24(S)-Hydroxycholesterol induces RIPK1-dependent but MLKL-independent cell death in the absence of caspase-8

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Abstract

24(S)-hydroxycholesterol (24S-OHC), which is enzymatically produced in the brain, is known to play an important role in maintaining brain cholesterol homeostasis. We have previously reported that 24S-OHC induces a type of non-apoptotic programmed necrosis in neuronal cells expressing little caspase-8. Necroptosis has been characterized as a type of programmed necrosis in which activation of receptor-interacting protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like (MLKL) is involved in the signaling pathway. In the present study, we investigated the involvement of these three proteins in 24S-OHC-induced cell death. We found that RIPK1 but neither RIPK3 nor MLKL was expressed in human neuroblastoma SH-SY5Y cells, while all three proteins were expressed in human T lymphoma caspase-8-deficient Jurkat (Jurkat^{Cas8^{-/-}}) cells. In Jurkat^{Cas8^{-/-}} cells, tumor necrosis factor α (TNF α)-induced cell death was significantly suppressed by treatment with respective inhibitors of RIPK1, RIPK3, and MLKL. In contrast, only RIPK1 inhibitor showed significant suppression of 24S-OHC-induced cell death, and even this was less prominent than was observed in TNF α -induced cell death. In Jurkat^{Cas8^{-/-}} cells, knockdown of either RIPK1 or RIPK3 caused moderate but significant suppression of 24S-OHC-induced cell death, but no such effect was observed as a result of knockdown of MLKL. Collectively, these results suggest that, for both SH-SY5Y cells and Jurkat^{Cas8^{-/-}} cells, 24S-OHC-induced cell death is dependent on RIPK1 but not on MLKL. We therefore conclude that, in the absence of caspase-8 activity, 24S-OHC induces a necroptosis-like cell death which is RIPK1-dependent but MLKL-independent.

Keywords: 24S-hydroxycholesterol, cell death, necroptosis, receptor-interacting protein kinase 1, mixed lineage kinase domain-like

Introduction

The brain is the most cholesterol-rich organ and contains about 25% of the total amount cholesterol in the human body [1]. Brain cholesterol is locally synthesized and its metabolism is separated from the peripheral circulation by the blood-brain barrier. A large amount of the excess cholesterol is converted into 24(S)-hydroxycholesterol (24S-OHC) by cholesterol 24-hydroxylase (CYP46A1) [2]. 24S-OHC can cross the blood-brain barrier and enter the peripheral circulation and be converted into bile acids in the liver, like other side-chain-oxidized cholesterol [3]. It has been reported that free 24S-OHC is present at concentrations of up to 30 μ M in human brain homogenates [4]. Net efflux of 24S-OHC across the blood-brain barrier and into the peripheral circulation has been estimated to be about 6 mg/24 h and net uptake of 24S-OHC by the liver has been estimated to be about 7 mg/24 h [4].

While 24S-OHC is an important physiological molecule for maintaining brain cholesterol homeostasis, recent evidence has shown that 24S-OHC possesses a potent neurotoxicity which may contribute to the development of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [5-7]. The levels of 24S-OHC in the plasma and in the cerebrospinal fluid have been reported to increase significantly in AD and in PD patients [8, 9]. Selective expression of CYP46A1 around neuritic plaques has also been reported [10]. However, some other studies have shown that 24S-OHC levels were diminished in the brains of AD patients [5]. The difference in the stages of disease may account for the conflicting results. Furthermore, we have recently reported that high concentrations of 24S-OHC elicit caspase-independent programmed cell death in human neuroblastoma SH-SY5Y cells and in rat primary cortical neuronal cells [11]. We demonstrated that necroptosis, a form of programmed necrosis [12], may account for 24S-OHC-induced cell death. We

further showed that acyl-CoA:cholesterol acyltransferase 1 (ACAT1) esterified 24S-OHC and that lipid droplet-like structures were formed at an early stage in 24S-OHC-treated cells [13]. Since selective ACAT1 inhibitor or ACAT1 siRNA inhibited 24S-OHC-induced cell death, we postulated that ACAT1-mediated esterification of 24S-OHC followed by formation of lipid droplets might be an initial key event in 24S-OHC-induced cell death; however, the molecular mechanisms responsible for execution of cell death remained unclear.

The idea that cell death could occur by a process called necroptosis, defined as programmed necrosis, shattered the traditional thinking by which cell death had been classified as either apoptosis or necrosis [12]. Unlike apoptosis, in which the caspases play a central role in the cell death machinery, necroptosis occurs through a caspase-independent cell death mechanism. Tumor necrosis factor α (TNF α) has been used to study the mechanism of necroptosis [14]. Binding of TNF α to TNF receptor promotes assembly of membrane-associated complex I, which activates canonical nuclear factor- κ B (NF- κ B) and facilitates cell survival [15]. When the NF- κ B pathway is blocked by suppression of cellular inhibitor of apoptosis protein (cIAP)-1/2 with cIAP antagonist (smac mimetic), caspase-8-dependent apoptosis is induced [16, 17]. When caspase-8 is inhibited, the cell death pathway is switched from apoptosis to necroptosis, this necroptosis being such that kinase activity of receptor-interacting protein kinase 1 (RIPK1) is activated, leading to formation of a necrosome complex comprising RIPK1 and RIPK3 [18-20]. Within the complex, these interact with each other through their RIP homotypic interaction motifs (RHIMs) [21]. The phosphorylated RIPK1 phosphorylates RIPK3, and then the phosphorylated RIPK3 phosphorylates mixed lineage kinase domain-like (MLKL) protein [22, 23]. The phosphorylated MLKL translocates from cytosol to the plasma membrane, where it

directly disrupts plasma membrane integrity, resulting in induction of necrotic cell death [24, 25]. Thus, the three proteins, RIPK1, RIPK3, and MLKL, as well as the activation of these by phosphorylation, are considered to play key roles in necroptosis signaling [23, 25, 26].

We showed in a previous study that 24S-OHC induced RIPK1-dependent cell death in neuronal cells. We found that caspase-8 expression was at undetectable levels in the neuronal cells used in our study, which we understood to be the reason why 24S-OHC was observed to induce necroptosis not apoptosis [11]. We also found that 24S-OHC induced apoptosis in human T lymphoma Jurkat cells which endogenously expressed caspase-8, and that 24S-OHC induced RIPK1-dependent non-apoptotic cell death in the presence of pan-caspase inhibitor [13].

In the present study, we investigate the mechanism of non-apoptotic cell death, for which purpose we use caspase-8-deficient Jurkat (Jurkat^{Cas8^{-/-}}) cells and we focus especially on the pathway downstream of RIPK1. We examine whether RIPK3 and MLKL are involved in the 24S-OHC-induced cell death machinery, and we show that 24S-OHC induces RIPK1-dependent but MLKL-independent cell death. We further show with respect to RIPK3 that 24S-OHC induces RIPK3-independent cell death in SH-SY5Y cells, and that 24S-OHC induces RIPK3 protein-dependent but RIPK3 kinase activity-independent cell death in Jurkat cells. We conclude that caspase-8-independent cell death induced by 24S-OHC may be a novel necroptosis-like type of cell death.

Experimental

Materials. 24S-OHC was obtained from Enzo Life Sciences (Farmingdale, NY, USA) or kindly provided by Kowa Company, Ltd (Aichi, Japan), this having been dissolved

in ethanol (Wako, Osaka, Japan) and stored at -20°C. Ethanol was used as a vehicle for control treatments. Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham's F-12 (DMEM/F-12) was obtained from Life Technologies (Carlsbad, CA, USA). RPMI-1640 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was from Hyclone (Thermo Scientific, Logan, UT, USA). Human TNF α was purchased from Peprotech (Rocky Hill, NJ, USA) and was dissolved in distilled water. Birinapant (smac mimetic) was from ChemieTek (Indianapolis, IN, USA). Necrostatin-1 (Nec-1) was from Sigma-Aldrich. Necrosulfonamide (NSA) and GSK'872 were from Merck Millipore (Darmstadt, Germany). Smac mimetic, Nec-1, NSA and GSK'872 were dissolved in dimethyl sulfoxide (DMSO, Wako). The following antibodies were from commercial sources: anti-RIPK1 (Cat# 610459), BD Biosciences (San Jose, CA, USA); anti-RIPK3 (Cat# ab72106), Abcam (Cambridge, MA, USA), anti-MLKL (Cat# M6697), and anti- β -actin (Cat# A5441), Sigma-Aldrich. All other chemicals, of analytical grade, were obtained from Sigma-Aldrich or Wako.

Cell lines and cell culture. SH-SY5Y human neuroblastoma cells, Jurkat human T lymphoma cells, caspase-8-deficient Jurkat cells (clone I 9.2, Jurkat^{Cas8^{-/-}}), and HT-29 human colorectal adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were routinely maintained in DMEM/F-12 (SH-SY5Y, HT-29) or RPMI-1640 (Jurkat, Jurkat^{Cas8^{-/-}}) medium containing 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin; Life Technologies). For HT-29 cells, we added 2 mM L-Glutamine (Life Technologies). Cells were grown at 37°C under an atmosphere of 95% air and 5% CO₂.

Cell treatment. For treatment, Jurkat^{Cas8^{-/-}} cells were seeded at a density of 3.0×10^5 cells/ml. To examine the toxicity of TNF α in the presence of smac mimetic or 24S-OHC in Jurkat^{Cas8^{-/-}} cells, cells were treated with 20 ng/ml TNF α and 100 nM smac mimetic or 50 μ M 24S-OHC, respectively, for the indicated periods. Control cells were treated with DMSO or ethanol, respectively. To evaluate the effects of Nec-1, GSK'872, or NSA, the cells were pretreated with 100 μ M Nec-1, 10 μ M GSK'872, or 1 μ M NSA for 1 h before further treatment.

RNA isolation and real-time PCR. Total RNA isolation and real-time PCR were conducted as described previously [27]. Briefly, total RNA was isolated by using Tripure Isolation Reagent (Roche Applied Science, Mannheim, Germany) along with chloroform (Wako) according to the manufacturer's instructions. Synthesis of cDNA was carried out using random hexamer and ReverTra Ace (Toyobo, Osaka, Japan). The quantitative detection of differentially expressed genes was performed with the 7900HT Fast Real Time PCR system (Applied Biosystems) and SYBR green master mix (Life technologies) using the following condition: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Human *RPL32* (ribosomal protein L32) was used for the normalization of each reaction. The following primers were used (Life technologies): human *RIPK1* forward, 5'-TCTGAGGCAGCCAACTTTCTG-3'; reverse, 5'-GGCTACGGCAGCTGAAGTG-3'; human *RIPK3* forward, 5'-CGTGTACGAAGCAGTGTGCAA-3'; reverse, 5'-GGGCAGCTCAGCCAATGA-3'; and human *MLKL* forward, 5'-CCCCTTGTGAAGCCCAAGA-3'; reverse, 5'-TGACTGGTGCCGATGAAC-3'; human *RPL32* forward, CCCCTTGTGAAGCCCAAGA; reverse

TGACTGGTGCCGGATGAAC.

Preparation of cellular samples and western blot analysis. A whole cell extract was prepared as described previously [13]. Briefly, cells were suspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP-40, 1% deoxycholate, 0.1% SDS) containing PhosSTOP (Roche Applied Science) and a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) at 4°C for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 12,000 x g for 15 min. Protein samples were subjected to SDS-PAGE and were transferred to a PVDF membrane for 1 h at 100 V. Immunoblotting was visualized with enhanced chemiluminescence (Millipore, Billerica, MA, USA).

Determination of cell viability. For determination of cell viability of Jurkat^{Cas8^{-/-}} cells, WST-8 assay, lactate dehydrogenase (LDH) assay, and flow cytometry analysis were used. WST-8 assay was performed using Cell Counting Kit-8 according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). LDH activity assay was performed as described previously [28]. Data are expressed as percentage of total LDH activity, after subtraction of background determined from the culture medium alone. Flow cytometry using propidium iodide staining was conducted as described previously [11]. Briefly, control and treated cells were stained with 5 µl of 250 µg/ml propidium iodide (PI), followed by analysis using a BD FACS Aria™ II cell sorter (Becton, Dickinson and Company, Franklin Lakes, NJ USA) with a 488 nm argon laser.

Knockdown of RIPK1, RIPK3 or MLKL by small interfering Small interfering RNAs (siRNA) targeting human *RIPK1* RNA. (5'-AUCAAUCUGAGACUGUGUGAAGCCC-3') and *RIPK3* (5'-GCAGUUGUAUAUGUUAACGAGCGGUCG-3') were obtained from Life Technologies and Hokkaido System Science (Sapporo, Japan) respectively as described previously [11, 13]. siRNA targeting human *MLKL* (5'-CAAACUCCUGGUAACUCA-3') [23] was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Stealth RNAi negative control (NC) with medium GC content (48 %) (the sequence was not available) was obtained from Life technologies. Jurkat^{Cas8^{-/-}} cells were transfected with siRNA specific for RIPK1, RIPK3, or MLKL for 72 h by using a Neon Transfection System according to the manufacturer's instructions (Life Technologies). To confirm knockdown efficiency for RIPK1 and MLKL, expression levels of mRNA and protein were analyzed by real-time PCR and western blot analysis.

Statistical analysis. Data are reported as mean $\pm$ S.D. of at least three independent experiments unless otherwise indicated. The statistical significance of the difference between the determinations was calculated by analysis of variance using ANOVA, Tukey-Kramer multiple comparisons test. The difference was considered significant when the P value was < 0.05 .

Results

1. The different levels of expression of RIPK1, RIPK3, and MLKL in various cell lines

To elucidate the mechanism of 24S-OHC-induced cell death, we first examined the expression levels of RIPK1, RIPK3, and MLKL in SH-SY5Y cells, wild-type Jurkat cells, and caspase-8-deficient Jurkat (Jurkat^{Cas8^{-/-}}) cells. Human colon adenocarcinoma (HT-29) cells were used as positive control because these have been utilized in studies of the mechanism of necroptosis [23, 29, 30]. Real-time PCR measurement showed that *RIPK1* mRNA was highly expressed in all examined cells (Figure 1A). In contrast, *RIPK3* mRNA was barely expressed in SH-SY5Y cells and in Jurkat cells. The expression levels of *MLKL* mRNA were also low in SH-SY5Y cells. Consistent with real-time PCR results, western blot results showed significant expression of RIPK1 but not of RIPK3 or of MLKL in SH-SY5Y cells (Figure 1B). HT-29 cells and Jurkat^{Cas8^{-/-}} cells showed high expression of RIPK1, RIPK3, and MLKL. Taken together with our previous findings [11], our present results suggested that 24S-OHC-induced cell death in SH-SY5Y cells is dependent on RIPK1, but is independent of RIPK3 and MLKL. To further confirm the signaling pathway of 24S-OHC-induced caspase-independent cell death, we chose Jurkat^{Cas8^{-/-}} cells for a study in which we further examined the respective roles of RIPK3 and MLKL in cell death induced by either TNF α or 24S-OHC.

2. 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells was suppressed by selective inhibitor of RIPK1 but not of RIPK3 or MLKL

Necroptosis has been reported to be specifically inhibited by the RIPK1 inhibitor necrostatin-1 (Nec-1) [31], the RIPK3 inhibitor GSK'872 [32, 33], and the MLKL inhibitor necrosulfonamide (NSA) [23]. Nec-1 is a selective kinase inhibitor of RIPK1 which inhibits autophosphorylation of RIPK1. GSK'872 selectively inhibits kinase activity of RIPK3, resulting in inhibition of MLKL phosphorylation. NSA is

known to block RIPK3/MLKL complex formation, resulting in inhibition of MLKL phosphorylation. To examine the role of RIPK1, RIPK3, and MLKL in cell death of Jurkat^{Cas8^{-/-}} cells, we first evaluated the effect of Nec-1, GSK'872, or NSA on TNF α -induced cell death by LDH assay. The results showed that in the presence of cIAP antagonist (smac mimetic), TNF α -induced cell death in Jurkat^{Cas8^{-/-}} cells was significantly suppressed by Nec-1, GSK'872, or NSA (Figure 2A). These results suggest that TNF α -induced necroptosis in Jurkat^{Cas8^{-/-}} cells is dependent on RIPK1, RIPK3, and MLKL as previously reported for other cell lines [22-24]. In contrast, 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells was significantly suppressed by Nec-1, but not by GSK'872 or NSA (Figure 2B). These results indicate that 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells depends on the phosphorylation of RIPK1 but not that of MLKL.

3. 24S-OHC-induced cell death was suppressed by knockdown of RIPK1 or RIPK3 in Jurkat^{Cas8^{-/-}} cells.

To further confirm the role of endogenous RIPK1 and RIPK3 in 24S-OHC-induced Jurkat^{Cas8^{-/-}} cell death, we tested the effect of siRNA knockdown of RIPK1 or RIPK3 on 24S-OHC-induced cell death. Marked declines in constitutive RIPK1 mRNA and protein levels were observed, confirming that we had successfully knocked down RIPK1 after 72 h transfection of RIPK1 siRNA (Figure 3A, 3B). As expected, cell death induced by TNF α and smac mimetic was suppressed almost completely by RIPK1 siRNA (Figure 3C). Under these conditions, the cytotoxicity of 24S-OHC was significantly decreased (Figure 3D), in similar fashion as observed with Nec-1 treatment. We have previously reported that RIPK3 siRNA significantly suppressed cell death induced by 24S-OHC in the presence of pan-caspase inhibitor in

Jurkat cells [13]. In the present study, we found that knockdown of RIPK3 significantly suppressed cell death induced by 24S-OHC in Jurkat^{Cas8^{-/-}} cells (Figure 3E). Taken together with the suppressive effect of Nec-1 on 24S-OHC-induced cell death, these results suggest that the proteins RIPK1 and RIPK3 are both involved in the mechanism underlying cell death induced by 24S-OHC, and that the kinase activity of RIPK1 but not that of RIPK3 plays an important role in this pathway.

4. 24S-OHC-induced cell death was independent of MLKL in Jurkat^{Cas8^{-/-}} cells.

MLKL has emerged as a crucial and specific protein acting as executor in the downstream pathway of necroptosis [23, 25]. Interaction between the N-terminal four-helical bundle domain of MLKL and phosphatidylinositol phosphates at the plasma membrane leads to membrane permeabilization and results in necrotic cell death. To investigate the possible role of MLKL protein in 24S-OHC-induced cell death, we performed siRNA knockdown of MLKL in Jurkat^{Cas8^{-/-}} cells. The results of real-time PCR analysis and western blot analysis showed that we had successfully knocked down MLKL after 72 h transfection as compared with negative control (Figure 4A, 4B). Knockdown of MLKL resulted in significant suppression of TNF α -induced cell death (Figure 4C). In contrast, knockdown of MLKL did not affect 24S-OHC-induced cell death (Figure 4D). Taken together, these results indicate that 24S-OHC-induced cell death is independent of MLKL.

Discussion

Potent neurotoxicity of 24S-OHC [11], and involvement of 24S-OHC in the development of chronic neurodegenerative diseases such as AD and PD [8, 34], have been reported. We have previously shown that 24S-OHC induces neuronal cell death

through a RIPK1-dependent necroptotic pathway in the absence of caspase-8 activity [11]. It has been shown that during the process of typical necroptosis a necrosome complex is formed in which RIPK1 binds and activates RIPK3, resulting in phosphorylation of MLKL [23]. MLKL functions in the downstream pathway of RIPK1 and RIPK3, and modulates ion channel activities at plasma membranes to cause necrosis [25, 35]. In the present study, we examined the involvement of these three components (RIPK1, RIPK3, and MLKL) of the necroptosis pathway to elucidate the mechanism of 24S-OHC-induced cell death. We found that neither mRNA nor proteins of RIPK3 and MLKL were expressed in SH-SY5Y cells (Figure 1). There was also no expression of protein for RIPK3 or MLKL in 24S-OHC-treated cells (data not shown). These results indicate that 24S-OHC-induced cell death in neuronal cells is dependent on RIPK1 but is independent of RIPK3 and of MLKL. Consistent with our observations, it has been reported that expression of RIPK3 and MLKL was at undetectable levels in mouse brain [36, 37]. Based on our current and previous findings, we conclude that 24S-OHC causes RIPK1-dependent necroptosis-like cell death in SH-SY5Y cells.

In a previous study, we showed that 24S-OHC could induce either apoptotic or non-apoptotic cell death in Jurkat cells, and we showed that caspase-8 played a key role in determining which type of cell death was induced [13]. To further investigate the mechanism of 24S-OHC-induced non-apoptotic cell death, we used caspase-8-deficient Jurkat cells. In the context of the necroptosis pathway, the kinase activity of RIPK1 is crucial [14]. Results of our present study indicated that Nec-1 or RIPK1 siRNA significantly suppressed 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells (Figure 2 and 3), suggesting that RIPK1 kinase activity is involved in the mechanism of 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells, which is similar to what we had

previously observed in SH-SY5Y cells with little expression of caspase-8 [11]. Interestingly, although GSK'872, a specific kinase inhibitor of RIPK3, did not show protective effect with respect to cell death (Figure 2), RIPK3 siRNA significantly suppressed 24S-OHC-induced cell death (Figure 3). In our previous study, we showed that protective effect of RIPK3 siRNA was observed in Jurkat cells treated with 24S-OHC in the presence of pan-caspase inhibitor [13]. In the present study, we observed that, in the presence of caspase inhibitor, RIPK3 protein was expressed in Jurkat cells at levels similar to levels which we observed in Jurkat^{Cas8-/-} cells (data not shown). Our previous and present studies therefore indicate that RIPK3 protein itself but not its kinase activity plays an important role in caspase-independent cell death induced by 24S-OHC in Jurkat cells.

The results of MLKL siRNA and NSA treatment showed that 24S-OHC induced cell death independently of MLKL (Figure 4). These data suggest that, in Jurkat^{Cas8-/-} cells, 24S-OHC induces a type of necroptosis-like cell death which is RIPK1-/RIPK3-dependent, but which is MLKL-independent. Several studies have indicated that formation of the heterodimeric amyloid structures of RIPK1 and RIPK3 through their RHIMs mediate necroptosis [38, 39]. Further experiments will be required to address whether RHIMs-mediated oligomerization of RIPK1 and RIPK3 occurs and whether this activates the downstream pathway of 24S-OHC-induced cell death in Jurkat cells. It has been reported that the kinase activity of phosphorylated RIPK3 is important for activation of MLKL in the necroptotic pathway following stimulation by many different stimuli, including TNF α [17, 35], CD95L (also known as FASL or APO-1L) [40], and TNF-related apoptosis-inducing ligand (also known as APO-2L) [40]. However, since we found in the present study that 24S-OHC-induced cell death in Jurkat^{Cas8-/-} cells was independent of activation of RIPK3, this

suggests that MLKL protein is not indispensable for 24S-OHC-induced cell death signaling.

Since we found in the present study that MLKL protein was not involved in execution of cell death in either SH-SY5Y cells or Jurkat^{Cas8^{-/-}} cells, one possible hypothesis is that there may be certain proteins involved in the downstream pathway which trigger disruption of membrane integrity during 24S-OHC-induced cell death. In line with this hypothesis, it is possible that RIPK1 may phosphorylate some other substrate to initiate the 24S-OHC-induced cell death machinery. Unlike the situation observed with necroptosis induced by TNF α and smac mimetic, neither Nec-1 nor RIPK1 siRNA exhibited strong inhibitory effect on 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells (Figure 3). Since we had previously observed significant occurrence of 24S-OHC-induced cell death in SH-SY5Y cells even treated with Nec-1 or RIPK1 siRNA [11], it is therefore plausible that RIPK1-dependent cell death accounts for some instances of 24S-OHC-induced cell death while RIPK1-independent cell death accounts for others. Further experiments will be required to address these possibilities.

Our previous study showed that esterification of 24S-OHC by ACAT1 and formation of lipid droplet-like structures were observed as initial events in 24S-OHC-induced cell death in SH-SY5Y cells and in Jurkat cells [13]. In the same study, we showed that ACAT1 inhibitor or ACAT1 siRNA significantly suppressed formation of lipid droplet-like structures and of cell death. In that study, we concluded that formation of lipid droplet-like structures occurred upstream of the switching point at which the determination was made as to whether cell death would be apoptotic and necroptotic. Unlike the situation in which cell surface death receptor is stimulated by its ligand [30, 35], 24S-OHC esterification and subsequent formation of lipid droplet-

like structures following 24S-OHC treatment occur as intracellular events. It is therefore thought to be likely that the cell death machinery which is activated in 24S-OHC-induced cell death is different from that which is activated in death receptor-mediated cell death.

Taken together, these results suggest that 24S-OHC, a brain-specific oxidation product of cholesterol, induces a novel type of necroptosis-like cell death which is different from the death receptor-mediated type of necroptosis.

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Abbreviations

24S-OHC, 24(S)-hydroxycholesterol; ACAT1, acyl-CoA:cholesterol acyltransferase 1; AD, Alzheimer's disease; cIAP, cellular inhibitor of apoptosis protein; CYP46A1, cholesterol 24-hydroxylase; Jurkat^{Cas8^{-/-}}, caspase-8-deficient Jurkat; LDH, lactate dehydrogenase; MLKL, mixed lineage kinase domain-like; Nec-1, Necrostatin-1; NF- κ B, canonical nuclear factor- κ B; NSA, Necrosulfonamide; PD, Parkinson's disease; RHIMs, RIP homotypic interaction motifs; RIPK, receptor-interacting protein kinase; TNF α , tumor necrosis factor α ; TNFR, tumor necrosis factor receptor

Figure legends

Figure 1. The different levels of expression of RIPK1, RIPK3, and MLKL in various cell lines. (A) The mRNA levels of *RIPK1*, *RIPK3*, and *MLKL* in HT29, SH-SY5Y, Jurkat^{Cas8^{-/-}}, and Jurkat cells were quantified by real-time PCR. After normalization to the levels for *RPL32*, results are shown. (B) Total protein lysates of HT29, SH-SY5Y, Jurkat^{Cas8^{-/-}}, and Jurkat cells were subjected to western blot analysis using appropriate antibodies as indicated.

Figure 2. 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells was suppressed by selective inhibitor of RIPK1 but not of RIPK3 or MLKL. (A and B) Jurkat^{Cas8^{-/-}} cells were pretreated with or without 100 μ M Nec-1, 10 μ M GSK'872, or 1 μ M NSA for 1 h and then treated with 20 ng/ml TNF α and 100 nM smac mimetic (TS) for 24 h (A) or 50 μ M 24S-OHC for 16 h (B). Cell viability was measured by LDH assay (A) or flow cytometry (B). **P<0.01, when compared with cells treated with TS or 24S-OHC in the absence of inhibitor.

Figure 3. 24S-OHC-induced cell death was suppressed by knockdown of RIPK1 or RIPK3 in Jurkat^{Cas8^{-/-}} cells. (A and B) Jurkat^{Cas8^{-/-}} cells were transfected with RIPK1 (siRIPK1) or negative control (NC) siRNA oligo for 72 h. (A) The mRNA levels of *RIPK1* were quantified by real-time PCR. *P<0.05, when compared with NC siRNA. (B) Total protein lysates were subjected to western blot analysis using appropriate antibodies as indicated. (C and D) The cells were challenged with 20 ng/ml TNF α and 100 nM smac mimetic (C) or 50 μ M 24S-OHC (D) for 24 h. Cell viability was measured by WST-8 assay. **P<0.01, when compared with NC siRNA + TS or 24S-OHC. (E) Jurkat^{Cas8^{-/-}} cells were transfected with RIPK3 (siRIPK3) or NC

siRNA oligo for 72 h and were then treated with 50 μ M 24S-OHC for 24 h. Cell viability was measured by WST-8 assay. ** $P < 0.01$, when compared with NC siRNA+24S-OHC.

Figure 4. 24S-OHC-induced cell death was not suppressed by knockdown of MLKL in Jurkat^{Cas8^{-/-}} cells. (A and B) Jurkat^{Cas8^{-/-}} cells were transfected with MLKL (siMLKL) or NC siRNA oligo for 72 h. (A) The mRNA levels of *MLKL* were quantified by real-time PCR. * $P < 0.05$, when compared with NC siRNA. (B) Total protein lysates were subjected to western blot analysis using appropriate antibodies as indicated. (C and D) The cells were challenged with 20 ng/ml TNF α and 100 nM smac mimetic (C) or 50 μ M 24S-OHC (D) for 24 h. Cell viability was measured by WST-8 assay. ** $P < 0.01$, when compared with NC siRNA + TS or NC siRNA + 24S-OHC.

Figure 5. 24S-OHC induces necroptosis-like cell death in the absence of caspase-8 activity. When TNF α binds to its receptor, TNFR, it promotes the assembly of a membrane-associated complex I which activates NF- κ B and facilitates cell survival. Blocking of NF- κ B by smac mimetic drives cells to necroptosis pathway in the absence of caspase-8. The kinase activity of RIPK1 triggers the autophosphorylation, followed by the phosphorylation of RIPK3, resulting in the recruitment of MLKL to the necrosome complex. The phosphorylated RIPK3 activates the phosphorylation of MLKL that promotes the translocation of MLKL to the plasma membrane and directly disrupts their integrity and causes necroptosis. Unlike TNF α -induced necroptosis, the esterification of 24S-OHC by ACAT1 resulting in formation of lipid droplet-like structures is the initial event of 24S-OHC-induced cell death. In the current study, we

show that 24S-OHC induces RIPK1-dependent but MLKL-independent necroptosis-like cell death. With respect to RIPK3, 24S-OHC induced RIPK3-independent cell death in SH-SY5Y cells, and RIPK3 protein-dependent but RIPK3 kinase activity-independent cell death in Jurkat cells.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.