New aspects of 24S-hydroxycholesterol in modulating neuronal cell death

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ABSTRACT

24(S)-hydroxycholesterol (24S-OHC), which is enzymatically produced in the brain, has been known to play an important role in maintaining cholesterol homeostasis in the brain and has been proposed as a possible biomarker of neurodegenerative disease. Recent studies have revealed diverse functions of 24S-OHC and gained increased attention. For example, 24S-OHC at sub-lethal concentrations has been found to induce adaptive response via activation of the liver X receptor signaling pathway, thereby protecting neuronal cells against subsequent oxidative stress. It has also been found that physiological concentrations of 24S-OHC suppress amyloid- β production via down-regulation of amyloid precursor protein trafficking in neuronal cells. On the other hand, high concentrations of 24S-OHC have been found to induce a type of non-apoptotic programmed cell death in neuronal cells expressing little caspase-8. Since neuronal cell death induced by 24S-OHC has been found to proceed by a unique mechanism which is different from but in some ways similar to necroptosis—necroptosis being a type of programmed necrosis induced by tumor necrosis factor α —neuronal cell death induced by 24S-OHC has been called “necroptosis-like” cell death. 24S-OHC-induced cell death is dependent on formation of 24S-OHC esters but not on oxidative stress. This review article discusses newly reported aspects of 24S-OHC in neuronal cell death and sheds light on the possible importance of controlling 24S-OHC levels in the brain for preventing neurodegenerative disease.

Keywords

24(S)-hydroxycholesterol, CYP46A1, cholesterol homeostasis, cell death, neurodegenerative disease

Abbreviations

24S-OHC, 24(S)-hydroxycholesterol; 5,6-epoxyC, cholesterol-5,6-epoxide; 7-OOHC, 7-hydroperoxycholesterol; 7-OHC, 7-hydroxycholesterol; A β , amyloid- β ; ACAT1, acyl-CoA:cholesterol acyltransferase 1; AD, Alzheimer's disease; Apo A-I, apolipoprotein A-I; APP, amyloid precursor protein; c-FLIP, cellular FLICE inhibitory protein; cIAP, cellular inhibitor of apoptosis protein; COPII, coat protein complex II; cum-OOH, cumene-hydroperoxide; CSF, cerebrospinal fluid; CYP46A1, cholesterol 24-hydroxylase; DCFH-DA, dichlorodihydrofluorescein diacetate; GRP78, glucose-regulated protein 78; HD, Huntington's disease; Jurkat^{Cas8^{-/-}}, caspase-8-deficient Jurkat; LCAT, lecithin-cholesterol acyltransferase; LXR, liver X receptor; MLKL, mixed lineage kinase domain-like; MRI, magnetic resonance imaging; PD, Parkinson's disease; RIPK, receptor-interacting protein kinase; ROS, reactive oxygen species; RXR, retinoid X receptor; TNF α , tumor necrosis factor α

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Introduction

The brain is the most cholesterol-rich organ and contains about 25% of the total amount of cholesterol in the body [1]. Brain cholesterol is locally synthesized and its levels are not influenced by the nutritional intake of cholesterol [2,3]. The blood-brain barrier prevents cholesterol transport between the brain and the peripheral circulation [1, 4-7]. Excess brain cholesterol due to either increased synthesis or cell death needs to be eliminated from the brain into the peripheral circulation by a suitable elimination pathway. To maintain a steady-state level of brain cholesterol, excess cholesterol is converted to 24(S)-hydroxycholesterol (24S-OHC) by the neuronal enzyme cholesterol 24-hydroxylase (CYP46A1) [8, 9]. 24S-OHC penetrates the blood-brain barrier by diffusion or by way of organic anion transporting polypeptide 2 [10]. The content of 24S-OHC in the brains of mice lacking CYP46A1 was less than 5% of that of wild-type mice [11], suggesting that CYP46A1 is responsible for production of 24S-OHC in the brain. About 30 μ M of free 24S-OHC has been detected in human brain homogenates [12]. Various oxysterols, including 24S-OHC, can also down-regulate cholesterol synthesis by blocking the proteolytic activation of a transcription factor, sterol regulatory element-binding protein [13]. 24S-OHC is known to be an endogenous ligand of the liver X receptor (LXR) transcription factors which regulate cholesterol efflux [14]. 24S-OHC in circulation is transported by low-density lipoprotein to the liver, where it is metabolized to bile acids [15]. It is therefore clear that 24S-OHC is an important physiological molecule for cholesterol metabolism in the human body.

Cholesterol metabolism is thought to be involved in the pathogenesis of Alzheimer's disease (AD), and the ϵ 4 allele of apolipoprotein E has been shown to be a

genetic risk factor for late-onset AD [16]. A polymorphism in the *CYP46A1* gene which causes increase in 24S-OHC levels has been reported to be associated with AD [17]. In addition to the concentration of 24S-OHC measured in total brain homogenates [12], Heverin *et al.* measured the levels of 24S-OHC in four different brain areas of health controls and AD patients [18]. They found that 24S-OHC levels in all four areas were about 20 ng/mg tissue in control and were decreased in AD patients. If we assume that a volume of 1 mg tissue is 1 μ l, we can calculate the level of 24S-OHC in brain tissue as approximately 50 μ M. Several studies reported that 24S-OHC levels were elevated in the plasma [19,20] and cerebrospinal fluid (CSF) [21] of AD patients. However, other studies have reported lower levels of plasma 24S-OHC in AD patients [22,23]. The potential role of 24S-OHC as a biomarker for diagnosis of Parkinson's disease (PD) has also been investigated [24,25]. As with AD, conflicting reports have been observed for PD. While one study reported unchanged levels of 24S-OHC in plasma but significantly increased levels of 24S-OHC in the CSF of patients with PD [24], another study showed markedly reduced levels of 24S-OHC in the plasma of PD patients [25]. The increased levels of 24S-OHC reported in the CSF of PD patients was correlated with longer disease duration [24]. The level of 24S-OHC in plasma from patients with Huntington's disease (HD) was observed to decrease in correspondence to the progress of the disease as quantified by magnetic resonance imaging (MRI) measurement of striatal atrophy and striatal volume [26]. Similar to what was observed in HD, levels of 24S-OHC in plasma were found to decrease in proportion to the degree of brain atrophy as measured by MRI in AD and multiple sclerosis [27]. Neurodegeneration, with loss of neurons, would be expected to lead to reduction in the levels of 24S-OHC in plasma. It is postulated that plasma levels of 24S-OHC may thus be regarded as a surrogate marker

for the number of metabolically active neurons located in the gray matter of the brain [28]. Hughes *et al.* examined a total of 28 studies on plasma 24S-OHC and neurodegenerative disease and concluded that plasma 24S-OHC might be higher in the early stages of cognitive impairment and lower in advanced stages of AD compared with normal controls due to loss of neurons [29].

Although it has long been suspected that 24S-OHC might play an important role in the pathogenesis of neurodegenerative disease, the exact functions of 24S-OHC have only recently begun to be investigated. In this review, we summarize the dose-dependent and diverse functions of 24S-OHC, and we explore new models for understanding the role of 24S-OHC in neuronal cell death with a view toward gaining a better grasp of neurodegenerative disease pathologies.

Oxysterols: oxidation products of cholesterol

Cholesterol is oxidized enzymatically and non-enzymatically to produce several kinds of oxysterols [30-32]. In non-enzymatic oxidation, cholesterol is attacked by free radicals such as hydroxyl radical, alkoxyl radical, and peroxy radical which are formed in the decomposition of hydrogen peroxide and hydroperoxides by metal ions such as iron and copper [33]. The free radical-mediated oxidation of cholesterol generates 7-hydroperoxycholesterol (7-OOHC), 7-hydroxycholesterol (7-OHC), 7-ketocholesterol (7-KC), and cholesterol-5,6-epoxide (5,6-epoxyC) as major products. Both α and β forms of 7-OOHC, 7-OHC, and 5,6-epoxyC are formed. 7 β -OHC has been reported to derive in the brain from the oxidation of cholesterol by amyloid- β (A β), and to a lesser extent by amyloid precursor protein (APP) [34]. Singlet oxygen attacks

carbon at the 5 position to generate 5 α -hydroxycholesterol specifically. In enzymatic oxidation of cholesterol, several cholesterol hydroxylases produce 24S-OHC, 25-OHC, and 27-OHC as summarized in Table 1 [33,35]. 7 α -OHC is also produced via an enzymatic mechanism [33,36]. Many of the cholesterol hydroxylases except 25-hydroxylase are cytochrome P450 enzymes. A unique enzyme, 11 β -hydroxysteroid dehydrogenase type 1, catalyzes interconversion between 7-KC and 7 β -OHC [33,37].

Production of 24S-OHC in health and disease

In the adult brain, cholesterol is mainly synthesized in astrocytes, from which it is delivered to neurons via apoE-dependent transportation [38]. CYP46A1 expression is normally restricted to certain neuronal cell types which include hippocampal principal neurons and some interneuron classes [39]. Abnormal induction of CYP46A1 by glial cells in the brains of AD patients has been reported [40,41].

Transcriptional and post-transcriptional regulation of CYP46A1 activity, as well as 24S-OHC synthesis, are poorly understood. The careful promoter assay conducted by Ohyama *et al.* failed to determine the transcriptional regulation sites [42]. However, they showed that oxidative stress induced by stimulation of neuronal cells with inflammatory cytokines or tertiary-butyl hydroperoxides caused a significant increase in transcriptional activity. The precise mechanisms are not well known yet. In addition to various direct influences, it is reasonable to suppose that 24S-OHC synthesis will be affected by other cholesterol metabolic pathways, including neurosteroid biosynthesis. Neurosteroids are produced in neurons, astrocytes, and oligodendrocytes—the particular enzymes which are expressed being specific to the type of cell [43,44]. The first step in synthesis of neurosteroids from cholesterol is

action of a mitochondrial cholesterol side-chain cleavage enzyme, such as 20- or 21-hydroxylase [45,46]. Since these enzymes and CYP46A1 both take cholesterol as substrate, they will presumably compete with each other for cholesterol [47]. Because CYP46A1 resides in the endoplasmic reticulum (ER) while cholesterol side-chain cleavage enzymes reside in mitochondria, cholesterol trafficking to specific organelles could control which pathway is favored under different conditions [48].

In mouse models of AD, neuronal overexpression of CYP46A1 has been found to reduce the amyloid pathology [49]. On the other hand, knockout of the *CYP46A1* gene did not affect amyloid formation in mouse models of AD, although deletion of this gene provided a survival advantage in this mouse model [50]. The CC genotype of the IVS3+43C → T polymorphism was associated with increased 24S-OHC/cholesterol ratio in the CSF of AD patients, indicating that the CYP46A1 gene locus may predispose an individual to AD by increasing the 24S-OHC/cholesterol ratio in the brain [17]. Antifungal agents such as voriconazole have been found to be high-affinity inhibitors of CYP46A1 [51,52], which result might cause one to expect that voriconazole would control 24S-OHC function in the brain [48]. However, increase in CYP46A1 expression in the retina of voriconazole-treated rats has been shown [53], indicating that further investigation will be needed before it will be possible to draw conclusions regarding the compounds which control CYP46A1 activity.

Mechanism of protection of neuronal cells by 24S-OHC

It has been demonstrated that certain lipid peroxidation products not only exhibit cytotoxic effect but can also exhibit cytoprotective effect if there is induction of an adaptive response [33,54]. Adaptive response has been defined as a state occurring

when a sub-lethal concentration of an oxidation product significantly enhances cell tolerance with respect to a subsequent oxidative stress [55,56]. Since the highly cytotoxic potential of 7-KC has been demonstrated in neuronal cells [57-59], 7-KC is suspected to be involved in the pathogenesis of neurodegenerative disease. In the case of 24S-OHC, stimulation of human neuroblastoma cell line (SH-SY5Y cells) with sub-lethal concentrations of 24S-OHC was found to induce adaptive response and protect the cells against subsequent cytotoxic stress induced by 7-KC [60]. The cytoprotective effect of 24S-OHC disappeared in LXR β -knocked-down cells, suggesting that LXR β played a key role in 24S-OHC-induced adaptive response. LXR forms a functional heterodimer with retinoid X receptor (RXR) and regulates transcription of several genes involved in the cholesterol efflux pathway [61]. Co-treatment with 24S-OHC and 9-*cis* retinoic acid (the latter being a ligand of RXR) significantly enhanced adaptive response to prevent 7-KC-induced cell death [60]. Taken together, these results indicate that sub-lethal concentration of 24S-OHC protected neuronal cells against subsequent oxidative stress as a result of induction of an adaptive response in which the LXR/RXR was activated.

Accumulation of A β is believed to be the earliest observable event in AD development [62]. A β or A β aggregation has been known to cause neuronal cell death [63,64]. 24S-OHC has been shown to more preferentially than 27-OHC favor the processing of APP to the non-amyloidogenic pathway [65]. The processing of APP into A β by β -secretase and γ -secretase is thought to occur in the trans-Golgi network and endosomes [66-68]. APP budding via coat protein complex II (COPII) vesicles from the ER was reduced by 70% in 24S-OHC-treated cells [69]. Proteomics and immunoblotting analysis revealed that 24S-OHC induced expression of glucose-

regulated protein 78 (GRP78), an ER chaperone, resulting in the formation of the APP/GRP78 complex. These results suggest that 24S-OHC down-regulates APP trafficking via enhancement of the complex formation of APP with up-regulated GRP78 in the ER, resulting in suppression of A β production.

Mechanism of induction of neuronal cell death by 24S-OHC

There are many studies in the literature which report acceleration of neuronal cell death as a result of A β accumulation [70-72]. 24S-OHC, in some cases, has been shown to enhance A β accumulation. For example, increase in A β accumulation as a result of enhancement of APP expression by 24S-OHC has been shown in neuroblastoma cells [73]. Results of *in vitro* experiments suggest that the balance between levels of 24S-OHC and 27-OHC may be of importance for generation of A β peptides [6]. It has been reported that high CSF concentrations of cholesterol, 24S-OHC, and 27-OHC are associated with increase in production of both sAPP forms, i.e., both sAPP α and sAPP β , in AD patients [74]. Enhancement of A β production via ABCA1 induction by 24S-OHC has also been reported [75].

In addition to the enhancing effect of 24S-OHC on A β production, acceleration of A β cytotoxicity by oxidative stress and/or 24S-OHC has been reported [76,77]. It has also been reported that 24S-OHC in the close vicinity of amyloid plaques appears to enhance the adhesion of large amounts of A β to the plasma membrane of neurons and then to amplify the neurotoxic action of A β by locally increasing reactive oxygen species (ROS) steady-state levels [78,79]. The interaction of the A β molecule with the hydrophobic part of 24S-OHC has been observed [80].

Direct cytotoxicity of 24S-OHC in differentiated SH-SY5Y cell has been shown by Kölsch *et. al* [81]. 24S-OHC caused apoptosis as indicated by caspase-3 activation and partially prevented by radical scavenging antioxidant vitamin E in SH-SY5Y cells differentiated by treatment with retinoic acid [82]. When we treated undifferentiated SH-SY5Y cells with 24S-OHC, we observed that 24S-OHC exhibited cytotoxicity in a concentration-dependent manner (Fig. 1)[60,83,84]. 24S-OHC at concentrations higher than 10 μ M significantly caused cell death in which caspase-3 and caspase-9 were not activated [84]. Cell death induced by 24S-OHC was not suppressed by several kinds of antioxidants such as α -tocotrienol, an analogue of α -tocopherol BO-653, deferoxamine, curcumin, and N-acetylcysteine (Fig. 2 A)[84]. We used dichlorodihydrofluorescein diacetate (DCFH-DA) (Fig. 2 B, C) and 2-(4-Diphenylphosphanyl-phenyl)-9-(3,6,9,12-tetraoxatridecyl)-anthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10-tetraone, Liperfluo (Fig. 2 D, E) to detect free radical and lipid hydroperoxides [85], respectively. No increase in the ROS levels in cells treated with by 24S-OHC was observed while significant increase was shown by treatment with cumene-hydroperoxide (cum-OOH). This discrepancy may be explained by different cell conditions between undifferentiated and differentiated with retinoic acid which was shown to induce caspase-8 in neuroblastoma cell [86].

As summarized in Fig. 3, our recent studies have revealed that 24S-OHC induces a type of non-apoptotic programmed cell death in neuronal cells expressing little caspase-8 [84]. The term “necroptosis” has been used to describe 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 human T-lymphoma cell line, Jurkat cells upon exposure to tumor necrosis factor

α (TNF α) [87,88]. RIPK1 has been known to be activated by autophosphorylation [89]. It has been shown that RIPK1 phosphorylation is involved in 24S-OHC-induced cell death in SH-SY5Y cells [84]. We recently found that RIPK1 but neither RIPK3 nor MLKL was expressed in neuronal SH-SY5Y cells [90]. Using caspase-8-deficient Jurkat (Jurkat^{Cas8^{-/-}}) cells expressing these three proteins, we showed that TNF α -induced cell death was dependent on RIPK1, RIPK3, and MLKL [90] as reported in the literature [87,88]. In contrast, of three kinase inhibitors tested which respectively targeted the kinase activities of RIPK1, RIPK3 and MLKL, only RIPK1 kinase inhibitor was found to cause significant suppression of 24S-OHC-induced cell death in Jurkat^{Cas8^{-/-}} cells. Almost as surprising was the fact that in Jurkat^{Cas8^{-/-}} cells, knockdown of either RIPK1 or RIPK3 caused moderate but significant suppression of 24S-OHC-induced cell death, but knockdown of MLKL showed no such effect. Taking into consideration especially the fact that RIPK3 inhibitor did not display inhibitory effect, we concluded that 24S-OHC-induced cell death was dependent on RIPK1 kinase activity and RIPK3 protein (*not* kinase activity) but not on MLKL kinase activity in Jurkat^{Cas8^{-/-}} cells. Taken collectively, these results indicate that in the absence of caspase-8 activity, 24S-OHC induces necroptosis-like cell death which is RIPK1-dependent but MLKL-independent (Fig. 3).

We previously showed that 24S-OHC induces caspase-dependent apoptosis in Jurkat cells which endogenously express caspase-8 [91]. When caspase-8 was inactivated in Jurkat cells by treatment with the pan-caspase inhibitor ZVAD-fmk (ZVAD), 24S-OHC was found to induce caspase-independent cell death, and it was furthermore found that this was significantly inhibited by RIPK1 kinase inhibitor or knockdown of RIPK1 or RIPK3 as had separately been observed in Jurkat^{Cas8^{-/-}} cells.

These results suggest that 24S-OHC can induce either apoptosis or necroptosis-like cell death, which of the two is induced being determined by caspase-8 activity in T-lymphoma Jurkat cells (Fig. 3).

Regardless of the presence or absence of ZVAD, 24S-OHC treatment was found to induce formation of lipid droplet-like structures and cell death in Jurkat cells and SH-SY5Y cells, and it was furthermore found that this was suppressed by treatment with an inhibitor of acyl-CoA:cholesterol acyltransferase 1 (ACAT1) [91]. ACAT1 catalyzes esterification of cholesterol with unsaturated fatty acid at the ER [92]. Analysis using gas chromatography-mass spectrometry revealed that the 24S-OHC-treated cells exhibited accumulation of 24S-OHC esters but not of cholesteryl esters, and that accumulation of 24S-OHC esters was reduced when ACAT1 was inhibited either by ACAT1 inhibitor or by siRNA for ACAT1 [91]. Together with the disappearance of lipid droplet-like structures which resulted from inhibition of ACAT1 activity, recovery of cell viability was observed. Collectively, these results suggest that ACAT1-catalyzed 24S-OHC esterification and the resulting formation of lipid droplet-like structures are initial key events in 24S-OHC-induced cell death. Therapeutic strategies which might employ ACAT1 inhibitors to treat neurodegenerative disease could therefore be promising. Marca *et al.* showed esterification of 24S-OHC by lecithin-cholesterol acyltransferase (LCAT) activity of apolipoprotein A-I (ApoA-I) suppressed neurotoxicity of 24S-OHC by preventing 24S-OHC to enter into cell [93]. This protecting mechanism using LCAT activity against cytotoxicity of 24S-OHC may play a role when released 24S-OHC from neuron attacks itself or other cells.

It is still unclear whether the esterified form of 24S-OHC is itself cytotoxic or

whether it is the formation of lipid droplet-like structures that triggers cell death signaling. It is possible to hypothesize that certain proteins which suppress cell death, e.g., cellular FLICE inhibitory protein (c-FLIP)[94] or cellular inhibitor of apoptosis (cIAP)[95], might collect within lipid droplet-like structures and cause induction of cell death. Another possibility is that the lipid droplet-like structures could serve as an intracellular platform to permit association of certain proteins which might then presumably promote cell death signaling.

Conclusion

The different forms of cell death which are induced by 24S-OHC may contribute to different pathogeneses. 24S-OHC has diverse functions as described above and as summarized in a graphic abstract, and these diverse functions may account for some of the conflicting results observed in connection with the pathologies of various neurodegenerative diseases. Under normal conditions or during early stages of neurodegenerative disease, it would appear that low levels of 24S-OHC act protectively, but that as 24S-OHC levels increase it would appear that a point is then reached at which 24S-OHC no longer acts protectively but instead functions causally to induce cell death. Following such a period, since a large number of neuronal cells will have died, this fact may itself be sufficient to explain why 24S-OHC levels are thereafter observed to decrease. It is difficult to determine the range of physiological and pathological concentrations of 24S-OHC in the brain. While it is hoped that control of 24S-OHC levels in the brain might one day play a role in preventing neurodegenerative disease, we must at the same time remember that it is likely to be only as a result of gaining a clear understanding of the “big picture” of cell death signaling that we will be able to

establish effective therapeutic strategies.

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Figure legends

Figure 1. Cell death induced by 24S-OHC in SH-SY5Y cells. SH-SY5Y cells were treated with variable concentrations of 24S-OHC for 24 h, and cell viability was measured by WST-8 assay. Each bar represents mean $\pm$ SD (n = 3). *, P < 0.05, **, P < 0.01, when compared with vehicle control.

Figure 2 Oxidative stress induced by 24S-OHC in SH-SY5Y cells. (A) Effects of antioxidants against 24S-OHC toxicity in SH-SY5Y cells were assessed. After treatment of cells with various antioxidants, α -tocotrienol, 10 μ M; deferoxamine, 10 μ M; BO-653, 10 μ M; curcumin, 10 μ M; and N-acetylcysteine, 2 mM for 24 h, cells were treated with 24S-OHC for 24 h, and the viability was measured by MTT assay. (B)-(E) Increase in intracellular ROS levels were measured using fluorescent probes, DCFH-DA (B,C) and Liperfluo (D,E) by flowcytometry. Cells were treated with ethanol (control; white), 50 μ M 24S-OHC (gray) or 100 μ M cumene-hydroperoxide (cum-OOH; black) for 6 h. mean $\pm$ S.D. (n=3), **p< 0.01 Tukey, ANOVA when compared with vehicle control.

Figure. 3 Summary of the proposed mechanism of cell death induced by 24S-OHC in neuronal cells and T-lymphoma cells. 24S-OHC is esterified by ACAT1 at ER resulting in formation of lipid droplet-like structures. In SH-SY5Y cells expressing little caspase-8, 24S-OHC induces necroptosis-like cell death which is dependent on RIPK1 phosphorylation. In Jurkat cells, 24S-OHC can induce either apoptosis or necroptosis-like cell death, which of the two is induced being determined by caspase-8 activity.

While $\text{TNF}\alpha$ induces necroptosis which is dependent on kinase activity of RIPK1, RIPK3, and MLKL, 24S-OHC induces necroptosis-like cell death which is dependent on RIPK1 kinase activity and RIPK3 protein (*not* kinase activity) but not on MLKL kinase activity.

Table 1 Enzymes involved in oxysterol synthesis

Enzymes	Oxysterol
cholesterol 7 α -hydroxylase (CYP7A1)	7 α -OHC
cholesterol 24-hydroxylase (CYP46A1)	24S-OHC
cholesterol 25-hydroxylase	25-OHC
sterol 27-hydroxylase (CYP27A1)	27-OHC
11 β -hydroxysteroid dehydrogenase type 1	7-KC, 7- β -OHC

Figure1

Fig. 1

Figure2

Fig. 2

Figure3

Fig. 3

Graphical Abstract