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Acetoacetyl-CoA synthetase is essential for normal neuronal development

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ABSTRACT

Cholesterol and fatty acids are essential, abundant components of neuronal tissue. Acetoacetyl-CoA synthetase (AACS) is a ketone body-utilizing enzyme for the synthesis of cholesterol and fatty acids and is highly expressed in the brain. In this study, we investigated the regulation of AACS during neurite outgrowth to clarify the physiological role of AACS in neurogenesis. Messenger RNA levels and the expression of AACS were increased during neurite outgrowth in Neuro-2a cells. The expression of HMG-CoA reductase, a key enzyme of cholesterol biosynthesis, was also increased. ChIP assays showed that the amount of SREBP-2, a key transcription factor of cholesterol synthesis, interacted with the AACS promoter was increased during neurite outgrowth, and knockdown of SREBP-2 down-regulated the mRNA levels of AACS in Neuro-2a cells. The expression of AACS in the brains of mouse embryos was dramatically increased between E16.5 and E18.5. Moreover, knockdown of AACS in primary neurons caused decreases in the expression of MAP-2 and NeuN, which are markers of neuronal differentiation, as well as synaptopodin, a marker of spine apparatus. These results suggest that AACS is regulated by SREBP-2 and involves in the normal development of neurons.

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1. Introduction

Cholesterol and fatty acids are critical constituents of cellular membranes and are found at particularly high concentrations in neural tissue [1]. They play a key role in embryonic and fetal development, especially in neuronal development [2,3]. The generation of acetyl-CoA in the cytosol is the first step in lipid biosynthesis. Therefore, acetyl-CoA is a crucial substrate for the cholesterol and fatty acids synthesis pathway. Cytosolic acetyl-CoA is produced by ATP-citrate lyase (ACL), which cleaves citrate to produce acetyl-CoA and oxaloacetate in the cytosol [4,5]. Acetyl-CoA is also produced from acetoacetyl-CoA by the action of cytosolic acetoacetyl-CoA thiolase [6].

Ketone bodies, including acetoacetate and β-hydroxybutyrate, are known to be significant energy sources for various tissues. This is particularly true for the brain, which has no substantial non-glucose-derived energy source [7]. Recent studies showed that ketone bodies are involved in insulin secretion and neonatal metabolic homeostasis, suggesting that they play an important role not only in the fasting state but also in the normal state [8,9]. Mitochondrial succinyl-CoA:3-ketoacid CoA transferase (SCOT; EC 2.8.3.5) is the enzyme thought to be responsible for ketone body utilization for energy production [10]. However, it has been shown that acetoacetate is activated to its CoA ester by a cytosolic acetoacetate-specific ligase, acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16), for the direct production of acetyl-CoA, which is then used for the synthesis of physiologically important lipidic substances, such as cholesterol and fatty acids, in the cytosol [11]. We purified AACS as a discrete enzyme from the bacterial strain *Zoogloea ramigera* I-16-M [12] and from rat liver for the first time [13], and we demonstrated that its activity in rats is remarkably changed under certain physiological or pathological conditions, such as development [14], pravastatin administration [15], and streptozotocin (STZ)-induced diabetes [16].

Recently, we found that AACS mRNA was highly expressed in the brains of humans and rats and enriched in neuronal-like cells in the hippocampus and cortical regions [17,18], where the localization profile of AACS mRNA was different from that of SCOT mRNA. These results suggest that ketone body utilization via AACS is an important pathway in neural tissue. To clarify the physiological role of AACS in neurons, we examined the expression of AACS during neurite outgrowth and embryogenesis and the effects of AACS knockdown on neuronal development.

2. Materials and methods

2.1. Cell culture

Mouse Neuro-2a cells (JCRB No. IFO50081) were purchased from the Health Science Research Resources Bank (Japan). The cells

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were maintained in Eagle's minimal essential medium (E-MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C in an atmosphere of 5% CO_2. Cells were seeded at 5.0×10^4 cells/mL, incubated for 24 h, and then treated with serum-free E-MEM containing retinoic acid (Sigma).

2.2. Analysis of RNA

Total RNA was purified from Neuro-2a cells using an RNeasy mini kit (Qiagen) or an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Total RNA from Neuro-2a cells was analyzed by real-time RT-PCR as described previously [19]. cDNA and gene-specific primers were added to the SYBR Green PCR Master Mix (SYBR Premix Ex Taq, Takara) and subjected to PCR amplification in an Applied Biosystems StepOne (Applied Biosystems). The amplified transcripts were quantified using the standard curve method with β-glucuronidase (Gusb) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls. The real-time PCR primers were designed based on data from GenBank (according to accession numbers) using Primer Express software (Applied Biosystems).

2.3. Western blot analysis

Mouse hepatocytes were lysed in RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1% sodium lauryl sulfate and 0.1% sodium deoxycholate]. Cell debris was removed by centrifugation at 14,000*g* for 15 min at 4 °C, and the resulting supernatant (cell lysate) was used for Western blot analysis. Protein concentrations in the cell lysates were measured using a Bio-Rad protein assay kit.

For Western blotting, 15 μg of protein was separated using SDS polyacrylamide gel electrophoresis (SDS–PAGE, 7.5% gel) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed with specific antibodies and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. HRP was detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.4. Microscopic procedures

Neuro-2a cells were grown on cover glasses. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and blocked with 3% (w/v) bovine serum albumin (BSA) for 30 min. After blocking, the cells were incubated overnight at 4 °C in Can Get Signal immunostain solution A (Toyobo) containing anti-AACS antibody. Then, the cells were washed three times with 0.1% BSA in PBS and incubated with Alexa Fluor 488 secondary antibody (Invitrogen) at room temperature for 1 h in the dark. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution.

For DNA visualization, preparations were stained with propidium iodide (Calbiochem). After incubation with the secondary antibody, the cells were incubated with 1 mg/mL RNase in PBS. Then, the cells were stained with propidium iodide for 15 min. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution. Digital images of fixed cells were taken with a Confocal Laser Scanning Microscope (Bio-Rad).

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out as described previously [20]. Briefly, Neuro-2a cells were fixed in normal culture medium with formaldehyde at a final concentration of 1% for 10 min at 37 °C. After centrifugation, the cells were suspended in 50 mM Tris–HCl (pH 8.0) containing 1% SDS, 10 mM EDTA and protease inhibitor cocktail (Sigma). Chromatin was sheared by sonication on ice. After centrifugation to remove the cell debris, the supernatant was diluted with 50 mM Tris–HCl (pH 8.0) containing 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate and protease inhibitor cocktail (Sigma). Aliquots of the supernatant were incubated for 15 min at 4 °C with protein G-sepharose beads (GE Healthcare). Immunoprecipitation was carried out overnight at 4 °C with the following antibodies (1 μg): anti-normal rabbit IgG (Santa Cruz Biotechnology) and anti-SREBP-2 (Abcam). The immunoprecipitated DNA–protein complex was eluted with 200 μL of 10 mM Tris–HCl (pH 8.0) containing 300 mM NaCl, 5 mM EDTA and 0.5% SDS. DNA-protein cross-links were reversed at 65 °C for 4 h and then subjected to PCR amplification. The sequences of the PCR primers included the following: AACS, forward 5′-GAATGAACGAACGAACGAGG-3′ and reverse 5′-ACCACGCCCTCTTCTGTAAC-3′.

2.6. RNA interference

To knock down SREBP-2 gene expression, cells were transfected with a validated pool of siRNA duplexes directed against mouse SREBP-2 (Dharmacon). Neuro-2a cells were transfected with the indicated siRNAs (40 nM) using LipofectAMINE RNAiMAX Reagent (Invitrogen). After 24 h, total RNA was extracted and analyzed using real-time PCR.

2.7. Primary neuron

Mouse primary cortical cultures were prepared from gestational day 14 embryos as described previously with minor modifications [21]. Briefly, brains were dissected and digested for 20 min in 15 units papain (Worthington) and 0.01% DNase1 (Roche). Cells were cultured in Neurobasal medium (Miltenyi Biotec) containing 2% MACS Supplement B-27 (Miltenyi Biotec), 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen) on plastic dishes coated with poly-L-lysine. After 3 days, the cells were exposed to 4 μM Cytosine β-D-arabinofuranoside to inhibit the proliferation of non-neuronal cells.

2.8. Virus production and transduction

Lenti-X 293T cells (Takara) were seeded in 100 mm poly-L-lysine plates (Iwaki) and transfected with pGreenPuro shRNA vector (System Biosciences) and packaging mix (Invitrogen) according to the manufacturer's protocols. The media were replaced with 10% FBS/D-MEM at approximately 14 h post-transfection, and the viral supernatants were collected 48 h after transfection.

To knock down AACS expression, primary neurons were infected with lentiviruses encoding shRNA sequences against 2 different mouse AACS sequences (shAACS #1, GTTCAGTGGAATCGTCTAC; shAACS #2, CCGTGTGGTCGGCTATCTA) or with control viruses encoding shRNA sequences against pGL3 (shcontrol #1, CTTACGCTGAGTACTTCGA) and LacZ (shcontrol #2, ATCGCTGATTTGTGTAGTC). After 2 days, the culture media were replaced. Subsequently, the medium was changed every 5 days, and protein was extracted at the indicated time.

3. Results

3.1. Induction of a ketone body-utilizing enzyme during neurite outgrowth

Previously, we demonstrated that AACS is highly expressed in the brains of mice and rats and showed neuron-specific expression of the AACS gene in the cerebral cortex [18,20]. These results suggest the involvement of ketone body utilization in neuronal functions. To clarify the physiological role of AACS in neurons,

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