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INCREASED EXPRESSION OF ACYL-COENZYME A: CHOLESTEROL ACYLTRANSFERASE-1 AND ELEVATED CHOLESTERYL ESTERS IN THE HIPPOCAMPUS AFTER EXCITOTOXIC INJURY

J.-H. KIM,[a] S.-M. EE,[a] J. JITTIWAT,[b] E.-S. ONG,[c] A. A. FAROOQUI,[d] A. M. JENNER[e] AND W.-Y. ONG[a,f]*

[a]*Department of Anatomy, National University of Singapore, Singapore 119260*

[b]*Department of Physiology, Khon Kaen University, Thailand*

[c]*Department of Epidemiology and Public Health, National University of Singapore, Singapore 119260*

[d]*Department of Molecular and Cellular Biochemistry, The Ohio State University, USA*

[e]*Illawara Health and Medical Research Institute, University of Wollongong, NSW 2522, Australia*

[f]*Neurobiology and Ageing Research Programme, National University of Singapore, Singapore 119260*

Abstract—Significant increases in levels of cholesterol and cholesterol oxidation products are detected in the hippocampus undergoing degeneration after excitotoxicity induced by the potent glutamate analog, kainate (KA), but until now, it is unclear whether the cholesterol is in the free or esterified form. The present study was carried out to examine the expression of the enzyme involved in cholesteryl ester biosynthesis, acyl-coenzyme A: cholesterol acyltransferase (ACAT) and cholesteryl esters after KA excitotoxicity. A 1000-fold greater basal mRNA level of ACAT1 than ACAT2 was detected in the normal brain. ACAT1 mRNA and protein were upregulated in the hippocampus at 1 and 2 weeks after KA injections, at a time of glial reaction. Immunohistochemistry showed ACAT1 labeling of oligodendrocytes in the white matter and axon terminals in hippocampal CA fields of normal rats, and loss of staining in neurons but increased immunoreactivity of oligodendrocytes, in areas affected by KA. Gas chromatography-mass spectrometry analyses confirmed previous observations of a marked increase in level of total cholesterol and cholesterol oxidation products, whilst nuclear magnetic resonance spectroscopy showed significant increases in cholesteryl ester species in the degenerating hippocampus. Upregulation of ACAT1 expression was detected in OLN93 oligodendrocytes after KA treatment, and increased expression was prevented by an antioxidant or free radical scavenger *in vitro*. This suggests that ACAT1 expression may be induced by oxidative stress. Together, our results show elevated ACAT1 expression and increased cholesteryl esters after KA excitotoxicity. Further studies are necessary to determine a possible role of ACAT1 in acute and chronic neurodegenerative diseases. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

*Correspondence to: W.-Y. Ong, Department of Anatomy, National University of Singapore, Singapore 119260. Tel: +65-65163662; fax: +65-67787643.
E-mail address: wei_yi_ong@nuhs.edu.sg (W.-Y. Ong).

Abbreviations: ACAT, acyl-coenzyme A: cholesterol acyltransferase; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; KA, kainate; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline.

Key words: cholesterol, cholesteryl esters, neuroinflammation, cholesterol oxidation products, oxidative stress, ACAT1.

Cholesteryl esters are formed by cholesterol and fatty acyl coenzyme A such as arachidonoyl-coenzyme A, and hydrolyzed back to cholesterol continuously, forming the cholesterol/cholesteryl ester cycle (Brown et al., 1980). Hydrolysis of cholesteryl esters provides cholesterol and fatty acids for membrane formation and maintenance, lipoprotein trafficking, lipid detoxification, evaporation barriers, and fuel in times of stress or nutrient deprivation (Turkish and Sturley, 2009). On the other hand, overproduction of cholesteryl esters may be associated with the pathology of obesity, atherosclerosis (Leon et al., 2005; Puglielli et al., 2001; Zhao et al., 2009), and Alzheimer's disease (AD) (Puglielli et al., 2001). Cholesterol is crucial for the integrity of the cell membrane, cell signaling, and gene transcription (Bjorkhem and Meaney, 2004) and disruption in homeostasis is observed in neurodegenerative diseases such as Niemann-Pick C disease, multiple sclerosis (Leoni et al., 2005), and AD (Adibhatla and Hatcher, 2008).

The equilibrium between free and esterified cholesterol is controlled by acyl-coenzyme A: cholesterol acyltransferase (ACAT), an integral membrane protein localized to the endoplasmic reticulum (ER) (Chang et al., 2009). Two isoforms of ACAT, ACAT1 and ACAT2 have been identified in neural and non-neural tissues (Rudel et al., 2001). ACAT1 is a 56 kDa protein present in the brain, liver, adrenal glands, and macrophages (Lee et al., 1998). ACAT2 is mainly expressed in hepatocytes and intestinal mucosal cells (Rudel et al., 2001). ACAT not only plays a role in the reesterification process of cholesterol (Zhang et al., 2003) but also acts as a cholesterol sensor, and expression is induced by increased cholesterol concentrations (Brown and Jessup, 2009). Use of ACAT inhibitors has been explored in the treatment of atherosclerosis (Alegret et al., 2004; Leon et al., 2005) and models of AD (Hull et al., 2006; Huttunen and Kovacs, 2008). ACAT is reported to modulate Aβ formation through control of free cholesterol and cholesteryl ester levels (Cordy et al., 2006).

Increased levels of cholesterol and cholesterol oxidation products (COPs) have been observed in rat hippocampus after excitotoxic injury induced by the potent glutamate analog, kainate (KA) (He et al., 2006; Kim et al., 2009; Ong et al., 2003, 2010b). This is a model of spinal cord injury, head injury, stroke, and neurodegenerative diseases. The hippocampus is one of the most vulnerable brain regions to

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KA, which causes neuronal cell loss in cornu ammonis 1 (CA1) and CA3 pyramidal cells and interneurons of the dentate hilus (Ben-Ari, 1985; Nadler et al., 1978). Increased cholesterol and COPs may enhance exocytosis in surviving neurons resulting in propagation of excitotoxic injury (Ma et al., 2010; Zhang et al., 2009). Thus far however, it is unclear whether increased cholesterol after neuronal injury is in the free or esterified form. The present study was carried out, to elucidate the expression of ACAT isoforms in the normal brain and after KA lesions. The levels of free, esterified, and total cholesterol were also determined in the lesioned hippocampus.

EXPERIMENTAL PROCEDURES

Kainate injection

Adult male Wistar rats weighing 200 g each were purchased from Centre for Animal Resources (CARE) of the National University of Singapore. They were anaesthetized by ketamine and xylazine cocktail (prepared with 7.5 ml ketamine (75 mg/kg), 5 ml xylazine (10 mg/kg), and 7.5 ml sterile water) and KA (Tocris Bioscience, MO, USA). 1 μl of 1 mg/ml was i.c.v. injected into the right lateral ventricle through a small craniotomy as previously described (Kim and Ong, 2009). The animals were assessed according to the Racine scale of seizure severity (Racine, 1972) and found to have scores of at least 3 out of 5. Rats were deeply anesthetized and sacrificed at 1 day, 1 week, and 2 weeks after injection as described in the experiments below. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

RT-PCR analyses

Twenty rats were used for this portion of the study, consisting of untreated controls, and animals injected with KA and sacrificed 1 day, 1 week, and 2 weeks after injection (five rats per group). The lesioned right hippocampi were quickly removed and immersed in RNAlater® (Ambion, TX, USA), snap frozen in liquid nitrogen, and kept at −80 °C till analyses. Total RNA was extracted and isolated using TRizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNeasy® Mini Kit (Qiagen, Inc., CA, USA) was used to purify the RNA. The samples were then reverse transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, CA, USA). Reaction conditions were 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 s. Real-time PCR amplification was then carried out in the 7500 Real time PCR system using TaqMan® Universal PCR Master Mix (Applied Biosystems). ACAT1, ACAT2, and β-actin probes were used according to the manufacturers' instructions (Applied Biosystems). The PCR conditions were: an initial incubation of 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were carried out in triplicate. The threshold cycle, CT, which correlates inversely with the levels of target mRNA, was measured as the number of cycles at which the reporter fluorescence emission exceeds the preset threshold level. The amplified transcripts were quantified using the comparative CT method (Livak and Schmittgen, 2001), with the formula for relative fold change$=2^{-\Delta\Delta CT}$. The mean was calculated and possible significant differences between KA injected and control hippocampal specimens were analyzed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. $P<0.05$ was considered significant.

Western blot analyses

Twelve rats were used for this portion of the study, consisting of untreated controls, and animals injected with KA and sacrificed 1 day, 1 week, and 2 weeks after injection (three rats per group). The hippocampus was harvested and homogenized in 10 volumes of ice-cold buffer containing 0.32 M sucrose, 4 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 0.25 mM dithiothreitol. After centrifugation at 1000 g for 10 min, the supernatant was collected and protein concentrations in the preparation were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA). Total proteins (40 μg) were resolved in 10% sodium dodecyl sulfate (SDS) polyacrylamide gels under reducing conditions and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Non-specific binding sites on the PVDF membrane were blocked by incubation with 5% non-fat milk for 1 h. The PVDF membrane was then incubated overnight with rabbit polyclonal antibody to ACAT1 (Cayman, MI, USA, 1:500 dilution in Tris–buffered saline (TBS] at 4 °C. After washing with 0.1% Tween-20 in TBS, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin IgG (1:2000 dilution) (Amersham) for 1 h at room temperature. The protein was visualized with an enhanced chemiluminescence kit (PiPCe, Rockford, IL, USA). Loading controls were carried out by incubating the blots at 50 °C for 30 min with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–hydrochloride, pH 6.7), followed by reprobing with a mouse monoclonal antibody to β-actin (Sigma-Aldrich, MO, USA; diluted 1:10,000 in TTBS) and horseradish peroxidase-conjugated anti-mouse IgG (Pierce, Rockford, IL, USA, 1:2000 in TTBS). Exposed films containing blots were scanned and the densities of the bands measured using Gel-Pro Analyzer 3.1 program (Media Cybernetics, Silver Spring, MD, USA). The densities of the ACAT1 bands were normalized against those of β-actin, and the mean ratios calculated. Possible significant differences between the values from the KA-injected and control rats were analyzed using one-way ANOVA with Bonferroni's multiple comparison post-hoc test. $P<0.05$ was considered significant.

Immunoperoxidase labeling

Three untreated control rats and three 1 week post-KA injected rats were used for this portion of the study. The rats were deeply anesthetized and perfused through the left cardiac ventricle with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The fixed brain tissues were removed and sectioned coronally at 100 μm using a Vibrating microtome. The sections were divided for Cresyl Fast Violet (Nissl) and immunohistochemical staining as follows: Sections were washed for 3 h in phosphate-buffered saline (PBS) to remove traces of fixative and then incubated overnight with a rabbit polyclonal antibody to ACAT1 (Cayman, MI, USA, diluted 1:200 in PBS). The sections were then washed three times in PBS and incubated for 1 h at room temperature in a 1:200 dilution of biotinylated horse anti-rabbit IgG (Vector, Burlingame, CA, USA). The sections were reacted for 1 h at room temperature with an avidin-biotinylated horseradish peroxidase complex, and visualized by treatment for 5 min in 0.05% 3,3-diaminobenzidine tetrahydrochloride solution in Tris–buffer containing 0.05% hydrogen peroxide. The color reaction was stopped with several washes of Tris–buffer. Some sections were mounted on glass slides and lightly counterstained with Methyl Green before coverslipping. Control sections were incubated with antigen-absorbed antibody instead of primary antibody (prepared by incubating 200 μg/ml immunizing peptide with ACAT1 antibody overnight).

Double immunofluorescence labeling

Four rats were used for this portion of the study. The rats were injected with KA and sacrificed after 1 week by deep anaesthesia and perfusion through the left cardiac ventricle with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected out, and blocks containing the hippocam-