm. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells. (D) A549 cells were pretreated with various concentrations of ouabain for 1 h and then incubated with (filled circles) or without (open circles) TNF-α (2.5 ng/ml) for 4 h in the presence of ouabain. The cells were pulsed with [^{3}H] L-serine for 5 min in the control incubation buffer. Radioactivity (%) is shown as means ± S.D. (n = 3). Control absolute values for non-stimulated cells and TNF-α-stimulated cells were 10 769 ± 1290 and 12 025 ± 511 cpm, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, compared with non-treated cells.

TNF-α-induced expression of c-FLIP$_L$ almost completely at concentrations higher than 1 μM (Fig. 7B). These results indicate that ouabain does not affect the TNF-α-induced mRNA expression but inhibits the TNF-α-induced protein expression.

3.8. Ouabain inhibits de novo protein synthesis and Na^+-dependent amino acid transport

A549 cells were pretreated with ouabain for 1 h and then pulsed with [^{3}H]L-leucine for 2 h. Ouabain inhibited the incorporation of [^{3}H]L-leucine into proteins at concentrations higher than 1 μM (Fig. 8A). In addition, A549 cells were treated with ouabain for 1 h, and then pulsed with [^{3}H]L-serine or [^{3}H]L-glutamine for 5 min. Ouabain inhibited the uptake of L-serine and L-glutamine into the cell strongly at concentrations of 1–10 μM (Fig. 8C and supplementary Fig. 5). TNF-α stimulation did not influence the inhibitory effects of ouabain on the incorporation of L-leucine into proteins as well as the uptake of L-serine (Fig. 8B and D). These results indicate that ouabain inhibits *de novo* protein synthesis and the Na^+-dependent amino acid transport in much the same manner as odoroside A.

4. Discussion

Odoroside A and ouabain were found to inhibit cell-surface ICAM-1 expression in response to TNF-α and IL-1 stimulation at comparable concentrations. In this study, the molecular mechanism by which odoroside A and ouabain inhibit TNF-α-induced cell-surface expression of ICAM-1 was characterized. While odoroside A and ouabain did not affect the early NF-κB signaling pathway, they potently blocked TNF-α-induced ICAM-1 expression at the protein level. Consistent with these results, odoroside A and ouabain prevented *de novo* protein synthesis by blocking the Na^+-dependent amino acid transport. As a direct molecular target, odoroside A was found to inhibit the ATPase activity of Na^+/K^+-ATPase as potently as ouabain.

Amino acid transport across the plasma membrane in mammalian cells is catalyzed by a broad range of discrete systems that consist of different gene products and distinct substrate specificities [32]. Na^+/K^+-ATPase serves as a primary active transporter that maintains transmembrane Na^+ and K^+ gradients. Secondary active transporters of amino acids are known to couple amino acid transport to the electrical and chemical gradients initiated by the primary active transport [32]. We have shown that odoroside A profoundly inhibited the uptake of glycine, L-glutamine, and L-serine into the cell but had little influence on the uptake of L-leucine, L-phenylalanine, and L-lysine. As the former three amino acids were transported into A549 cells in an Na^+-dependent manner, our present results indicate that odoroside A blocks the Na^+-dependent amino acid transport system across the plasma membrane. This is further supported by the finding that odoroside A inhibits Na^+/K^+-ATPase at the enzyme level.

Ouabain exerted much the same inhibitory effects as odoroside A at the enzyme level as well as at the cell level. The IC_{50} values of odoroside A and ouabain on Na^+/K^+-ATPase activity were several times higher than those on the TNF-α- and IL-1-induced ICAM-1 expression. The difference of their IC_{50} values between the enzyme level and the cell level may be partly due to that high concentrations of odoroside A and ouabain are required to inhibit Na^+/K^+-ATPase activity in the cell-free system where purified enzymes were concentrated.

While the 1 h pretreatment with odoroside A and ouabain prevented the uptake of L-serine at the IC_{50} values of approximately 1 μM, the 5 h preincubation with these compounds led to much stronger inhibitory effects on the uptake of L-serine. The similar tendency was observed with the incorporation of L-leucine into proteins. Therefore, it seems that the relatively long times of exposure of cells to odoroside A or ouabain are required to inhibit the expression of NF-κB-inducible proteins. As a possible explanation, odoroside A and ouabain may bind and inhibit Na^+/K^+-ATPase in non-depolarized cells slowly. It is also possible that transmembrane Na^+ and K^+ gradients and/or intracellular pools of amino acids are gradually decreasing under conditions where Na^+/K^+-ATPase activity is partially inhibited by odoroside A and ouabain at low concentrations.

Odoroside A and ouabain did not affect the early NF-κB signaling pathway; rather, they inhibited the induction of ICAM-1 expression at the protein level upon TNF-α stimulation. The inhibition of protein synthesis is supported by the finding that odoroside A and ouabain block Na^+-dependent amino acid transport as a result of inhibiting Na^+/K^+-ATPase activity. However, these findings seem to be inconsistent with previous studies showing that oleandrin and digoxin inhibit TNF-α-induced NF-κB activation by blocking signaling events upstream of IκB degradation [17] and by blocking TRADD recruitment to TNF-R1 [18]. It has been also shown that oleandrin inhibits IL-8-, FMLP-, EGF-, or NGF-, but not IL-1- or TNF-α-induced NF-κB activation in macrophages [33]. Therefore, it is speculated that different cell types (A549 cells versus other cell lines) and/or different structures of compounds (odoroside A and ouabain versus other cardenolide glycosides) may determine whether the TNF-α-induced early NF-κB activation or later translation process is primarily influenced or not. This may be in line with the notion that the signal transduction activity of Na^+/K^+-ATPase is mediated in a manner independent of its activity as an ion pump [16].

It has been shown that ouabain activates the NF-κB pathway at low concentrations that only partially inhibit Na^+/K^+-ATPase [19]. Moreover, ouabain (0.1–10 nM) has been reported to protect from serum-deprivation-induced apoptosis and stimulate kidney cell proliferation in an NF-κB-dependent manner [20]. However, in contrast to these studies, ouabain does not seem to induce the NF-κB pathway in A549 cells, since ouabain did not induce the early NF-κB signaling events or the expression of NF-κB-inducible proteins at concentrations of 0.01–10 μM. Although we cannot rule out the possibility that ouabain is able to induce the NF-κB pathway at concentrations less than 10 nM, the ability of ouabain to activate the NF-κB pathway may be cell-type-specific.

Certain protein synthesis inhibitors elicit ribotoxic stress response that induces the activation of the MAP kinase superfamily [34]. We have recently shown that protein synthesis inhibitors such cycloheximide are able to induce ERK and p38 MAP kinase and thereby trigger ectodomain shedding of TNF-R1 in A549 cells, leading to the blockade of both the NF-κB signaling pathway and the apoptosis signaling pathway [29,35,36]. However, unlike these protein synthesis inhibitors, it seems most likely that odoroside A and ouabain do not induce the TNF-R1 shedding, as TNF-α-induced NF-κB activation occurred even in the presence of odoroside A and ouabain.

c-FLIP is expressed in various types of tumor cells, and is a major anti-apoptotic protein that render resistant to death receptor-mediated apoptosis by preventing caspase-8 activation [27,28]. Cycloheximide is frequently used to sensitize cells to TNF-α-induced apoptosis mainly by downregulating c-FLIP [27,28]. Similar to cycloheximide [29], odoroside A and ouabain were able to downregulate the expression of c-FLIP. Therefore, Na^+/K^+-ATPase inhibitors, such as odoroside A, may be more suitable to promote TNF-α-induced apoptosis in that they are unable to elicit ribotoxic stress response and the resultant TNF-R1 shedding. It was recently shown that cardenolide glycosides exert antiproliferative and/or apoptotic effects in various types of cancer cells [16]. In those cases, the ability of such cardenolide glycosides as odoroside A to inhibit protein synthesis by blocking the Na^+-dependent amino acid transport may contribute to their anticancer properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.06.027.

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