NeuroToxicology 59 (2017) 88–97

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

27-Hydroxycholesterol regulates cholesterol synthesis and transport in C6 glioma cells

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ARTICLE INFO

Article history:
Received 30 May 2016
Received in revised form 1 February 2017
Accepted 2 February 2017
Available online 3 February 2017

Keywords:
27-hydroxycholesterol
Cholesterol
Synthesis
Transport
Brain
Astrocyte

ABSTRACT

The oxysterol 27-Hydroxycholesterol (27-OHC) is a major cholesterol metabolite that can cross the blood brain barrier (BBB) from peripheral circulation to the brain. Currently, the role of 27-OHC on cholesterol homeostasis in astrocytes and the underlying mechanisms are not defined. Since all brain cholesterol is essentially synthesized in brain itself and astrocytes as net producers of cholesterol are essential for normal brain function, here we investigated the effects of 27-OHC on cholesterol synthesis and transport in C6 glioma cells. C6 cells were treated with 5, 10 and 20 μM 27-OHC for 24 h and the cell viability and apoptosis, the cholesterol levels and metabolism-related mediators, genes and proteins were subsequently assessed using cell-counting kit (CCK)-8, Amplex red, ELISA, real-time PCR and Western blot, respectively. We found that 27-OHC decreased cholesterol levels by down-regulating the expression of sterol-regulated element binding protein-1 (SREBP-1a), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CR) and low density lipoprotein receptor (LDLR) and promoted cholesterol transport by up-regulating the expression of peroxisome proliferator-activated receptors-γ (PPAR-γ), liver X receptor-α (LXR-α), ATP-binding cassette transporter protein family member A1 (ABCA1) and apolipoprotein E (ApoE)genes. Our results suggested that 27-OHC may represent a sensitive modulator of cholesterol metabolism disorder by suppressing cholesterol synthesis and stimulating cholesterol transport in astrocytes.

1. Introduction

Disturbances in brain cholesterol homeostasis may lead to learning and memory deficits and even to neurodegenerative diseases such as Alzheimer's disease (AD) (Di Paolo and Kim, 2011). Some studies have indicated that high levels of brain cholesterol are responsible for β-amyloid peptide (Aβ) accumulation in AD patients and dysfunctional cholesterol metabolism may increase the risk of developing AD (Nicholson and Ferreira, 2010). Despite lack of conclusive evidence, a substantial body of literature supports the contention that high levels of plasma cholesterol can also influence the pathogenesis of AD (Kivipelto et al., 2001; Refolo et al., 2000; Solomon et al., 2009). However, the question as to how plasma cholesterol contributes to the development of AD remains to be answered in view of the fact that the brain makes its own cholesterol in situ and little or no circulating cholesterol enters into the brain owing to the impermeability of the blood brain barrier (BBB) (Pfrieger and Ungerer, 2011). At the present state of knowledge, several hypotheses have been advanced to explain the question and it may be possible that oxysterols, side-chain oxidized metabolites of cholesterol, may represent the link between circulating cholesterol and the development of AD.

In marked contrast to cholesterol, oxysterols have the ability to cross the BBB into the brain. One of these metabolites, 27-hydroxycholesterol (27-OHC), is the quantitatively most important cholesterol metabolite originating mainly from the peripheral circulation and is formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27A1) enzyme (Marwarha and Ghribi, 2014). Heverin et al. have reported a substantial continuous flux of 27-OHC from the circulation into the brain and the levels of 27-OHC in cerebrospinal fluid (CSF) are associated with the corresponding levels in periphery (Heverin et al., 2005), indicating the correlation between hypercholesterolemia and increased flux

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http://dx.doi.org/10.1016/j.neuro.2017.02.001
0161-813X/

of 27-OHC into the brain. The levels of 27-OHC have been demonstrated a significant increase in AD brains (Heverin et al., 2004). On the other hand, the oxysterol 24S-hydroxycholesterol (24-OHC), which is also involved in the pathogenesis of AD, is synthesized in neurons from cholesterol by CYP46A1 enzyme that is expressed exclusively in the brain (Hughes et al., 2013).

Currently, the role of these two oxysterols and the mechanism by which they are important to the pathogenesis of AD have not been clearly defined. Under in vitro conditions, 27-OHC was reported to enhance production of β amyloid (Aβ) in human neuroblastoma SH-SY5Y cells and 24-OHC may favor the non-amyloidogenic pathway (Prasanthi et al., 2009). In addition, our team has also shown that 27-OHC may represent a pathogenic factor in AD by regulating Nrf2 signaling pathway and increasing oxidative stress in astrocyte cells (Ma et al., 2015). These studies have revealed some potential mechanism. However, it has been reported that oxysterols have diverse physiological and biochemical functions, including maintaining cholesterol homeostasis and regulating nuclear receptors (Javitt, 2007). Within the mature brain, astrocytes, in particular, synthesize and release cholesterol (Pfrieger, 2003). Oxysterols can suppress cholesterol synthesis in response to high levels of cholesterol by interacting with the absence of activated sterol regulatory element-binding proteins (SREBPs) and degradation of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CR). Besides, oxysterols can also bind liver X receptor (LXR) and then affect the expression of several genes involved in cholesterol metabolism (Leoni and Caccia, 2013). Excessive formation of 27-OHC followed by hypercholesterolemia may contribute to disturbances in brain cholesterol homeostasis and subsequently pathogenesis of AD. Although evidence has provided that 24S-OHC induces cholesterol efflux via an LXR-controlled pathway in astrocytes, which may be relevant for neurological diseases (Abildayeva et al., 2006), it remains less understood that whether 27-OHC may induce similar disruptive brain cholesterol homeostasis in astrocyte cells.

In this study we tested the hypothesis that 27-OHC contributes to the disruptive cholesterol homeostasis in astrocytes. For this purpose the effects of 27-OHC on cholesterol metabolism in C6 glioma cells were detected. The levels of total cholesterol (TC), free cholesterol (FC) and cholesterol esterase (CE), as well as the mRNA and protein of HMG-CoA reductase (HMG-CR), sterol regulatory element-binding protein-1a (SREBP-1a), low-density lipoprotein receptor (LDLR), peroxisome proliferator-activated receptor-γ (PPAR-γ), liver X receptor-α (LXR-α), ATP-binding cassette transporter protein family member A1(ABCA1) and apolipoprotein E (ApoE) in C6 cells treated with 27-OHC were especially determined. Additionally, cell viability and apoptosis assays were also performed to assess the cytotoxicity of 27-OHC; ELISA assay kits were used for evaluation of HMG-CR, low-density lipoprotein (LDL), cholesterol ester transfer protein (CETP) and microsomal triglyceride transfer protein (MTTP).

2. Materials and methods

2.1. Materials and cell lines

The C6 rat glioma cell line was obtained from Peking Union Medical Center Laboratory, Beijing, China. 27-OHC was purchased from Santa Cruz Biotechnology Company (USA). The stock solution was prepared by dissolving 27-OHC in ethanol to 1000 μM and stored at −80 °C until use. Before each cell treatment, 27-OHC was first diluted in saline with 8% ethanol (v/v) and then added to culture medium to a final concentration of 5, 10 and 20 μM, containing 0.04%, 0.08% and 0.16% ethanol (v/v). DMEM, fetal bovine serum (FBS) and penicillin (10,000 units/ml)/streptomycin (10,000 μg/mL) (P/S) were purchased from Gibco Biotechnology Company (USA). The study design has been approved by the Ethics Committee of Capital Medical University (AEEI-2014-047).

2.2. Cell culture

The C6 cells were cultured in DMEM supplemented with FBS (10%) and penicillin (100 U/ml)/streptomycin (100 U/ml) at 37 °C in an atmosphere of CO_2 (5%)/air (95%). The cells were seeded at an appropriate density (1×10^6 cells/cm^2) in culture dishes. The culture medium was replaced every 2 days. Cells incubated with DMEM were used as control group and others were treated with 5, 10, and 20 μM 27-OHC for 24 h.

2.3. CCK-8 assay kit

The 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-8/CCK-8) assay was used to measure cell viability. Briefly, C6 cells were seeded at 5×10^4 cells/ml (150 μl per well) in 96-well plates for 24 h in DMEM. Then, the cells were incubated with 0 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, or 300 μM 27-OHC for 24 h. After treatment with 27-OHC for 24 h, CCK-8 reagent (10 μl) in DMEM (100 μl) was added to each well, and the cultures were incubated at 37 °C for 4 h. The absorbance was measured with a microplate reader (Tecan, Switzerland) at a wavelength of 450 nm.

2.4. Annexin V-FITC/PI assay kit

C6 cells were treated with increasing concentrations of 27-OHC (0 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM) for 24 h, washed with PBS and trypsination. The apoptosis was detected with Annexin V-FITC/propidium iodine (PI), according to the guidelines of assay kit (Beijing Keygen Biotech, China). Laser excitation wavelength was 488 nm, the green signal from Annexin V-FITC was examined at 525 nm and the red signal from PI was measured at 620 nm. Annexin V^-/PI^- cells are vitality; Annexin V^+/PI^- cells are in early apoptosis; Annexin V^+/PI^+ cells are necrotic or in late apoptosis. Each sample was set up in triplicate within the assay, and at least three independent experiments were performed.

2.5. Amplex red cholesterol assay

The cholesterol content in the C6 cells was tested using the Amplex Red Cholesterol Assay kit (Invitrogen) according to the manufacturer's instructions. Briefly, the C6 cells were seeded at density of 1×10^6cells/cm^2 in culture dishes. After 24 h incubation of 27-OHC at 37 °C in a CO2 (5%)/air (95%) atmosphere, sub-confluent cultures were rinsed three times in PBS to eliminate residual growth medium. Whole cell extracts were prepared by 0.5 ml RIPA lysis buffer (0.5% sodium deoxycholate, 0.1%SDS, 1% NP-40, 150 mM NaCl, 1 mM DTT, 50 mM Tris–HCl (pH 7.4) and phenylmethanesulfonyl fluoride (PMSF). After vigorous shaking for 40 min at 4 °C, lysates were clarified at 15,000 g for 20 min at 4 °C and supernatants containing protein were evaluated using BSA as a standard for protein concentration by BCA assay. Cell extracts were diluted with 1 × cholesterol reaction buffer (0.1 M potassium phosphate, pH 7.4, 0.05 M NaCl, 5 mM cholic acid, and 0.1% Triton X-100). Then, 50 μl of 150 μM Amplex Red reagent (1 U/ml horseradish peroxidase, 1 U/ml cholesterol oxidase, and 1 U/ml cholesterol esterase) was added to 50 μl samples in 96-well plates. After a 60-min incubation at 37 °C in the dark, the sample fluorescence was measured using a microplate reader (Tecan, Switzerland) at 530/25 nm excitation and 590/35 nm emission wavelengths. The total cholesterol (TC) content was determined by measuring the cholesterol concentration following digestion with cholesterol esterase (CE). To measure the free cholesterol (FC), CE