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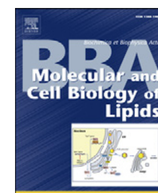

Cholesterol efflux is differentially regulated in neurons and astrocytes: Implications for brain cholesterol homeostasis

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ABSTRACT

Disruption of cholesterol homeostasis in the central nervous system (CNS) has been associated with neurological, neurodegenerative, and neurodevelopmental disorders. The CNS is a closed system with regard to cholesterol homeostasis, as cholesterol-delivering lipoproteins from the periphery cannot pass the blood–brain-barrier and enter the brain. Different cell types in the brain have different functions in the regulation of cholesterol homeostasis, with astrocytes producing and releasing apolipoprotein E and lipoproteins, and neurons metabolizing cholesterol to 24(S)-hydroxycholesterol. We present evidence that astrocytes and neurons adopt different mechanisms also in regulating cholesterol efflux. We found that in astrocytes cholesterol efflux is induced by both lipid-free apolipoproteins and lipoproteins, while cholesterol removal from neurons is triggered only by lipoproteins. The main pathway by which apolipoproteins induce cholesterol efflux is through ABCA1. By upregulating ABCA1 levels and by inhibiting its activity and silencing its expression, we show that ABCA1 is involved in cholesterol efflux from astrocytes but not from neurons. Furthermore, our results suggest that ABCG1 is involved in cholesterol efflux to apolipoproteins and lipoproteins from astrocytes but not from neurons, while ABCG4, whose expression is much higher in neurons than astrocytes, is involved in cholesterol efflux from neurons but not astrocytes. These results indicate that different mechanisms regulate cholesterol efflux from neurons and astrocytes, reflecting the different roles that these cell types play in brain cholesterol homeostasis. These results are important in understanding cellular targets of therapeutic drugs under development for the treatments of conditions associated with altered cholesterol homeostasis in the CNS.

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1. Introduction

The regulation of cholesterol homeostasis in the brain has lately become an area of intense investigation, as cholesterol and regulators of cholesterol homeostasis have been implicated in the pathogenesis of genetic and neurodegenerative diseases such as Niemann–Pick Type C and Alzheimer's diseases [1]. Furthermore, we recently proposed that developmental neurotoxicants, such as alcohol and retinoic acid, may exert some of their deleterious effects in the brain by affecting cholesterol homeostasis [2].

Cholesterol levels and trafficking in the brain are regulated by endogenous mechanisms, as the blood–brain-barrier is impermeable to lipoproteins, preventing blood cholesterol from reaching the brain parenchyma. While both astrocytes and neurons synthesize cholesterol *de novo* (though in different amounts), they greatly differ in the mechanisms they adopt to control intracellular cholesterol levels and cholesterol trafficking within the brain [3]. As in the periphery, cholesterol is circulated in the brain associated with lipoproteins, which are produced by astrocytes, but not neurons; astrocytes and microglia, but not neurons, also express apolipoprotein E (apo E). Lipoproteins produced and released by astrocytes are discoidal in shape and contain apo E, phospholipids, and cholesterol, but lack in the core lipids (cholesterol esters or triglycerides). In contrast, lipoproteins found in the cerebrospinal fluid are round, contain a cholesterol ester core, are similar to plasma high density lipoproteins (HDL) [4], and are derived from the remodeling of astrocyte-secreted lipoproteins after they extract cholesterol from other cell types [5]. Lipoproteins can exit the brain through the cerebrospinal fluid, which thus represents an important route of brain cholesterol elimination [6]. Astrocyte-released lipoproteins can extract cholesterol from neurons (Fig. 1)[7], but can also interact with lipoprotein receptors on the neuronal membrane and trigger neuritogenesis and synaptogenesis;

Abbreviations: ABC, ATP-binding cassette; ACM, astrocyte conditioned medium; Apo, apolipoprotein; DIV, day in culture; HC, hydroxycholesterol; LXR, liver X receptor; PDL, poly-D-lysine; RA, retinoic acid; RXR, retinoid X receptor; siRNA, small interfering RNA

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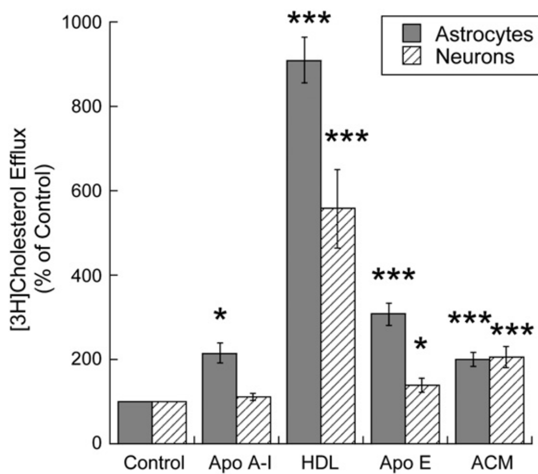

Fig. 1. Cholesterol acceptor-mediated cholesterol efflux from neurons and astrocytes. Primary rat cortical neurons and astrocytes were labeled with 1 μCi/ml [^{3}H]cholesterol for 24 h followed by a 6 h incubation with or without apo A-I (10 μg/ml), HDL (50 μg/ml), apo E_3 (15 μg/ml) or ACM. [^{3}H]Cholesterol was quantified in the medium and in the cellular lipids. In the absence of acceptors, 1.36 ± 0.137% and 1.403 ± 0.102% of total [^{3}H] cholesterol was found in the medium of astrocytes and neurons respectively. Data, expressed as percent of control, represent the mean (± SE) of 6–9 independent determinations; *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to control by the Dunnett's post hoc test.

whether this effect is due to the activation of signaling pathways activated by the binding of lipoproteins to lipoprotein receptors or to the uptake, by neurons, of cholesterol and other lipids from lipoproteins remains controversial [1,8,9]. Excess intracellular cholesterol in neurons activates the enzyme cholesterol 24-hydroxylase (a cytochrome P450, CYP46A1), which is expressed in neurons, but not in astrocytes [10,11], thereby metabolizing cholesterol to 24(S)-hydroxycholesterol (24(S)-HC) which freely exits neurons and the brain. Hydroxysterols, such as 24(S)-HC, through the activation of Liver X Receptors (LXR), inhibit cholesterol synthesis in many cell types, and upregulate the levels of the cholesterol transporters ATP binding cassette A1 (ABCA1) and ABCG1 and cholesterol efflux [12,13]. Interestingly, it has been reported that neuronal 24(S)-HC increases the levels of apo E, ABCA1 and ABCG1, and cholesterol efflux in astrocytes [14,15].

From this brief overview it is apparent that several functions regulating cholesterol levels in the brain are compartmentalized in either neurons or astrocytes, that the behavior of one cell type affects the response of the other, and that both cell types together contribute to the regulation of cholesterol levels in the brain. In the present study we investigated whether cholesterol transporter-mediated cholesterol efflux is also differentially regulated in astrocytes and neurons.

The ATP binding cassette (ABC) transporter proteins have been described as key players in regulating cholesterol efflux in the periphery. ABCA1 facilitates cholesterol efflux to lipid-free apo A-I and apo E and is involved in the biogenesis of lipoproteins, while the half-transporters ABCG1 and ABCG4 are involved in the further lipidation of nascent lipoproteins [16]. Various studies have shown that ABCA1, ABCG1 and ABCG4 are expressed in both astrocytes and neurons [17,18].

The regulation of cholesterol efflux in the brain has been extrapolated from the model of reversed cholesterol transport characterized in the cardiovascular system; however, several aspects of cholesterol efflux specific to the brain are not fully elucidated. Of particular relevance is the fact that no detailed studies have been carried out on the role of cholesterol transporters in mediating cholesterol efflux in primary neurons in cultures. It has been generally assumed that the presence of ABCA1 and ABCG1 cholesterol transporters in cells indicate their involvement in cholesterol efflux; we show here that, while this is indeed the case in astrocytes, neurons represent an exception to this rule, and that the upregulation of ABCA1 and ABCG1 transporters in neurons does not result in upregulation of cholesterol efflux.

In a previous study, we found that cholesterol efflux in cortical astrocytes is regulated by ABCA1 and ABCG1 and that ethanol and the LXR plus Retinoid X Receptors (RXR) agonists up-regulate ABCA1 and ABCG1, and increase cholesterol efflux, leading to cellular cholesterol depletion [14,15,19]. In the present study we compared ABCA1- and ABCG1-mediated cholesterol efflux in astrocytes and neurons by upregulating the levels of these two cholesterol transporters with LXR/RXR agonists and with ethanol, by inhibiting ABCA1 activity and by silencing ABCA1 and ABCG1 expression. Interestingly, we found that in cortical neurons, in contrast to what observed in astrocytes, ABCA1 and ABCG1 are not involved in acceptor-mediated cholesterol efflux. In addition, we found that while the expression of ABCA1 and ABCG1 was comparable in neurons and astrocytes, the expression of another cholesterol transporter, ABCG4, is much higher in neurons than in astrocytes. Silencing ABCG4 did not affect cholesterol efflux in astrocytes but decreased lipoprotein-mediated cholesterol efflux in neurons. Cholesterol efflux thus appears to be differentially regulated in astrocytes and neurons.

2. Materials and methods

2.1. Materials

ABCA1 and ABCG1 antibodies were from Novus Biologicals (Littleton, CO). Glial fibrillary acidic protein (GFAP), neuronal specific enolase (NSE) antibodies, Alexa fluor-488 and Alexa fluor-555 secondary antibodies, TRIzol reagent, Amplex red cholesterol assay kit, Stealth RNAi™ siRNAs selectively targeting rat ABCG1 and ABCG4, tissue culture medium, fetal bovine serum (FBS), B27 supplements, lipofectamine RNAiMAX Transfection Reagent, and Opti-MEM I were from Invitrogen (Carlsbad, CA). βIII-tubulin antibody was from Chemicon International (Temecula, CA). Lipoprotein-deficient serum (LPDS) was from Biomedical Technologies, Inc. (Stoughton, MA). Sulfo-N-hydroxysuccinimide-biotin, non-targeting and ABCA1 specific small interfering RNA (siRNA) were from Thermo Fisher Scientific (Pittsburgh, PA). Protein agarose A immunoprecipitation kit was from Roche Applied Science (Indianapolis, IN). GeneAmp® RNA PCR kit and rat ABCA1, ABCG1 and ABCG4 primers were from Applied Biosystems (Carlsbad, CA). [^{3}H]Cholesterol and [^{3}H]acetate were from Perkin Elmer (Covina, CA). Apo A-I, HDL and low density lipoprotein (LDL) were from EMD Biosciences (La Jolla, CA). Recombinant apo E_3 was from Leinco Technologies, Inc. (St. Louis, Missouri). TLC plates were from Macherey-Nagel (Bethlehem, PA). Rat astrocyte nucleofector kit and rat neuron nucleofector kit were from Lonza (Walkersville, MD). i-Fect™ siRNA Transfection Reagent was from Neuromics (Edina, MN). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Time-pregnant Sprague–Dawley rats were purchased from Charles River (Wilmington, MA).

2.2. Cell culture

Primary cortical astrocytes were prepared from E21 Sprague–Dawley fetuses, as previously described [20]. Astrocytes were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (FBS/DMEM medium). The treatments were carried out in serum-free DMEM supplemented with 0.1% Bovine Serum Albumin (BSA) and antibiotics.

Primary cortical neurons were prepared from E21 Sprague–Dawley fetuses following a method optimized in our laboratory by modifying a previously described method [21]. Briefly, cortices were dissected and incubated in 2 mg/ml papain with 80 μg/ml DNAase and 5 mM $MgCl_2$ for 30 min at 37 °C. Cells were then centrifuged at 150×*g*, the

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