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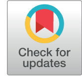
7-Oxygenated cholesterol molecules differentially affect the expression of zonula occludens-1 in vascular smooth muscle cells and monocyte/macrophage cells



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A B S T R A C T

To investigate the effects of 7-oxygenated cholesterol molecules on the expression of tight junction proteins, we examined the outcomes effects of 7-ketocholesterol (7K), 7α-hydroxycholesterol (7αOH-Chol) and 7β-hydroxycholesterol (7βOHChol) on the expression of the tight-junction protein zonula occludens-1 (ZO-1) using vascular cells. Vascular smooth muscle cells (VSMCs) constitutively express ZO-1, and this expression remained unaffected in the presence of cholesterol. However, the level of ZO-1 protein decreased after exposure to 7K and, to a lesser extent, 7αOHChol and 7βOHChol. ZO-1 was translocated to the nucleus following treatment with 7K; this translocation was inhibited by z-VAD-fmk, a pan-caspase inhibitor. ZO-1 protein was found to disintegrate in the aorta of ApoE knockout mice fed a high cholesterol diet, whereas it remained intact in the wild-type control. THP-1 monocyte/macrophage cells, which show no expression of ZO-1, were not influenced by treatment with cholesterol, 7K, and 7βOHChol. However, the treatment of THP-1 cells with 7αOHChol resulted in ZO-1 expression, which largely remained localized on the cytoplasmic membrane. These results indicate the varying effects of 7-oxygenated cholesterol molecules on the expression and localization of ZO-1 depending on cell types, and suggest the contribution of 7-oxygeneted cholesterol molecules to the structural alteration of tight junctions.

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1. Introduction

Zonula occludens (ZO)-1, a peripheral membrane protein of approximately 220 kDa molecular weight, is a member of the membrane-associated guanylate kinase (MAGUK) family which possess several common structural domains of 80–90 amino acids (termed PDZ domains), one SH3 domain, and a domain homologous to yeast guanylate kinase [1]. ZO-1 plays important roles in the function of tight junctions (TJs). The tight junction protein (TJP) controls vascular permeability and is also involved in cell migration, cell differentiation, and cell-cell fusion [1–4]. Under high-glucose conditions, the expression of ZO-1 decreases in the rat glomerular epithelial cells (GECs) [5]. The levels are also found to be down-regulated in breast cancer and during corneal wound repair [6,7]. ZO-1 is found to be stabilized in the confluent Madin-Darby canine kidney (MDCK) cells, with an increase in expression being proportional to the cell density [8,9]. ZO-1 commonly exists in the cytoplasmic membrane and is linked to other TJPs [10]. However, the protein is detected in the nucleus during proliferation [11] and in specific phases of the cell cycle [12]. These findings indicate that expression and localization of ZO-1 can be regulated by environmental factors controlling the growth or differentiation of cells.

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7-Oxygenated cholesterol molecules, such as 7α-hydroxycholesterol (7αOHChol), 7-ketocholesterol (7K) and 7β-hydroxycholesterol (7βOHChol), are a class of abundant oxysterols present in atherosclerotic arteries and in the blood of hypercholesterolemic patients [13,14]. The 7-oxygenated derivatives are endogenous signaling molecules that differently affect cells in terms of cell differentiation and cell growth. 7αOHChol promotes differentiation of monocytic cells into mature dendritic cells (mDCs)-phenotype [15]. 7K induces differentiation of the lens epithelial cells (LECs) into fiber cells [16], and monocytic cells into macrophages/foam cells [17]. 7K also induces migration and proliferation smooth muscle cells (SMCs) [18], as well as cell apoptosis through mitochondrial and nuclear damage [19]. However, it is unknown whether 7-oxygenated cholesterol affects the expression of tight junction proteins.

In this study, we investigated the effects of 7-oxygenated cholesterol molecules on ZO-1 expression using vascular smooth muscle cells (VSMCs) and monocyte/macrophage cells. Our results indicate that depending on cell types, 7-oxygenated cholesterol molecules differentially regulate the expression and localization of ZO-1.

2. Materials and methods

2.1. Cell culture

Human VSMCs and human monocyte/macrophage THP-1 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). VSMCs were grown in Kaighn's modification of Ham's F-12 (F-12K) medium (ATCC) supplemented with 10% fetal bovine serum (FBS), and THP-1 cells were cultured in RPMI1640 supplemented with 10% FBS. Cells from passage 7 to passage 9 were used in the experiments.

2.2. Reagents

Cholesterol and 7K were purchased from Sigma-Aldrich (St. Louis, MO, USA). 7αOHChol and 7βOHChol were purchased from Steraloid (Newport, RI, USA). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk) was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against ZO-1 and heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and α-tubulin antibody was purchased from Calbiochem (La Jolla, CA, USA).

2.3. Western blot analysis

VSMCs or THP-1 cells were lysed with lysis buffer (1% SDS, 1 mM $NaVO_3$, 10 mM Tris-HCl, pH 7.4) containing protease inhibitor cocktail. An equal amount of protein from each sample was separated by SDS-PAGE, following which they were transferred to PVDF membrane. The non-specific binding of primary antibody was blocked using 5% skim milk in 0.1% Tween 20/TBS, after which the membrane was probed with the indicated primary antibodies at 4 °C overnight. Membranes were washed three times with 0.1% Tween 20/TBS for 15 min each, and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Bands were detected with the enhanced chemiluminescence (ECL) Western blotting detection system.

2.4. Isolation of cytoplasmic and nuclear fractions

Cytoplasmic and nuclear fractions were isolated from cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, Il, USA), following the manufacturer's instructions.

2.5. Preparation of murine aortic roots

Animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines, and all protocols were approved by the Pusan National University Animal Care and Use Committee. ApoE knockout and wild-type (C57BL/6) mice were fed a high-fat diet for 12 weeks, as previously reported [20]. The aortic roots isolated from the mice were embedded in OCT compound and snap-frozen in liquid nitrogen. Serial sections 4-μm-thick were fixed on silica-coated glass slides, and stored in deep-freezer until further use for immunohistofluorescent staining.

2.6. Immunofluorescent staining of ZO-1

VSMCs and THP-1 cells cultured as an adherent layer on cover-slips, and the frozen tissue sections were fixed with ice-chilled acetone for 10 min at room temperature and washed in PBS. After incubation with blocking solution (5% skim milk in PBS) for 1 h to reduce non-specific binding, cells and sections were incubated with anti-ZO-1 antibody for 2 h at room temperature, followed by incubation with green fluorescence-conjugated secondary antibody for 1 h in darkness. After washing in PBS, the probed cells and tissues were mounted on slide glasses and visualized by confocal microscopy.

2.7. Flow cytometric analysis

After incubation with cholesterol or 7-oxygenated cholesterol molecules, THP-1 cells were incubated with the ZO-1 antibody for 1 h, followed by incubation with fluorescent dye-conjugated secondary antibody for 40 min at room temperature. Cells were washed with PBS, re-suspended in 1% paraformaldehyde in PBS, and analyzed by flow cytometry.

2.8. Statistical analysis

Statistical analysis (one-way ANOVA) was performed using PRISM version 5.0 (GraphPad software, San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Reduction of ZO-1 expression in VSMCs by 7-oxygenated cholesterol molecules

ZO-1 protein was detected by Western blot analyses after exposing VSMCs to cholesterol, 7K, 7αOHChol, and 7βOHChol. VSMCs constitutively express ZO-1 protein, and we observed a decrease in the levels following treatment with the 7-oxygenated cholesterol derivatives. Of these, 7K most effectively reduced the ZO-1 expression, as indicated by the band densities expressed in a bar-graph (Fig. 1A, low panel). Furthermore, the decrease in the decrease levels was proportionate to the treatment duration, being reduced to 41.3% and 38.4% of control following treatment with 7K for 48 h and 72 h, respectively (Fig. 1B). On examining whether the 7-oxygenated cholesterol molecules affected the ZO-1 transcripts, we found that the level of ZO-1 transcript was not affected by these molecules (Supplementary Fig. 1). And, the cells did not showed the cell toxicity in the treated concentration of 7-oxygenated cholesterol derivatives (Supplementary Fig. 2). These results indicate that 7-oxygenated cholesterol derivatives affect the ZO-1 expression of VSMCs at the protein level.

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