ORP8 Mediates Oxysterol-induced ER Stress And Cell Apoptosis

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Abstract

OSBP-related protein 8 (ORP8) is localized to the endoplasmic reticulum (ER) and nuclear envelope and has been implicated in lipid homeostasis. Oxysterols and cholesterol act as ligands of ORPs [1] and oxysterols inhibit the growth of various cell types [2], but the mechanisms that occurs remain obscure. To gain insight into how oxysterols affect the ER stress and cell apoptosis, we utilized quantitative real-time PCR (qPCR) , cell apoptosis assay analyses ,our results shown that ORP8 overexpression, contrary to ORP8 silencing, mediated apoptosis-induced by 27-oxysterol in HepG2 cells, which coincided with increased ER stress response marker, C/EBP homologous protein (Chop).

Keywords

ORP8, ER stress, HepG2 apoptosis.

1. Introduction

OSBP-related proteins (ORPs) exist in eukaryotes from yeast to human [3, 4]. ORP8 is a member of the ORP family that contains a single C-terminal transmembrane domain targeting the protein to the endoplasmic reticulum (ER). This protein is relatively abundant in tissues with high levels of cholesterol such as brain, liver, kidney, and adipose tissue and has been reported to bind oxysterols and cholesterol [1]. A previous study indicated that ORP8 decreases cholesterol efflux in macrophages by suppressing ABCA1 expression, implying that it may play a role in the development of atherosclerotic lesions [5]. In hepatic cells, ORP8 functions as a negative regulator of intracellular cholesterol [6]. Other roles have been suggested for ORP8 including the inhibition of cell migration through its interaction with nucleoporin Nup62 [7] and the mediation of oxysterol interference of HepG2 cell cycle through its interaction with Astrin/SPAG5 [8].

Increasing data suggests that aberrant ORP expression or function may be associated with malignant tumor growth [9]. Increased expression of ORP3 mRNA has been found in B-cell-associated malignancies [10-13] and testicular cancer [14-16]. The ORP3 gene was identified as one of the common insertion sites of moloney murine leukemia virus in a mouse model of B-cell leukemia [10, 17]. ORP4 was identified as a putative marker for blood dissemination of solid tumors [18], and was reported to be up-regulated in leukocytes of patients with chronic myeloid leukemia [19, 20]. OSBP and ORP4L act as targets for antiproliferative natural products [21], while ORP5 expression was correlated with invasion and poor prognosis of human pancreatic cancer [22]. These observations suggest that ORPs may participate in signaling events that control cell death/survival decisions and proliferation, thereby regulating certain forms of cancer.

In the present study, we show that the expression of ORP8 may mediate oxysterol's cell toxicity and thus regulates cell apoptosis.

2. Materials and methods

2.1 Materials

Rabbit antibodies against human ORP8 were produced and affinity-purified by our lab;

Anti-actin monoclonal antibody was purchased from Proteintech;

Oxysterols and Hoechst 33342 were purchased from Sigma-Aldrich;

siORP8 or control non-targeting siRNA were produced by Invitrogen;

ORP8 cDNA was constructed by our lab;

One Step PrimeScript miRNA cDNA synthesis Kit (Takara Bio);

SYBR Premex EX Taq (Takara Bio);

Lipofectamine 2000 (Invitrogen);

HepG2 cells: cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO2.

2.2 Methods

ORP8 Overexpression. HepG2 cells were seeded on 6-well plates at 80–90% confluency for 24 h and then transfected with ORP8 cDNA or empty plasmid by using Lipofectamine 2000 for 12h, followed by 24h treatment with 15 M of 27-OHC.

RNA interference. HepG2 cells were seeded on 6-well plates at 30–50% confluency for 24 h and then transfected with siORP8 or control non-targeting siRNA (siORP8:GAG UGG UCU UGC AAA UUA UdTdT; siNT:UAG CGA CUA AAC ACA UCA AdTdT) by using Lipofectamine 2000 for 48h, followed by addition of 15 M of 27-OHC for 24 h.

Western blot analysis. Cellular total protein samples were mixed with Laemmli sample buffer, boiled for 10 min, and subjected to SDS-PAGE. Western blot analysis was conducted as described [23];

Cell apoptosis assays. For Hoechst 33342 staining, cells were trypsinized and harvested, then washed with pre-chilled phosphate buffer saline (PBS) three times and exposed to 10μg/ml Hoechst 33342 at room temperature in the dark for 10 min. Samples were imaged under the Nikon (Tokyo,Japan) Eclipse TE2000 fluorescence microscope. Propidium iodide (PI) staining followed by flow cytometry was used to detect cells with a sub-G1 DNA content. Cells transfected with ORP8 cDNA or empty vector were trypsinized, washed twice with PBS and fixed in 70% ethanol at a density of 0.5-1 x 106 cells/ml, resuspended in PI staining solution containing 50 μg/ml PI and incubated at room temperature for 30 min. Fluorescence was measured on a Becton-Dickinson (LOCATION OF COMPANY) FACSCalibur. Cells with sub-G1 DNA content were quantified using Cell QUEST software [23].

Quantitative real-time PCR. Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions. For mRNA quantitative PCR, RNA samples were reverse transcribed using random hexamer primers in the presence of RNase inhibitor (Takara Bio, Shiga, Japan). For miRNA quantitative PCR, RNA samples were directly reverse transcribed using the One Step PrimeScript miRNA cDNA synthesis Kit. qRT-PCR was performed with SYBR Premex EX Taq using the 7300 Sequence Detection System (Applied Biosystems, Beijing Silver Tower #2 DongSanHuan North Road, Invitrogen China Limited/Applied Biosystems China Limited). Relative quantification analysis was performed using the ΔΔCt method, with GAPDH and RUN48 as endogenous controls. Relative gene expression was presented as a ratio of target gene to reference control.

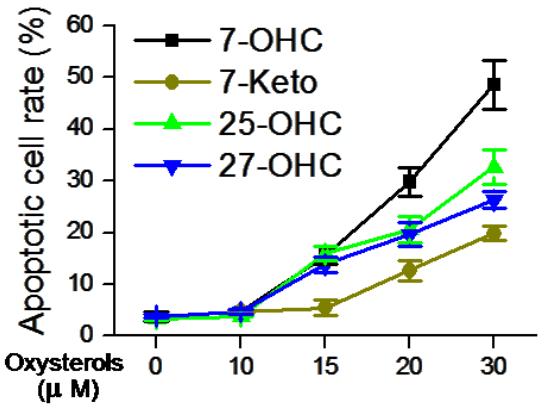

Fig. 1 Determination of apoptotic cell rate in the presence of various oxysterols

3. Results

3.1 The effect of oxysterols on the apoptosis of HepG2 cells

HepG2 cells were incubated with various oxysterols, resulting in the induction of apoptosis with increasing oxysterol concentrations.

3.2 ORP8 overexpression and ORP8 knockdown in HepG2 cells

Western blot assay indicated that both of ORP8 overexpression efficiency and ORP8 knockdown efficiency were over 100%.

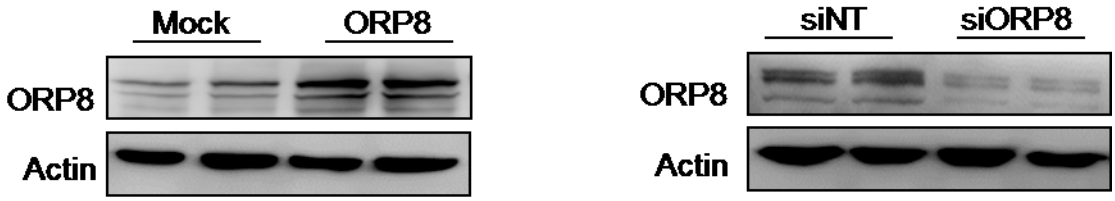

Fig. 2 Detection of transfection efficiency

3.3 Effects of ORP8 on Chop mRNA expression in HepG2 cells

The results of qPCR showed that Chop mRNA expression was potentiated by overexpression of ORP8 and inhibited by ORP8 silencing in HepG2 cells in the presence or absence of 27-OHC.

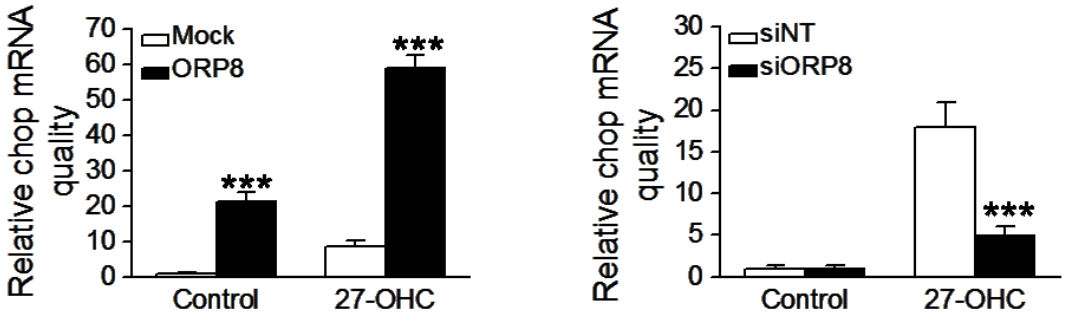

Fig. 3 Determination of Chop mRNA expression

3.4 Effects of ORP8 on apoptotic cell rate in HepG2 cells

Cell apoptosis array analysis indicated that apoptosis induced by 27-OHC was potentiated by overexpression of ORP8 and inhibited by ORP8 silencing in HepG2 cells.

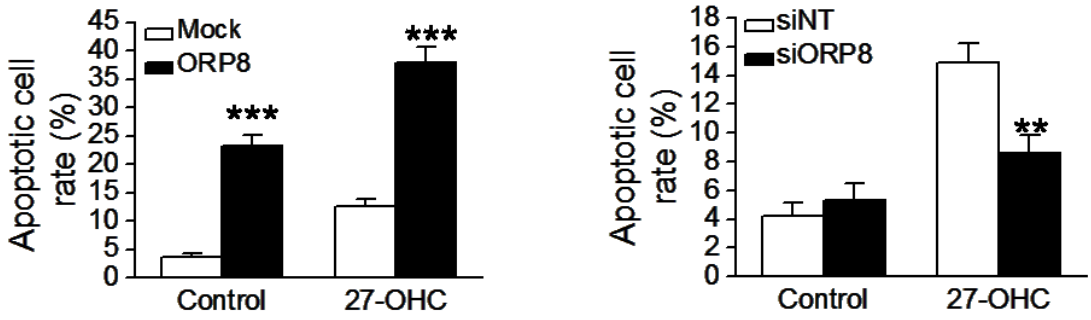

Fig. 4 Evaluation of apoptotic cell rate

4. Conclusion

These results offer primary clues that ORP8 mediates apoptosis-induced by oxysterol in HepG2 via a possible ER-stress response and may thereby serve as a novel target to understand the toxicity of oxysterols.

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