Original Article

24-Hydroxycholesterol Moderates the Effects of Amyloid-β on Expression of HMG-CoA Reductase and ABCA1 Proteins in Mouse Astrocytes

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Abstract

Background: Elevated brain cholesterol increases the risk of Alzheimer's disease. Production of 24-hydroxycholesterol (24s-OHC) by neurons prevents cholesterol accumulation in the brain. In this study, we investigated the effect of 24s-OHC on the HMG-COA reductase and ABCA1 which are involved in the brain cholesterol homeostasis with or without β-amyloid in astrocytes.

Methods and Materials: Astrocytes were treated with 24s-OHC with or without Aβ. Western blot and real-time polymerase chain reaction were done to detect protein and gene expression of β-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) and ABCA1, respectively. Cholesterol release was determined using a quantitation kit.

Results: Protein levels of HMGCR and ABCA1 were significantly increased by Aβ; however, the 24s-OHC was able to restore their levels and diminish the effect of amyloid-β. Aβ did not have a significant effect on HMGCR expression, while 24s-OHC reduced it by 68%. Aβ-induced ABCA1 expression did not increase cholesterol efflux as the lower levels of cholesterol in conditioned medium of Aβ-treated cells were found.

Conclusion: Our novel findings show that Aβ affects two key elements in the brain cholesterol homeostasis, HMGCR and ABCA1, which are crucial in cholesterol synthesis and efflux. Since 24s-OHC could suppress the Aβ effects on enhancement of HMGCR and ABCA1, therefore the cytochrome P450 46A1 (Cyp46A1), which is exclusively expressed in the central nervous system and responsible for producing of 24s-OHC, could consider as a therapeutic target in the cholesterol-related neurodegenerative diseases such as Alzheimer's disease.

Keywords: ABCA1, amyloid beta-peptides, brain, cholesterol, hydroxycholesterols, HMG-CoA

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Introduction

Alzheimer´s disease (AD) is the most abundant neurodegenerative disease of aging.[1] Formation of extracellular amyloid plaques resulting from deposition of Aβ outside the cell and neurofibrillary tangles comprising hyperphosphorylated tau protein are the main characteristics of AD.[2] The pathological activity of Aβ depends on its deformation from alpha isoform to beta sheet, leading to accumulation of Aβ-fibril form that is toxic for neurons. Several hypotheses have been proposed to explain development of AD; one of them is related to the cholesterol homeostasis in the brain and factors involved in this pathway,[3] as many studies have shown that disturbance of the brain cholesterol homeostasis may contribute to AD.[4]

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Cholesterol is a necessary component for neuronal function, so its homeostasis is important for maintaining brain activities such as memory and learning.[5] The blood-brain barrier (BBB) restricts transfer of cholesterol from peripheral blood to the brain and vice versa; therefore, brain cholesterol homeostasis is independent on circulating cholesterol. In astrocytes, cholesterol is synthesized from acetyl-CoA in several steps and β-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) is a main enzyme controlling cholesterol synthesis.[6]

Central nervous system (CNS) contains high amounts of cholesterol and excess cholesterol cannot cross the BBB.[7] Increased cholesterol polarity makes it suitable for passing through the BBB to be excreted from the brain, so it is modified to 24-hydroxyl cholesterol (24s-OHC) by a cytochrome oxidase P450 called cholesterol 24 hydroxylase (CYP46A1) which is mainly found in neurons.[8]

Astrocytes have many important roles in the central nervous system. These roles include water homeostasis, defense against oxidative stress, and energy storage. When the central nervous system is damaged, astrocytes communicate with the surrounding nervous system and ultimately, this astrocyte connection causes the repair and clearing of amyloid beta in AD.[9] Astrocytes, as the CNS supporting cells, produce and deliver cholesterol to the neurons where 24s-OHC is secreted as a signaling component to regulate cholesterol synthesis in astrocytes.[10] It is reported that 24s-OHC through both activation of liver X receptors (LXR) as its target genes and also modulation of the HMG-CoA–related degradation enzymes activity can regulate HMGCR and consequently cholesterol synthesis. Not only cholesterol synthesis but also other key elements in the brain cholesterol homeostasis are affected by 24s-OHC. For instance, it has been shown that 24s-OHC is able to increase the expression of apolipoprotein E (apoE) and ATP-binding cassette transporter A1 (ABCA1) which are two important proteins in cholesterol regulation.[11]

Epidemiological studies have shown a direct and positive correlation between cholesterol level and AD development.[12] We previously found a dramatic increase in expression of HMGCR and ABCA1 protein along with cholesterol synthesis when astrocytes were treated with Aβ.[13]

24s-OHC is a physiological inhibitor of HMGCR and consequently cholesterol synthesis in astrocytes; nevertheless, whether 24s-OHC is able to reverse the Aβ effect on the HMGCR protein expression and cholesterol synthesis in astrocytes has not been addressed. Thus, in the present research, the effect of Aβ and 24s-OHC on HMG-CoA reductase as the allosteric enzyme in the cholesterol biosynthesis, ABCA1 as a key element in the cell cholesterol release at their gene and protein levels was investigated in this study. Also, the amount of cholesterol in the cells and condition medium was determined.

Material and Methods

Materials

Aβ (Aβ1-42; Cat. No. A9810), 24s-OHC (Cat. No. 474737), and cholesterol extraction and assay kit (MAK043-1KT) were purchased from Sigma-Aldrich. Rabbit anti-HMG-CoA reductase antibody (Cat. No. 174830) and rabbit anti-GAPDH antibody (Cat. No. 181603) were purchased from Abcam. Anti-ABCA1 antibody (Cat. No. HJ1) was from Invitrogen. Fetal bovine serum and penicillin/streptomycin were from Gibco. Low glucose Dulbecco's Modified Eagle Medium and 0.25% trypsin-EDTA were from Bio-Idea, Iran. The P0 newborn of C57BL/6 mice was purchased from Animal laboratory of the Ahvaz Jundishapur University of Medical Sciences.

Primary cell culture of astrocytes, experimental groups

In this study, astrocytes were isolated from the P0 newborn of C57BL/6 mice. All experimental procedures were approved by the institute animal ethics committee (IAEC No. IR.AJUMS.REC.1395.633) in accordance with ethics guidelines of the US National Research Council Committee. Astrocytes were isolated as per a conventional method.[14] Briefly, after separating the brain, meninge was removed, the brain was minced, and then the small pieces were incubated with 1% trypsin for 3 minutes at room temperature. After pipetting the suspension, cell pellet was centrifuged at 1krpm for 3 minutes. Finally, cells were cultured in low-glucose Dulbecco's Modified Eagle Medium enriched with fetal bovine serum 10% at 37°C with 5% CO_2. In this method, the primary culture of astrocytes after a week had more than 97% astrocytes and about 3% oligodendrocytes and microglia. Four groups were used in this study. Group A control received DMSO as the vehicle, Group B received Aβ, Group C received 24s-OHC, and Group D received Aβ +24s-OHC.

Western blot

After treatment of the cells with 24s-OHC and Aβ for 24 hours, they were washed with phosphate buffered saline, scrapped with lysis buffer (RIPA plus protease inhibitor), and mechanically homogenized. Protein concentration was determined by Lowry protein assay and then samples were separated on 10% SDS-polyacrylamide gel and transferred to PVDF membranes. Membranes were incubated overnight with anti HMG-CoA reductase (1:5000; Abcam), ABCA1 (1:2000), and anti GAPDH (1:10000) at 4°C. After incubation time, the membranes were washed three times with tris-buffered saline/Tween (TBST) and then incubated with secondary antibody conjugated with horseradish peroxidase (1:4000; Sigma) and detection was performed using enhanced chemiluminescence substrates. The ImageJ software was used to quantify the western blot data.

Real-time polymerase chain reaction analysis of HMGCR and ABCA1

To determine the HMGCR and ABCA1 expression levels, cells were treated with DMSO (control), 1 μM Aβ, 5 μM 24s-OHC, or Aβ+24s-OHC for 24 hours ([15]) and total RNA was extracted using Resay kit (Qiagen, Germany). Concentration and purity of the extracted RNA were determined by Nanodrop

and cDNA was synthesized using a QuantiTect Reverse Transcription Kit (TAKARA, Japan). RNA expression levels were analyzed using comparative threshold cycle as per CT ($2^{-\Delta\Delta CT}$) comparison method and GAPDH as the internal controls. Primers[16] used for real-time polymerase chain reaction (RT-PCR) are listed in Table 1.

Astrocytes cholesterol content and release

To measure the cellular cholesterol, cells were treated with 24s-OHC with or without Aβ for 24 hours and at the end of incubation time, conditioned media were transferred to a glass tube and cells were washed with phosphate buffered saline. After drying cell plates with a dryer, cholesterol was extracted from the cells with hexane: isopropanol (3:2, v/v) solvent mixture or from the conditioned medium with chloroform: methanol (2:1, v/v) mixture[17] and analyzed by an extraction cholesterol assay kit (MAK043-1KT). The reaction mixtures were prepared as per the table described in the kit.

Statistical analysis

SPSS (Version 18) Software was used for statistical analysis and analysis of variance was used to analyze the western blotting data. Descriptive statistics presented data as mean ± standard deviation (SD) and $P < .05$ was considered as the significance level.

Results

Effect of 24s-OHC on HMGCR protein and gene expression in Aβ-treated astrocytes

To investigate the effect of 24s-OHC on protein levels of HMG-CoA reductase, as the main rate-limiting enzyme in cholesterol synthesis, astrocytes were treated with 5 μM of 24s-OHC[15] with or without 1 μM Aβ for 24 hours. The western blot results showed that protein expression of HMGCR was dramatically upregulated by Aβ; however, treating astrocytes with 24s-OHC resulted in decreased protein level of HMGCR. In co-treatment with Aβ, 24s-OHC was able to reduce the effect of Aβ on HMG-CoA reductase in comparison with the control group [Figure 1].

Increasing effect of Aβ on HMGCR and the regulatory effect of 24s-OHC were also examined by RT-PCR. Under conditions similar to those considered for western blotting, mouse astrocytes were seeded and treated, and total mRNA was extracted. The RT-PCR findings showed that Aβ did not have a significant effect on HMGCR gene expression but 24s-OHC significantly suppressed mRNA expression of HMGCR even in the presence of Aβ [Figure 2].

Table 1: Forward and reverse primer sequences of ABCA1, HMGCR, and GAPDH used in this study for real-time PCR assessments

Gene	Sequence	PCR product size (pb)
ABCA1 F	5/-CCCAGAGCAAAAAGCGACTC-3/	
ABCA1 R	5/-GGTCATCATCACTTTGGTCCTTG -3/	102
HMGCR F	5′-TGCTGCTTTGGCTGTATGTC-3′	
HMGCR R	5′-TGAGCGTGAACAAGAACCAG- 3′	127
GAPDH F	5/-ATGGTGAAGGTCGGTGTG -3/	
GAPDH R	5/-CATTCTCGGCCTTGACTG -3/	186

Effect of 24s-OHC on ABCA1 gene and protein expression in Aβ-treated mouse astrocytes

ABCA1 as the pivotal protein that is associated with cholesterol efflux was examined by western blot under conditions similar to those considered for HMGCR western blotting. Figure 3 shows ABCA1 protein expression in astrocytes treated with 24s-OHC with or without Aβ. Treatment with Aβ for 24 hours caused a substantial increase in ABCA1 protein expression in astrocytes. The Aβ-induced ABCA1 expression was markedly reduced when cells were co-treated with 24s-OHC, indicating that 24s-OHC has, somehow, a regulatory effect on ABCA1 expression in mice astrocytes.

RT-PCR results showed that Aβ and 24s-OHC did not have a significant effect on ABCA1 gene expression, suggesting that Aβ-induced ABCA1 protein enhancement and the regulatory effect of 24s-OHC is not induced through modulation of ABCA1 gene expression [Figure 4].

Effect of 24s-OHC on cholesterol release in the Aβ-treated astrocytes

To determine whether elevated protein level of ABCA1 in the previous experiment was associated with an increased activity

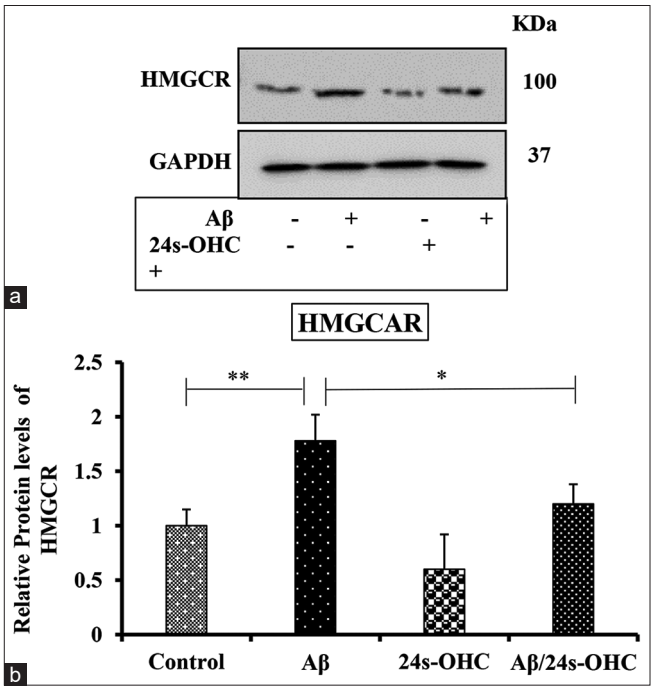

Figure 1: Effect of 24-hydroxycholesterol (24s-OHC) on HMGCR protein expression in the absence and presence of Aβ in mouse astrocytes. (a) Cells were treated with 24s-OHC (5 μM) and Aβ (1 μM) for 24 h, then harvested with RIPA buffer, and 100 μg/lane of cell lysate was subjected to SDS-PAGE and western blotting. (b) Data were analyzed using SPSS and are presented as mean ± SD of triplicate samples. *P value $<.05$ is significant

of this protein, cholesterol level in the culture media was measured after treating the cells under conditions similar to those considered for the previous experiment. Figure 5 shows a significant decrease (by about 40%) in cholesterol release in culture of the Aβ-treated cells. 24s-OHC has a nonsignificant mild effect on cholesterol release; however, it was able to repress the effect of Aβ and restore cholesterol release to near the background in 24s-OHC/Aβ group.

Discussion

Here, we investigated the effect of Aβ in the presence and absence of 24s-OHC on the gene expression and the protein levels of HMGCR and ABCA1 as the two critical players in the brain cholesterol homeostasis in mouse astrocytes. Our results showed that protein expression of HMGCR and particularly, ABCA1 increased in the cells treated with Aβ; however, ABCA1 or HMGCR gene expression was not influenced by Aβ treatment. Noteworthy, 24s-OHC was able to regulate HMGCR and ABCA1 protein levels and in case of HMGCR, we believe that this effect was mediated through suppression of its gene expression. Also, cholesterol release was suppressed by Aβ treatment, suggesting that Aβ-induced ABCA1 expression did not lead to induce its function to increase cholesterol efflux.

It has been shown that astrocytes cholesterol content is mainly regulated by HMG-CoA reductase as the main element in

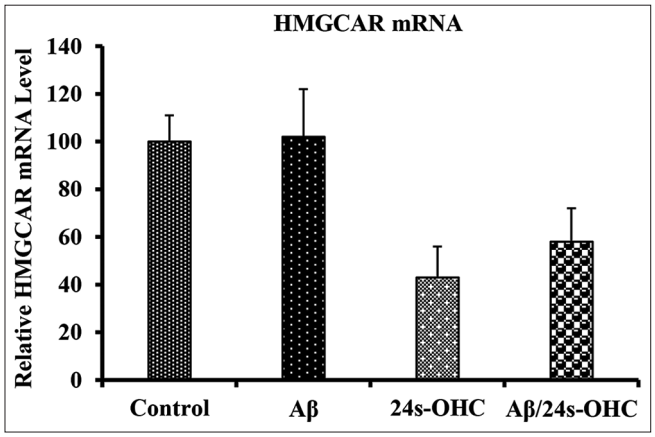

Figure 2: Effect of 24-hydroxycholesterol (24s-OHC) on HMGCR expression in the absence and presence of Aβ in astrocytes. After treatment with 24s-OHC (5 μM) in the absence and presence of Aβ (1 μM), total RNA was extracted and HMGCR gene expression was analyzed by real-time PCR. Values are reported as mean ± SD and the significance level is * P <.05. ** P <.01

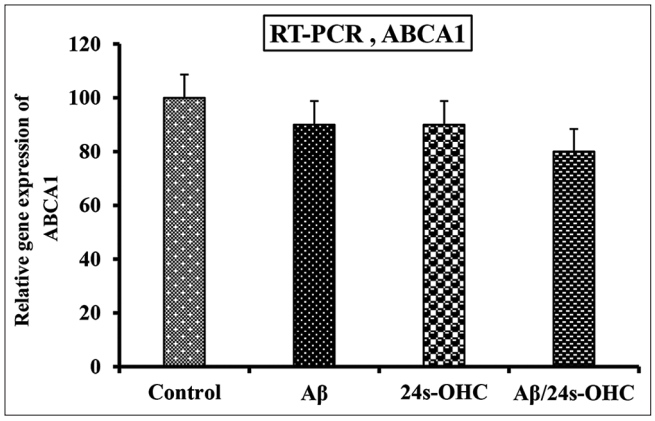

Figure 4: Effect of 24-hydroxycholesterol (24s-OHC) on ABCA1 expression in the absence and presence of amyloidβ (Aβ) in astrocytes. After treatment with 24s-OHC (5 μM) in the absence and presence of Aβ (1 μM), total RNA was extracted and ABCA1 mRNA was analyzed by real-time PCR. Values are reported as mean ± SD. The significance level is * P <.05. ** P <.01

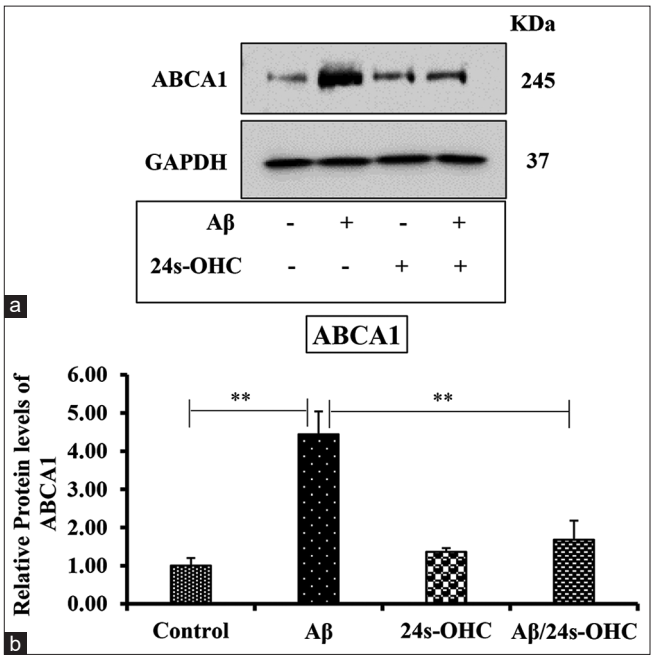

Figure 3: Effect of 24-hydroxycholesterol (24s-OHC) on ABCA1 protein level in the absence and presence of Aβ in astrocytes. (a) Cells were treated with 24s-OHC (5 μM) and Aβ (1 μM) for 24 h, then harvested with RIPA buffer, and 150 μg/lane of cell lysate was subjected to SDS-PAGE and western blotting. (b) Data were analyzed using SPSS and are presented as mean ± SD of the triplicate samples. *P value <.05 is significant

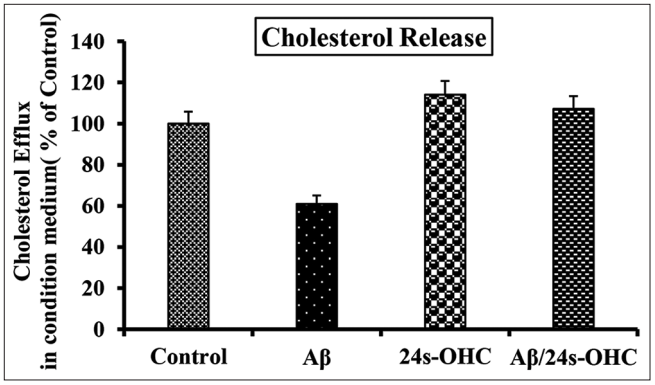

Figure 5: Effect of 24-hydroxycholesterol and Aβ on cell cholesterol content and cholesterol release in astrocytes culture media. Cells were incubated with 5 μM of 24s-OHC and 1 μM of Aβ. After 24 hours of incubation, cholesterol release in media was measured using cholesterol quantitation kit

cholesterol synthesis pathway, ABCA1 as a central protein in the cholesterol secretion, and CYP46A1, a crucial enzyme responsible for cholesterol elimination, that acts by converting cholesterol to 24s-OHC.[18]

Our novel findings showed that Aβ increases HMG-CoA reductase protein level and cell cholesterol content in mouse astrocytes which could be a reasonable explanation for the increased cholesterol accumulation in some cholesterol-related neurodegenerative diseases such as AD.[12] In contrast to protein level, Aβ had no a significant effect on HMGCR gene expression, so it could be concluded that Aβ inhibits the normal turnover of HMGCR by stabilizing it or inhibition of the particular proteases which are involved in the HMGCR degradation.

HMGCR activity is regulated by sterol and nonsterol metabolites.[19,20] In addition to the HMGCR activity, our data showed that treatment with 24s-OHC suppresses HMGCR gene and protein expression [Figures 1 and 2], suggesting a crucial regulatory effect of 24s-OHC on the cell HMGCR amount as well. Our data revealed that 24s-OHC is able to reduce Aβ-induced HMGCR gene and protein expression; however, it could not diminish the overall effect of Aβ [Figure 1].

How 24s-OHC can adjust the cell cholesterol content and moderate the effects of amyloid-β on the level of HMGCR [Figures 1 and 2]. We believe that it might be through the cytochrome P450 46A1 (Cyp46A1). Cyp46A1 is exclusively located in the CNS and can add a hydroxy group (-OH) to the cholesterol and convert it to the oxysterols such as 24s-OHC, 25s-OHC, and 27s-OHC.[21] Oxysterols including 24s-OHC are more soluble than cholesterol and they can enter the cells to regulate cholesterol synthesis through induction of HMGCR degradation. It is believed that certain sterols such as 24s-OHC potentially can trigger HMGCR ubiquitination which is necessary for proteasomal degradation.[22] Although there is a controversy about the role of 24s-OHC in the brain cholesterol homeostasis specially in AD,[23] our findings support the beneficiary and regulatory role of 24s-OHC on gene and protein expression of HMG-CoA reductase that is a rate-limiting enzyme in the cholesterol synthesis.

Unlike many reports emphasizing on the positive effect of 24s-OHC on cholesterol efflux,[24] we did not observe any significant changes in the cholesterol release in the cell treated with 24s-OHC [Figure 4]. These phenomena could be mediated by suppression of HMGCR expression or increasing of HMGCR degradation as explained above. Consistent with our findings, Rodríguez *et al*.[25] reported that accumulation of 24s-OHC in neuroblastoma cells decreased HMGCR expression and inhibited cholesterol synthesis in cultured cells.[26]

We next checked whether 24s-OHC could diminish Aβ-induced changes in ABCA1 protein expression in mice astrocytes. It is well known that 24s-OHC is a natural ligand for LXR and activation of LXR promotes cholesterol efflux by affecting ABCA1 expression leading to cell cholesterol reduction.[27,28] Although we have not provided any evidences on the effect of 24s-OHC through LXR, based on the literature's, it is safe to say that 24s-OHC probably regulates ABCA1 through LXR. The detailed impact of Aβ on ABCA1 has been characterized and reported in our recent publication.[29]

In the present study, we checked the effect of Aβ and 24s-OHC on ABCA1 protein level and function. We showed that treatment of astrocytes with Aβ significantly increased ABCA1 protein level; however, the Aβ-induced ABCA1 did not affect its function to increase cholesterol efflux. How Aβ increases the ABCA1 amount and its signaling pathways remain to be elucidated. Because ABCA1 gene expression does not change by Aβ [Figure 4], we speculate that the ABCA1 degradation might be affected by Aβ.

ABCA1 could be degraded by activated calpain[30,31] or ubiquitin-proteasome system as well. Suppression of ubiquitin/proteasome activities has been reported in the brain of Alzheimer's patients and in case of astrocytes, also Aβ inhibits the proteolytic activity of proteasomes in astrocytes.[32,33] Therefore, reduction in ABCA1 degradation could be a reasonable explanation for the sharp increase in ABCA1 protein level in astrocytes-treated with Aβ.[29]

In addition to the general pathways of ABCA1 degradation, cell cholesterol amount, as a post-translational factor, can influence the ABCA1 level.[34-36] So, our data showed that Aβ is able to reduce the release of cholesterol and lead to accumulation of cholesterol in the cell [Figure 5], which in turn, increases ABCA1 protein level [Figure 3]. In contrast to Aβ, 24s-OHC appears to have an indirect effect on reducing ABCA1 protein level by reducing the HMGCR as a crucial enzyme in the cholesterol synthesis pathway [Figure 1].

Taken together, the brain needs high amounts of cholesterol for its optimal functions and our novel findings show that HMGCR and ABCA1 which are two important elements involved in the cholesterol homeostasis are affected by Aβ. Interestingly, 24s-OHC as an excreted metabolite of cholesterol can probably invert Aβ effects and influence the brain cholesterol homeostasis.

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Conflicts of interest

There are no conflicts of interest.

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