7-Ketocholesterol Induces Endoplasmic Reticulum Stress in HT-29 Cells

Won-Hyo Lee[a], Choong Sik Lee[a], Kisang Kwon[b], Young-Sook Kwon[b], Seung-Whan Kim[c], Tae-Won Goo[d], and O-Yu Kwon[b,*]

[a] Department of Pathology, Chungnam National University, College of Medicine, Taejon 301–747, Korea
[b] Department of Anatomy, Chungnam National University, College of Medicine, Taejon 301–747, Korea. E-mail: lwhmd@naver.com
[c] Department of Emergency, Chungnam National University Hospital, Taejon 301–721, Korea
[d] Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon 441–100, Korea

* Author for correspondence and reprint requests

Z. Naturforsch. **64 c**, 307–310 (2009);
received October 7/November 20, 2008

7-Ketocholesterol (7-Kchol, oxidized cholesterol) is an important mediator of cell death in atherosclerosis mediated by up-regulated Nox 4 gene expression. In the current study using the human colon cancer HT-29 cell line, we have demonstrated that 7-Kchol promotes endoplasmic reticulum (ER) stress via gene up-regulation of ER chaperone and membrane kinases.

Key words: 7-Ketocholesterol, Endoplasmic Reticulum Stress, Human Colon Cancer HT-29 Cell Line

Introduction

Oxidized low-density lipoprotein (LDL) is known as a trigger of an apoptotic process which represents a major cause of plaque growth and rupture in vascular smooth muscle cells. Accumulation of this oxidized LDL induces the production of a lot of ROS (reactive oxygen species) that are one of the causes of inflammatory diseases, including atherosclerosis, diabetes, and hypertension where an oxidized cholesterol, 7-ketocholesterol (7-Kchol), is frequently detected at a high level. 7-Kchol triggers enhanced gene expression of Nox 4 and ROS overproduction, and induces endoplasmic reticulum (ER) stress by changing the ER lumen conditions, which finally lead to apoptosis (Pedruzzi *et al*., 2004; Chen *et al*., 2008).

ER stress mediates multiple molecular biological processes, called unfolded protein responses (UPRs), via ER membrane kinases [inositol-requiring enzyme 1 (IRE1), protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6)], which participate directly or indirectly in the UPRs of mammalian cells. The ER stress response in mammalian cells is triggered by the dissociation of immunoglobulin heavy-chain binding protein (Bip) from stress transducers, such as PERK, ATF6, and IRE1. Bip binds to ER luminal un-/misfolded proteins, which induces augmentation of the ER stress response. While activation (autophosphorylation and dimerization) of IRE1 activates the endonuclease domains, that cleave the X-box DNA-binding protein (XBP) mRNA, and generates an activated form of XBP1 by removing 23 nucleotides of the *Pst* I restriction enzyme site, activation of PERK results in phosphorylation of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) and inhibits translation initiation (Yoshida, 2007). ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, causing nuclear translocation of the *N*-terminal cytoplasmic domain, which contains the DNA-binding, dimerization, and transactivation domains, and subsequent binding to both ER stressresponse element (ERSE) and ATF6 sites to enhance ER molecular chaperone genes. UPR is rapidly sensitive to environmental or physical changes and associated with apoptosis (Schröder, 2008; Ron and Walter, 2007).

We investigated the expression patterns of the ER stress sensors at the cellular level that are induced by 7-Kchol against colon cancer HT-29 cells through the ER signaling pathway. Our data demonstrated that 7-Kchol up-regulates the ER chaperone, Bip, through ER membrane kinases. Therefore, control of ER chaperones by 7-Kchol, one of the oxidized cholesterols, may provide new opportunities at the molecular level for preventing and treating atherosclerosis.

Material and Methods

Human colon cancer HT-29 cells were routinely cultured in RPMI 1640 medium (McCoy's 5A modified media; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) on collagen-coated dishes in a humidified 5% CO_2 atmosphere at 37 °C. Total RNA was extracted using an SV Total RNA isolation system

(Promega, Madison, WI, USA). RT-PCR was performed using the forward primer (F) 5'-ACCACCAGTCCATCGCCATT-3' and reverse primer (R) 5'-CCACCCTGGACGGAAGTTTG-3' for IRE1; F 5'-AGTGGTGGCCACTAATGGAG-3' and R 5'-TCTTTTGTCAGGGGTCGTTC-3' for Bip; F 5'-CTAGGCCTGGAGGCCAGGTT-3' and R 5'-ACCCTGGAGTATGCGGGTTT-3' for ATF6; F 5'-ATCGAGTTCACCGAGCAGAC-3' and R 5'-TCACAGCTTTCTGGTCATCG-3' for PDI; F 5'-GGTCTGGTTCCTTGGTTTCA-3' and R 5'-TTCGCTGGCTGTGTAACTTG-3' for PERK; F 5'-ACATCAAATGGGGTGATGCT-3' and R 5'-AGGAGACAACCTGGTCCTCA-3' for GAPDH; F 5'-AAACAGAGTAGCAGCTCAGACTGC-3' and R 5'-TCCTTCTGGGTAGACCTCTGGGAG-3' for XBP1; and F 5'-CTCAGCGGAATCAATCAGCTGTG-3' and R 5'-AGAGGAACACGACAATCAGCCTTAG-3' for Nox 4. RT-PCR primers were supplied by Bioneer Co. (Taejon, Korea). Unless otherwise noted, all other chemicals were purchased from Sigma (St. Louis, MO, USA). The RT-PCR conditions were as follows: 30 cycles at 94 °C for 30 s; 58 °C for 30 s; and 72 °C for 1 min (but 10 min in the final cycle) using both above mentioned primers with *Taq* DNA polymerase. Immunoblotting analysis was performed according to the standard procedure. HT-29 cells were scraped, lysed by addition of SDS sample buffer [62.5 mM hydroxymethyl aminomethane-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125 mM DTT, and 0.03% (w/v) bromophenol blue], and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with the primary antibodies overnight at 4 °C. The blots were developed using an enhanced chemiluminescence Western blotting detection system kit (Amersham, Uppsala, Sweden). Rabbit anti-eIF2α antibody, eIF2α-P antibody, and goat anti-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-ATF6 antibody was obtained from Imgenex (San Diego, CA, USA).

Results and Discussion

Accumulation of un-/misfolded proteins in the ER lumen triggers an ER stress signal pathway through ER membrane stress transducers (IRE1, PERK, and ATF6) and an ER lumen regulator (Bip). Releasing Bip from the ER luminal stress-sensing domain of IRE1 induces autophosphorylation and dimerization of IRE1, which activates endonuclease domains that can cleave XBP1 mRNA and generate an activated form of XBP1 protein, finally binding to ERSE in the nucleus. PERK is also autophosphorylated and dimerized by releasing Bip, and then blocks total translation by phosphorylation of eIF2α. ATF6 consists of a *C*-terminal ER luminal stress-sensing domain and a cytosolic *N*-terminal basic leucine zipper (b-ZIP) domain, which is cleaved via S1P and S2P proteases, and translocated into the nucleus to bind to both ERSE and ATF6. We tested whether 7-Kchol stimulates ER stress signaling via IRE1, PERK, ATF6, and ER chaperones (Schröder and Kaufman, 2005).

The Ogier-Denis (Pedruzzi *et al*., 2004) group demonstrated that 7-Kchol induces early triggering of ER stress in human aortic smooth muscle cells, which is mediated by up-regulation of Nox 4 and ROS. As shown in Fig. 1A, firstly the expression of Nox 4 was checked by treatment of HT-29 cells with 7-Kchol (40 μg/ml), and the resulting expression was also up-regulated by 7-Kchol. Next, we tested the expression of the ER chaperone (Bip) and the ER signal associated membrane kinases (ATF6, IRE1, and PERK). Although the expression of Bip increased 7-Kchol-dependent, slightly increased mRNA levels of ATF6, IRE1, and PERK were detected. The results showed that 7-Kchol triggers the overproduction of Nox 4 and induces ER stress in HT-29 cells.

The first response to ER stress involves up-expression of the genes encoding ER chaperones, including Bip and GRP94, which increase the protein-folding activity and prevent protein aggregation. The second response to ER stress consists of three distinct ER membrane kinases that are downstream components of ER chaperones, and transmit stress signals from the ER to the nucleus in response to perturbation of protein folding in the ER. We checked the expression of ER membrane kinases.

AFT6 is constitutively expressed as a 90-kDa protein. ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, leading to nuclear translocation of the *N*-terminal cytoplasmic domain, which contains the DNA-binding, dimerization, and transactivation domains. There, the *N*-terminus binds to both ERSE and ATF6 to enhance ER molecular chaperone genes. 7-Kchol also enhanced ATF6 protein expression, and an

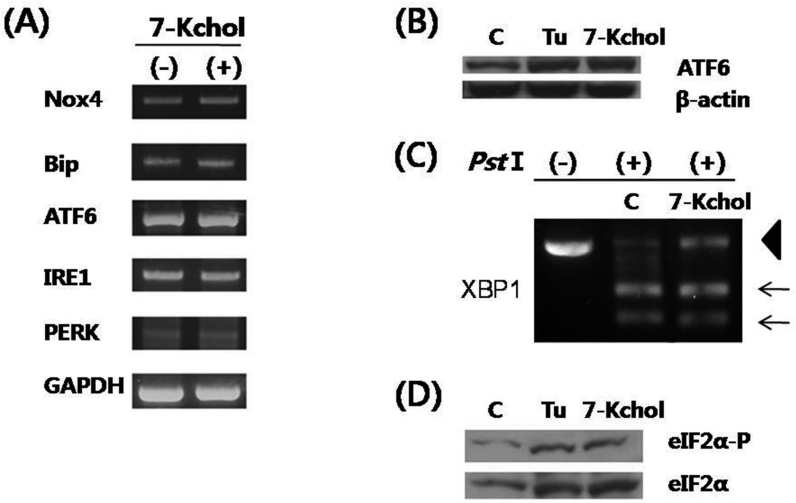

Fig. 1. The results of 7-Kchol treatment in the ER signal pathway. (A) 7-Kchol stimulated Nox 4 expression, and ER chaperone and membrane stress transducer gene expression in HT-29 cells treated with 7-Kchol. HT-29 cells were incubated with 0 or 40 μg 7-Kchol/ml for 16 h. All mRNA levels were measured by RT-PCR. (B) Immunoblotting analysis of ATF6. Cells were treated with 7-Kchol (40 μg/ml) or tunicamycin (Tu, 2 μg/ml) for 16 h. Cells without treatment, as a control, are indicated by C. Cell lysates were subjected to Western blotting with mouse anti-ATF6 monoclonal antibody. (C) XBP1 cDNA after *Pst* I digestion. HT-29 cells were treated with 7-Kchol (40 μg/ml) for 16 h and RT-PCR was performed. This fragment was further digested by *Pst* I to reveal a restriction site that was lost upon splicing of XBP1 by ER stress. The resulting XBP1 cDNA products were revealed on a 2% agarose gel. Unspliced XBP1 mRNA produced the two lower bands indicated by arrows (upper 290 bp and lower 183 bp), whereas spliced XBP1 mRNA have one 450 bp band (the arrow head). (D) Effects of 7-Kchol on phosphorylation of eIF2α protein. HT-29 cells were treated with 7-Kchol (40 μg/ml) and tunicamycin (Tu, 2 μg/ml) for 16 h. Cells were washed with PBS, scraped with a rubber policeman, and lysed in 100 μl of SDS sample buffer. After boiling for 5 min, 5 μl aliquots of each sample were subjected to SDS-PAGE (10% gel) and analyzed by immunoblotting with anti-phosphorylated-eIF2α antibody (eIF2α-P) and anti-eIF2α antibody (eIF2α). Although the experiments were performed in triplicate, only a representative blot is shown in this figure.

almost equal level of expression was detected by the ER-inducible drug tunicamycin (Tu, *N*-glycosylation inhibitor) (Fig. 1B). To confirm the induction of IRE1 gene expression by 7-Kchol, XBP1 mRNA splicing (the proximal step of IRE1) was tested by a RT-PCR assay. 7-Kchol treatment stimulated the XBP1 mRNA-splicing activity, indicating that IRE1 kinase activity triggers the attached RNase activity to produce spliced XBP1 mRNA; the resulting cDNA fragment is indicated by the arrow in Fig. 1C. Tunicamycin enhanced the phosphorylation of the eIF2α protein through PERK dimerization. 7-Kchol increased the expression and the phosphorylation of eIF2α (phosphorylation is the proximal step of PERK activation) (Fig. 1D).

In summary, we showed that 7-Kchol increased the expression of an ER chaperone and ER membrane stress transducers (IRE1, PERK, and ATF6) via typical UPRs in HT-29 cells. In addition, 7-Kchol enhanced the gene expression of Nox 4, which is one of the important factors of atherosclerosis induction. Thus, our findings suggest that 7-Kchol, an oxidized cholesterol, can trigger ER stress, which may provide new insight into the possible role of 7-Kchol in ER stress, and may help to develop novel drugs for atherosclerosis associated with ER stress.

Acknowledgement

This work was supported by a grant (Code # 20070401034024) from the BioGreen 21 Program, Rural Development Administration, Korea.

Chen K., Kirber M. T., Xiao H., Yang Y., and Keaney Jr. J. F. (2008), Regulation of ROS signal transduction by NADPH oxidase 4 localization. J. Cell Biol. **181**, 1129–1139.

Pedruzzi E., Guichard C., Ollivier V., Driss F., Fay M., Prunet C., Marie J. C., Pouzet C., Samadi M., Elbim C., O'dowd Y., Bens M., Vandewalle A., Gougerot-Pocidalo M. A., Lizard G., and Ogier-Denis E. (2004), NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. Mol. Cell. Biol. **24**, 10703–10717.

Ron D. and Walter P. (2007), Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. **8**, 519–529.

Schröder M. (2008), Endoplasmic reticulum stress responses. Cell. Mol. Life Sci. **65**, 862–894.

Schröder M. and Kaufman R. J. (2005), The mammalian unfolded protein response. Annu. Rev. Biochem. **74**, 739–789.

Yoshida H. (2007), Unconventional splicing of XBP-1 mRNA in the unfolded protein response. Antioxid. Redox Signal. **9**, 2323–2333.