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Review

Diverse functions of 24(S)-hydroxycholesterol in the brain

Noriko Noguchi *, Yoshiro Saito, Yasuomi Urano

Systems Life Sciences Laboratory, Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Kyoto 610-0394, Japan

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ABSTRACT

24(S)-hydroxycholesterol (24S-OHC) which is enzymatically produced in the brain plays important physiological roles in maintaining brain cholesterol homeostasis. We found that 24S-OHC at sub-lethal concentrations down-regulated amyloid precursor protein (APP) trafficking via enhancement of the complex formation of APP with up-regulated glucose-regulated protein 78, an endoplasmic reticulum chaperone. In accordance with this mechanism, 24S-OHC suppressed amyloid-β production in human neuroblastoma SH-SY5Y cells. Furthermore, 24S-OHC at sub-lethal concentrations induced adaptive responses via transcriptional activation of the liver X receptor signaling pathway, thereby protecting neuronal cells against the forthcoming oxidative stress induced by 7-ketocholesterol. On the other hand, we found that high concentrations of 24S-OHC induced apoptosis in T-lymphoma Jurkat cells which endogenously expressed caspase-8, and induced necroptosis – a form of programmed necrosis – in neuronal SH-SY5Y cells which expressed no caspase-8. In this Article, we show the diverse functions of 24S-OHC and consider the possible importance of controlling 24S-OHC levels in the brain for preventing neurodegenerative diseases.

Contents

1. 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]. In the adult brain, cholesterol is mainly synthesized in astrocytes and delivered to the neurons. The blood brain barrier prevents cholesterol transport between the brain and the blood [1–3]. To maintain a steady-state level of brain cholesterol, excess cholesterol is eliminated from the brain

Abbreviations: Aβ, amyloid-β; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; AD, Alzheimer's disease; APP, amyloid precursor protein; CHX, cycloheximide; 9cRA, 9-*cis* retinoic acid; COPII, coat protein complex II; CYP46A1, cholesterol 24-hydroxylase; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; 7KC, 7-ketocholesterol; LXR, liver X receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC, negative control; Nec-1, necrostatin-1; RIPK, receptor-interacting protein kinase; RXR, retinoid X receptor; 24S-OHC, 24(S)-hydroxycholesterol; TNFα, tumor necrosis factor α.

* Corresponding author. Address: Systems Life Sciences Laboratory, Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, 1-3 Miyakodani, Tatara, Kyotanabe, Kyoto 610-0394, Japan. Fax: +81 774 65 6262.
E-mail address: nnoguchi@mail.doshisha.ac.jp (N. Noguchi).

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after it is converted to 24(S)-hydroxycholesterol (24S-OHC) by the neuronal enzyme cholesterol 24-hydroxylase (CYP46A1) [4]. The content of 24S-OHC in the brains of mice lacking CYP46A1 was less than 5% of that of wild-type mice [5], suggesting that CYP46A1 is responsible for production of 24S-OHC in the brain. About 30 μM of free 24S-OHC has been detected in human brain homogenates [6]. 24S-OHC penetrates the blood brain barrier by diffusion or by using organic anion transporting polypeptide 2 [7]. 24S-OHC in circulation is transported by low-density lipoprotein to the liver, where it is metabolized to bile acids [8]. 24S-OHC is known to be an endogenous ligand of the liver X receptor (LXR) transcription factors which regulate cholesterol efflux [9]. 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 [10]. CYP46A1 and 24S-OHC therefore play pivotal roles in brain cholesterol homeostasis.

Cholesterol metabolism is thought to be involved in the pathogenesis of Alzheimer's disease (AD). For example, the ε4 allele of apolipoprotein E has been shown to be a genetic risk factor for late-onset AD [11]. Neurotoxicity of 24S-OHC has also been reported [12]. A polymorphism in the *CYP46A1* gene which causes increase in 24S-OHC levels has been reported to be associated with AD [13]. In mouse models of AD, neuronal overexpression of CYP46A1 reduces the amyloid pathology [14]. On the other hand, knockout of the *CYP46A1* gene did not affect amyloid formation in mouse models of AD, although deletion of this gene gives a survival advantage in this mouse model [15]. Some studies reported that 24S-OHC levels were elevated in the plasma [16,17] and cerebrospinal fluid [18] of AD patients. However, other studies have reported lower levels of plasma 24S-OHC in AD patients [19,20]. Even though 24S-OHC has been assumed to play an important role in the pathogenesis of neurodegenerative diseases such as AD, the exact functions of 24S-OHC have remained poorly understood. In this review, we summarize the diverse functions of 24S-OHC and provide what we hope are helpful insights to better understand and perhaps reconcile to some degree the various conflicting results that have been reported concerning the role of 24S-OHC in neurodegenerative diseases pathologies.

2. Concentration-dependent diverse functions of 24S-OHC

Since 24S-OHC has been shown to possess potent neurotoxicity [12], the effect of 24S-OHC on cell viability was evaluated using WST-8 assay. When SH-SY5Y cells, human neuroblastoma cells were treated with a variety of concentrations of 24S-OHC, cell death was induced in a concentration-dependent manner (Fig. 1). We found that 24S-OHC at concentrations less than 10 μM did not induce

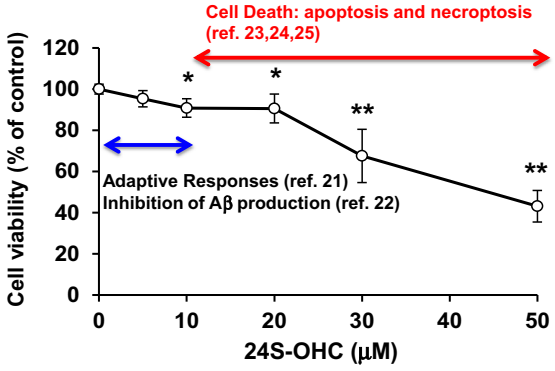

Fig. 1. Concentration dependent diverse functions of 24S-OHC. SH-SY5Y cells were treated with various concentrations of 24S-OHC for 24 h and cell viability was measured by WST-8 assay. Data represents mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, when compared with vehicle control.

significant cell death. We investigated the biological functions of 24S-OHC at concentrations less than 10 μM, and we explored molecular mechanisms underlying induction of cell death at concentrations of 24S-OHC higher than 10 μM. Our recent findings as reported at References [21–25], below, are summarized in Fig. 1.

3. 24S-OHC suppresses cytotoxicity of 7-ketocholesterol via adaptive responses

It has been demonstrated that certain lipid peroxidation products exhibit not only cytotoxic effects but also cytoprotective effects as a result of induction of adaptive responses [26]. Adaptive response has been defined as a state occurring when sub-lethal concentrations of oxidation products significantly enhance cell tolerance against a subsequent oxidative stress [27]. Oxidation products of cholesterol are generated enzymatically and also non-enzymatically in the brain [28]. When cholesterol is attacked by reactive oxygen species (ROS) at the 7-position, 7β-hydroxycholesterol, 7α- and 7β-hydroperoxycholesterol, and 7-ketocholesterol (7KC) are generated. 7α-Hydroxycholesterol is generated by both enzymatic and non-enzymatic oxidation [29]. Since the highly cytotoxic potential of 7KC has been demonstrated in neuronal cells [30–32], 7KC is suspected to be involved in the pathogenesis of neurodegenerative disease. We found that stimulation of SH-SY5Y cells with sub-lethal concentrations of 24S-OHC induced adaptive response and protected the cells against subsequent cytotoxic stress induced by 7KC (Fig. 2A) [21]. The cytoprotective effects of 24S-OHC disappeared in LXRβ-knockdowned cells (Fig. 2A), suggesting that LXRβ 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 [33]. We therefore assessed whether co-treatment of cells with 24S-OHC and RXR ligand 9-*cis* retinoic acid (9cRA) enhanced the adaptive response in sufficient degree to prevent 7KC-induced cell death. Co-treatment with 24S-OHC and 9cRA significantly enhanced adaptive response preventing 7KC-induced cell death. Together, these results suggest that activation of LXR pathway by 24S-OHC is involved in suppression of 7KC-induced cytotoxicity.

Next, we investigated target genes of LXRβ in an attempt to determine proteins responsible for 24S-OHC-induced adaptive response. Since expression of ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1), both of which are known to be target genes of LXRβ, was found to have increased in 24S-OHC-treated cells [21], we studied the effects of siRNA knockdown of either *ABCA1* or *ABCG1* on the adaptive response induced by 24S-OHC. While knockdown of *ABCA1* was found not to affect 24S-OHC-induced adaptive response, the protective effect of 24S-OHC was found to have diminished significantly in *ABCG1* siRNA-transfected cells. These results suggest that ABCG1 but not ABCA1 is involved in an LXRβ-mediated adaptive response induced by 24S-OHC resulting in suppression of cytotoxicity of 7KC (Fig. 2B).

Collectively, we concluded that low concentrations of 24S-OHC protected neuronal cells against forthcoming oxidative stress via induction of an adaptive response in which the LXR/RXR was activated.

4. 24S-OHC suppresses Aβ production by induction of ER chaperone protein

In mouse models of AD, an increase in 24S-OHC level was found to reduce AD pathology [14]. One of the most important characteristics of AD is the accumulation of amyloid-β (Aβ) peptides

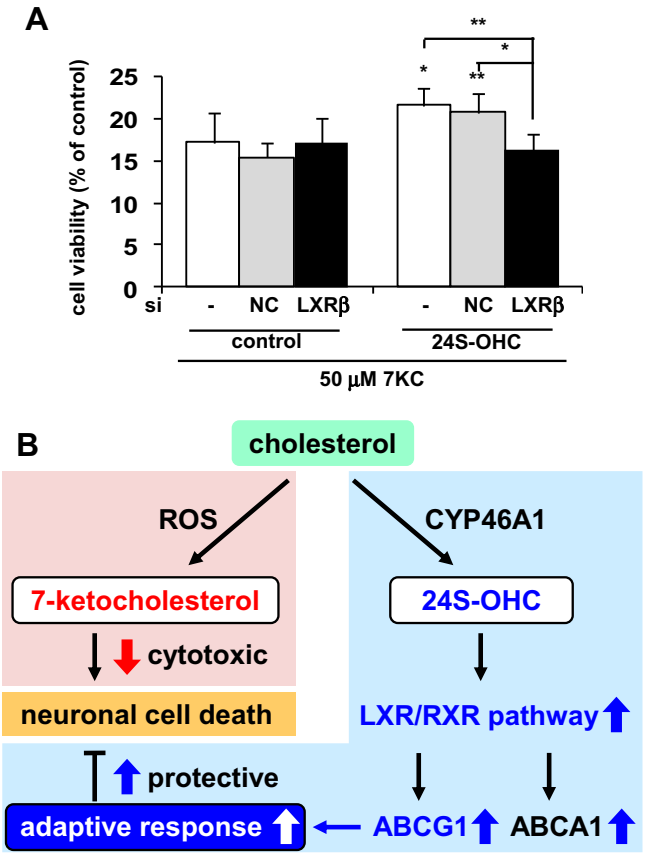

Fig. 2. Adaptive responses induced by 24S-OHC through LXR pathway reduce 7KC-caused cell death in SH-SY5Y cells. (A) SH-SY5Y cells were transfected with negative control (NC) or LXRβ siRNA and incubated for 24 h, and were then treated with 5 μM 24S-OHC for 24 h. The cells were challenged with 50 μM 7KC for 24 h. Cell viability was measured by WST-8 assay. Each bar represents mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, when compared with vehicle control. (B) Scheme of adaptive responses induced by 24S-OHC in which ABCG1 up-regulated by LXR activation is involved in suppression of cytotoxicity by 7KC.

in senile plaques [34]. Aβ is generated from APP by sequential proteolytic cleavage steps involving β- and γ-secretases [35]. The immature form of APP is synthesized and is *N*-glycosylated in the endoplasmic reticulum (ER), and this then moves from the ER to the Golgi via a secretory pathway and is *O*-glycosylated in the Golgi to become the mature form [36,37]. The mature APP is subsequently transported to the plasma membrane, where some of the mature APP is subjected to the endocytotic machinery. The processing of APP into Aβ by β- and γ-secretase is thought to occur in the trans-Golgi network and endosomes.

We investigated mechanisms underlying 24S-OHC affected Aβ production [22], at which time we found that APP budding via coat protein complex II (COPII) vesicles from the ER was diminished by 70% in 24S-OHC-treated cells. Proteomics and immunoblotting analysis revealed that 24S-OHC induced expression of glucose-regulated protein 78 (GRP78), an ER chaperone, through unfolded protein response pathways, and enhanced the formation of the APP/GRP78 complex. At this time, we also found that knockdown of GRP78 diminished the inhibitory effects of 24S-OHC on Aβ production.

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 Aβ production (Fig. 3).

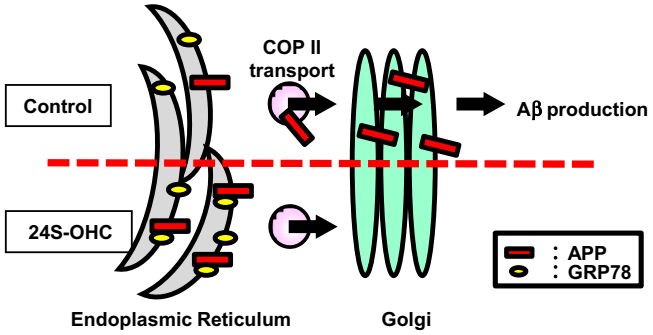

Fig. 3. 24S-OHC inhibits APP trafficking and reduces Aβ production. 24S-OHC treatment induces up-regulation of GRP78 through activation of unfolded protein response pathways and enhances complex formation of APP with GRP78 in the ER. Trafficking of APP from the ER to the Golgi via COPII vesicles is decreased, resulting in reduction of availability of APP to various secretases and suppression of Aβ production.

5. 24S-OHC induces apoptosis and necroptosis in a caspase-dependent manner

In addition to the well-known form of programmed cell death which is referred to as "apoptosis", there is another form of programmed cell death which is referred to as "necroptosis". Necroptosis has been described as being characterized by presence of necrotic cell death morphology but absence of apoptotic characteristics such as phosphatidylserine exposure and caspase activation [38].

24S-OHC at higher concentrations than 10 μM has been found to induce cell death in SH-SY5Y cells (Fig. 1) and primary cortical neuronal cells [23]. We characterized the form of cell death induced by 24S-OHC in SH-SY5Y cells [23]. Cells treated with 24S-OHC exhibited neither the nuclear fragmentation nor the caspase activation which are typical apoptosis. Moreover, 24S-OHC-treated cells showed necrosis-like morphological changes but did not induce the ATP depletion, one of features of necrosis. When cells were treated with necrostatin-1 (Nec-1), an inhibitor of receptor-interacting serine/threonine kinase 1 (RIPK1) which is required for necroptosis [39], 24S-OHC-induced cell death was significantly suppressed. The knockdown of RIPK1 by transfection of siRNA for *RIPK1* effectively attenuated 24S-OHC-induced cell death (Fig. 4A). These results suggest that 24S-OHC induces neuronal cell death by necroptosis, a form of programmed necrosis.

Since it had been reported that ROS play important roles in necroptosis [40], we investigated the roles of ROS in 24S-OHC-induced necroptosis using several kinds of fluorescent probes, and antioxidants and concluded that ROS were not involved in this form of cell death [23,24].

It has been shown that RIPK1 is inactivated by caspase-8, resulting in induction of apoptosis [23,25,41]. We found that caspase-8 was not expressed in either SH-SY5Y cells or primary neuronal cells, but that it was highly expressed in human T-lymphoma Jurkat cells [23]. We therefore treated Jurkat cells with 24S-OHC and investigated a form of cell death. 24S-OHC activated caspase-8 and caspase-3, and caused cell death (Fig. 4B), suggesting that 24S-OHC induced apoptosis in Jurkat cells. When Jurkat cells were treated with 24S-OHC in the presence of the pan-caspase inhibitor ZVAD, neither caspase-8 nor caspase-3 was activated; however, cell death was still induced (about 50% of cells tested died). Cell death was also induced when caspase-8-deficient Jurkat cells were treated with 24S-OHC [25]. It was found that this 24S-OHC-induced cell death could be prevented by treatment with siRNA for *RIPK3* [25] in the presence of ZVAD, suggesting that 24S-OHC was inducing

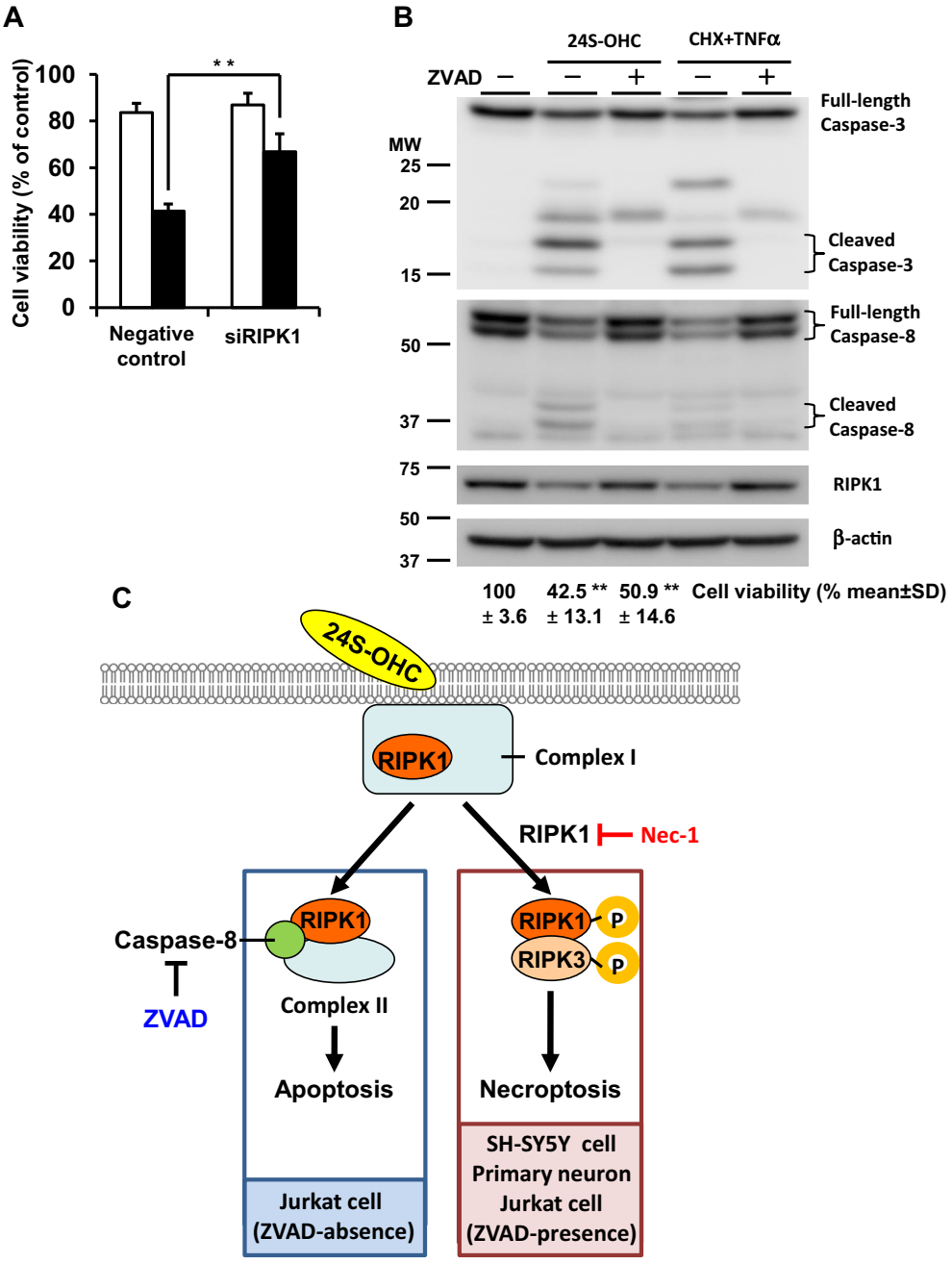

Fig. 4. 24S-OHC induces apoptosis and necroptosis depending on caspase-8 activity. (A) SH-SY5Y cells were pretreated with RIPK-1 siRNA for 4.5 days, and then treated with 24S-OHC for 24 h. Cell viability was determined by MTT assay. $^{**}P < 0.01$, when compared with the control. (B) Jurkat cells were pretreated with and without 20 μM ZVAD for 1 h and were then incubated with 50 μM 24S-OHC for 6 h or 24 h. As a control, cells were treated with 100 ng/mL CHX and 100 μg/mL TNFα. Whole cell lysates were immunoblotted with appropriate antibodies as indicated. Cell viability was determined by WST-8 assay. Data represents mean ± SD ($n = 3$). $^{**}P < 0.01$, when compared with vehicle control. (C) 24S-OHC induces RIPK-dependent necroptosis in neuronal cells in which there is little expression of caspase-8. In Jurkat cells expressing caspase-8, 24S-OHC induces apoptosis. 24S-OHC induces necroptosis in Jurkat cells when caspase-8 is inactivated genetically or by treating with the pan-caspase inhibitor ZVAD.

necroptosis in Jurkat cells under inactivation of caspases. In control experiments, tumor necrosis factor α (TNFα) and cycloheximide (CHX) induced activation of caspase-8 and caspase-3 in Jurkat cells, which was inhibited by ZVAD (Fig. 4B).

Collectively, these results suggest that 24S-OHC can induce apoptosis or necroptosis, which of the two is induced being determined by the caspase activity occurring in the affected cells (Fig. 4C). We speculate that these different forms of cell death which are induced by 24S-OHC may contribute to the different pathogeneses associated with AD, in as much as the diverse functions of 24S-OHC reported above may account for some of the conflicting results observed in connection with the pathologies of neurodegenerative diseases.

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