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Acyl-CoA dehydrogenase 9 (ACAD 9) is the long-chain acyl-CoA dehydrogenase in human embryonic and fetal brain ☆

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Abstract

We recently reported the expression and activity of several fatty acid oxidation enzymes in human embryonic and fetal tissues including brain and spinal cord. Liver and heart showed expression of both very long-chain acyl-CoA dehydrogenase (VLCAD) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) mRNA. However, while mRNA expression of LCHAD could be clearly detected in the retina and spinal cord, expression of VLCAD mRNA was low to undetectable in these tissues. Nevertheless, abundant acyl-CoA dehydrogenase (ACAD) activity was detected with palmitoyl-CoA as substrate in fetal central nervous tissue. These conflicting data suggested the presence of a different long-chain ACAD in human embryonic and fetal brain. In this study, using in situ hybridization as well as enzymatic studies, we identified acyl-CoA dehydrogenase 9 (ACAD 9) as the long-chain ACAD in human embryonic and fetal central nervous tissue. Until now, no clinical signs and symptoms of central nervous system involvement have been reported in VLCAD deficiency. A novel long-chain FAO defect, i.e., ACAD 9 deficiency with only central nervous system involvement, could, if not lethal during intra uterine development, easily escape proper diagnosis, since probably no classical signs and symptoms of FAO deficiency will be observed. Screening for ACAD 9 deficiency in patients with undefined neurological symptoms and/or impairment in neurological development of unknown origin is necessary to establish if ACAD 9 deficiency exists as a separate disease entity.

Keywords: Human embryo; Metabolism; Central nervous tissue; ACAD 9

The mitochondrial β-oxidation of fatty acids plays an indispensable role in energy metabolism in humans. During moderately intense exercise the heart and muscle preferentially use fatty acids as a substrate for energy production. During fasting, fatty acid oxidation (FAO) in the liver produces ketone bodies that are exported into the circulation and can be used by all peripheral tissues, but are preferentially used in the brain and in the heart [1]. Before the activated long-chain fatty acids, i.e., acyl-CoAs, can be oxidized, they have to be transported into the mitochondrion via the carnitine cycle, which involves the subsequent action of Carnitine Palmitoyl-CoA Transferase 1 (CPT1), Carnitine Acyl-Carnitine Translocase (CACT), and Carnitine Palmitoyl-CoA Transferase 2 (CPT2). Once inside the mitochondria, the fatty acyl-CoA esters undergo β-oxidation via a four-step mechanism, involving dehydrogenation, hydration, another dehydrogenation, and thiolytic cleavage. For each reaction of the β-oxidation spiral

☆ *Abbreviations:* ACAD, acyl-CoA dehydrogenase (EC 1.3.99); FAO, fatty acid oxidation; CPT1, Carnitine Palmitoyl-CoA Transferase 1 (EC 2.3.1.21); CPT2, Carnitine Palmitoyl-CoA Transferase 2 (EC 2.3.1.21); LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); MTP, mitochondrial trifunctional protein (EC 4.2.1.17, EC 1.1.1.35, EC 2.3.1.16); VLCAD, very long-chain acyl-CoA dehydrogenase (EC 1.3.99.13); ACAD 9, acyl-CoA dehydrogenase 9; LCAD, long-chain acyl-CoA dehydrogenase (EC 1.3.99.3).

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several chain-length specific iso-enzymes are known. Oxidation of long-chain fatty acids starts with the first dehydrogenation catalyzed by the very long-chain acyl-CoA dehydrogenase (VLCAD) enzyme. Palmitoyl-CoA (C16:0-CoA) is the preferred substrate for VLCAD. The next three steps in long-chain fatty acid metabolism are catalyzed by the mitochondrial trifunctional protein (MTP). This enzyme is made up of four α- and β-subunits, and harbors the activity of the next three enzymes required for the oxidation of long-chain fatty acids: the long-chain enoyl-CoA hydratase, the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), and the long-chain 3-keto-acyl-CoA thiolase. This last step results in a 2-carbon shortening of the fatty acid and the formation of 1 NADH, 1 $FADH_2$, and acetyl-CoA. Acetyl-CoA enters the Krebs' cycle, and NADH and $FADH_2$ enter the electron transport chain to produce the final product ATP, CO_2, and water. The importance of the mitochondrial long-chain FAO system is substantiated by the existence of different inborn errors of metabolism in which mitochondrial long-chain FAO is impaired [2–4]. In general, clinical signs and symptoms of FAO disorders are related to the lack of energy for many metabolic functions, resulting in hypoketotic hypoglycaemia, multiple organ failure with cardiomyopathy, liver disease, and skeletal myopathy. Symptoms of central nervous system involvement, i.e., peripheral neuropathy and pigmentary retinopathy, are only observed in LCHAD and MTP deficiency.

We recently reported the presence of several FAO enzymes in human embryonic and fetal tissues including brain and spinal cord, by demonstrating mRNA expression and enzyme activity. Furthermore, in the same tissues a broad range of acylcarnitines was found, implying an active FAO [5,6]. The observed pattern of expression of LCHAD and VLCAD during human embryonic development correlates well with the spectrum of clinical signs and symptoms reported in patients with VLCAD or LCHAD/MTP deficiency. Liver and heart showed an abundant expression of both VLCAD and LCHAD mRNA. Remarkably, while LCHAD mRNA was expressed in the developing brain, retina, spinal cord, and in the dorsal root ganglia, no clear expression of VLCAD could be detected in these tissues [5]. As the oxidation of fatty acids involves the consecutive action of four different enzymatic steps, all involved enzymes need to be present in order to allow a full cycle of the β-oxidation spiral. These conflicting data suggest the active presence of a different long-chain acyl-CoA dehydrogenase in embryonic and fetal brain.

Recently a novel homologue, ACAD 9, which belongs to the acyl-CoA dehydrogenase family was identified [7]. ACAD 9 shares approximately 47% amino acid identity and 65% similarity with human VLCAD. In addition, ACAD 9 and VLCAD are both bound to the inner mitochondrial membrane, which is a unique feature as the other ACADs are all located in the inner mitochondrial matrix. Like VLCAD, ACAD 9 shows activity towards palmitoyl-CoA (C16:0) and stearoyl-CoA (C18:0). However, it has an optimal activity with C16:1-CoA [7,8]. ACAD 9 mRNA was found to be ubiquitously expressed in most tissues of human adults, with a high level of expression in heart, skeletal muscle, kidney, liver, and in brain [7,7]. Presence of ACAD 9 in embryonic and fetal central nervous tissuses might explain the discrepancy between the lack of VLCAD mRNA expression in the embryonic and fetal central nervous system on the one hand, and the presence of enzymatic activity with palmitoyl-CoA on the other hand.

In this study, we identified ACAD 9 as the long-chain acyl-CoA dehydrogenase in human embryonic and fetal brain and central nervous tissue, using in situ hybridization as well as enzymatic studies.

Materials and methods

Human embryonic and fetal tissue. Human embryos and fetal tissues were collected from legally terminated pregnancies in agreement with the French law as well as the recommendations of the local Ethics Committee. Written informed maternal consent was obtained after termination of pregnancy. Tissues were prepared as described previously [9]. For the in situ hybridization studies in sections of intact embryos, two embryos, at Carnegie stage 18 and 23 (day 49 and 8.5 weeks of development), were used. For later hybridization studies tissues of two fetuses (17 and 20 weeks of development) were used. Every fetus was examined by a fetopathologist for evident pathology and malformations and fetal chromosomes were screened for common genetic abnormalities. If relevant pathology or disease was excluded, fetal tissue was used for this study. Also, separate frozen central nervous tissues of three different embryos (6, 7.5, and 8.5 weeks of development).

In situ hybridization probes. Templates used for the generation of hybridization probes for ACAD 9 were amplified by PCR from human genomic DNA. To generate a sense or an antisense RNA probe the following oligonucleotide primers were used: For the antisense: ACAD 9-forward with a T7 promotor: 5′-TAA TAC gAC TCA CTA TAg ggA gAA gTA TgA CCT ACC TCA CAg C-3″ and ACAD 9-reverse: 5′-TCC ATg gTT gCC TgT CAg CC-3′. For the sense: ACAD 9-forward: 5′-AgT ATg ACC TAC CTC ACA gC-3′ and ACAD 9-reverse with a T7 promotor: 5′-TAA TAC gAC TCA CTA TAg ggA gAT CCA Tgg TTg CCT gTC AgC C-3′. The inserts were verified by sequencing to exclude PCR-introduced errors.

Hybridization. Hybridization and post-hybridization washes were carried out according to standard protocols [10]. Slides were dehydrated, exposed to Biomax MR X-ray films (Amersham, UK) for 3 days, and dipped in Kodak NTB2 emulsion (Kodak, USA) for 3 weeks at +4 °C. Developed and toluidine blue counterstained slides were analyzed with dark and bright field illumination. Adjacent slides were hematoxylin/eosin/saffron stained for histological studies. No hybridization signal was detected with the $\alpha[^{35}S]$-labeled sense probes.

Enzymatic studies. Tissue samples, stored at −70 °C, were thawed. The activities of acyl-CoA dehydrogenase and 3-hydroxy-acyl-CoA dehydrogenase activity were measured as described elsewhere [11,12], using palmitoyl-CoA and 3-keto-palmitoyl-CoA as substrates, respectively. Control values for enzymatic activity in human liver were measured in our laboratory, using the same techniques as used for embryonic studies.

Results

ACAD 9 mRNA expression in human embryonic and fetal brain and spinal cord

At 7 and 8.5 weeks of development, specific ACAD 9 mRNA expression was observed in the spinal cord and

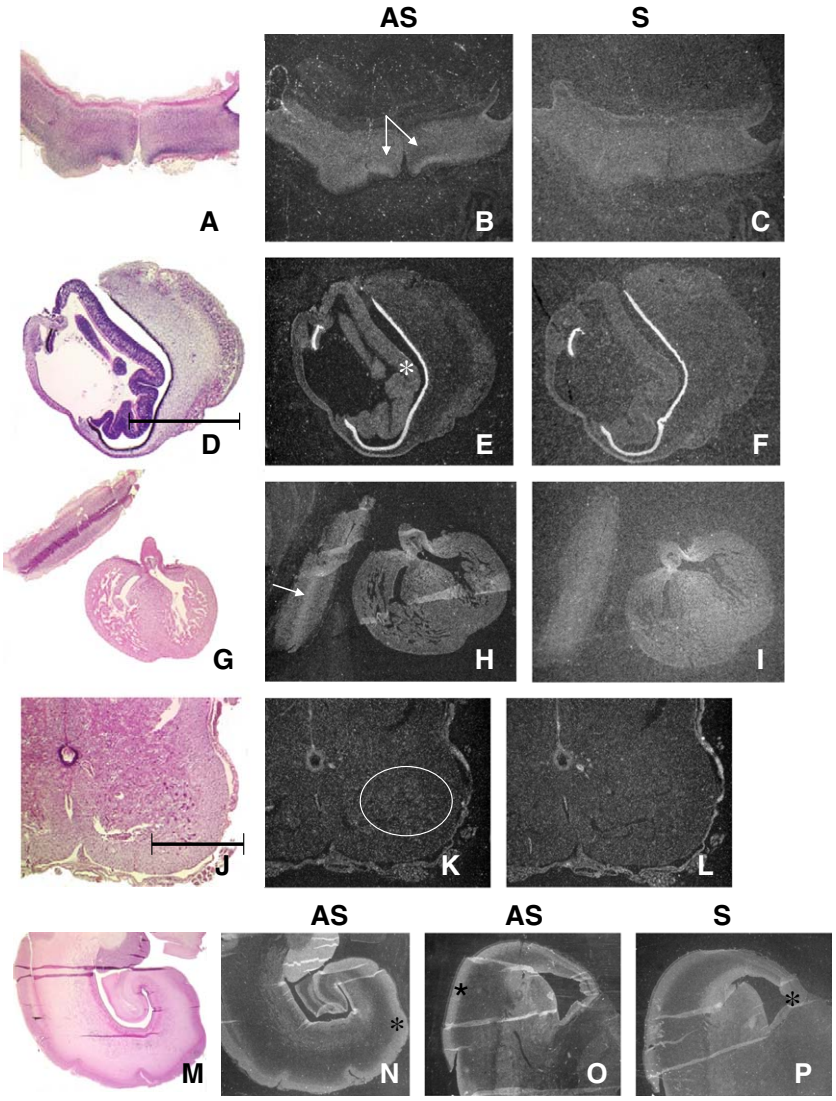

Fig. 1. ACAD 9 mRNA expression in human embryonic and fetal brain and spinal cord. (A, D, G, J, and M) Hematoxylin-eosin (HE) stained sections, adjacent to the slides hybridized with the ACAD 9 gene. The slides with the sense hybridized probe are shown as a control. (A–F) Section of the spinal cord and eye of a 7-week-old embryo. Expression in spinal cord (arrow) and retina (∗). (G–I) Section of the spinal cord and heart of an 8.5-week-old embryo. Expression in spinal cord (arrow). (J–L) Transverse section through the spinal cord of a 20-week-old fetus, showing expression in the motorneurons of the anterior horn (circle). (M–P) Section through part of the temporal brain of a 17-week-old fetus, showing expression in the cortex (∗). Scale bar (HE stained sections) = 1 mm.

neural retina (Fig. 1B, C, E, F, H, and I). At 20 weeks of development expression was conserved in the spinal cord. A more specific expression pattern could be detected in a subpopulation of cells in the anterior horn of the spinal cord (Fig. 1K and L). In the developing brain of a fetus at 17 weeks of development ACAD 9 expression was observed in the cortex (Fig. 1N, O, and P).

Acyl-CoA dehydrogenase and 3-hydroxy-acyl-CoA dehydrogenase activity measurements in human brain and spinal cord (6–8.5 weeks of development)

In human embryonic brain and spinal cord, low but significant 3-hydroxy-acyl-CoA dehydrogenase activity was measured with 3-keto-palmitoyl-CoA as substrate, with an activity of 23% and 18%, respectively, of the corresponding activity in human adult liver (Fig. 2). In addition, acyl-CoA dehydrogenase activity with palmitoyl-CoA could be clearly detected in embryonic brain and spinal cord at low to near-normal levels of 11% and 75%, respectively, when compared to adult liver (Fig. 2).

Discussion

In our previous work, we observed clear expression of LCHAD but not of VLCAD mRNA in human embryonic brain and spinal cord. However, despite the lack of VLCAD expression, enzymatic studies revealed palmitoyl-CoA dehydrogenase activity [5]. These data are well in line with the study of Tyni et al., in which immunohistochemistry

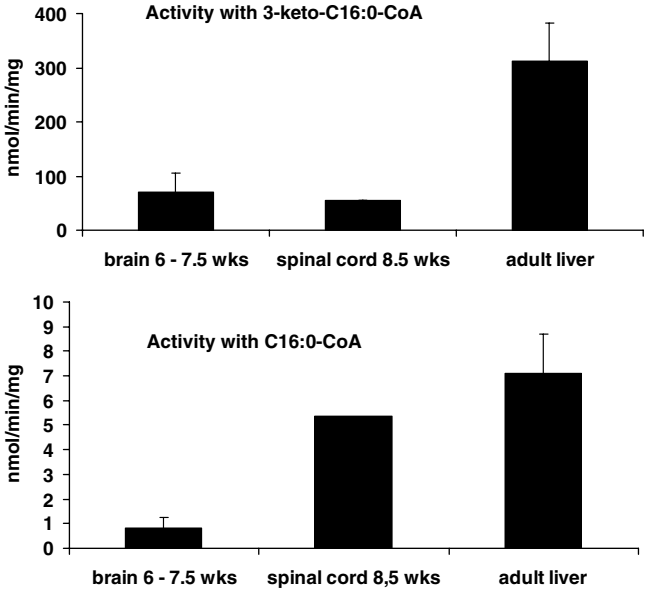

Fig. 2. Activity with 3-keto-C16:0-CoA and with C16:0-CoA in brain 6–7.5 weeks of development ($n = 2$), spinal cord 8.5 weeks of development ($n = 1$). Values are average ± standard deviation. Each tissue was analyzed in duplicate. Human adult liver: $n = 3$ for VLCAD and $n = 10$ for LCHAD.

of the human eye and brain using antibodies to FAO enzymes was performed [13]. This study showed labeling with LCHAD antibodies but not with VLCAD antibodies in brain [13]. These observations were puzzling given the notion that VLCAD and MTP, which harbors the activity of LCHAD, are believed to act as a metabolic unit catalyzing a complete cycle of long-chain fatty acid β-oxidation. The results described in this paper further substantiate that embryonic and fetal brain and spinal cord contain both long-chain acyl-CoA dehydrogenase activity and 3-hydroxy-acyl-CoA dehydrogenase activity as measured with palmitoyl-CoA and 3-keto-palmitoyl-CoA as substrates. Our finding reported in this paper which shows that ACAD 9 is expressed in embryonic and fetal central nervous system tissues may provide an explanation for the discrepancy between the enzymatic dehydrogenase activity with palmitoyl-CoA as substrate and the absence of VLCAD mRNA expression in these tissues.

The first step in long-chain FAO can be performed by three different enzymes: VLCAD, long-chain acyl-CoA dehydrogenase (LCAD), and ACAD 9. Although LCAD can use the long-chain fatty acid palmitoyl-CoA as a substrate, it is not its preferred substrate. LCAD plays an important role in the β-oxidation of long branched-chain acyl-CoAs [14,15] and of unsaturated fatty acid thioesters [16], and is only very weakly expressed in rat and mouse central nervous tissue [17,18]. However, ACAD 9 shows a high similarity with VLCAD and shows activity with palmitoyl-CoA as substrate [7,8]. Moreover, ACAD 9 was found to be abundantly expressed in adult human brain [7]. The expression of ACAD 9 in human embryonic and fetal brain and spinal cord as detected in our study correlates well with the observed activity with palmitoyl-CoA in these tissues. Since little to no expression of VLCAD could be detected in brain and spinal cord, we conclude that ACAD 9 might be the long-chain acyl-CoA dehydrogenase in the embryonic and fetal central nervous system.

The role of an active FAO in the human brain has not been fully elucidated. Probably, FAO might be of importance in maintaining a sufficient energy supply in the developing brain. In addition, FAO could be involved in homeostasis and lipid turn over of the fatty acid containing lipid membranes in the growing fetus.

Until now, no clinical signs and symptoms of central nervous system involvement have been reported in VLCAD deficiency which correlates well with the expression pattern of VLCAD. However, a deficiency of ACAD 9 might only result in central nervous system symptoms. A novel long-chain FAO defect with only central nervous system involvement might easily be missed, since no classical symptoms of long-chain FAO defects, such as hypoketotic hypoglycemia, cardiomyopathy, and skeletal muscle disease with rhabdomyolysis, will be observed, as VLCAD has overlapping substrate specificity with ACAD 9 in all other organs except for the brain and central nervous system. Another explanation could be that ACAD 9 deficiency is lethal during intrauterine development. Screening for ACAD 9 deficiency in patients with undefined neurological symptoms and/or an impairment in normal neurological development is necessary to establish whether this deficiency exists.

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