BBA - Molecular and Cell Biology of Lipids 1866 (2021) 158891

Contents lists available at ScienceDirect

BBA - Molecular and Cell Biology of Lipids

journal homepage: www.elsevier.com/locate/bbalip

LPIAT1/MBOAT7 contains a catalytic dyad transferring polyunsaturated fatty acids to lysophosphatidylinositol

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ARTICLE INFO

Keywords:
O-acyltransferase activity
PUFA
Arachidonic acid
NAFLD
Liver
Pichia pastoris

ABSTRACT

Human membrane bound O-acyltransferase domain-containing 7 (MBOAT7), also known as lysophosphatidylinositol acyltransferase 1 (LPIAT1), is an enzyme involved in the acyl-chain remodeling of phospholipids via the Lands' cycle. The *MBOAT7 rs641738* variant has been associated with the entire spectrum of fatty liver disease (FLD) and neurodevelopmental disorders, but the exact enzymatic activity and the catalytic site of the protein are still unestablished.

Human wild type MBOAT7 and three MBOAT7 mutants missing in the putative catalytic residues (N321A, H356A, N321A + H356A) were produced into *Pichia pastoris*, and purified using Ni-affinity chromatography. The enzymatic activity of MBOAT7 wild type and mutants was assessed measuring the incorporation of radiolabeled fatty acids into lipid acceptors.

MBOAT7 preferentially transferred 20:4 and 20:5 polyunsaturated fatty acids (PUFAs) to lysophosphatidylinositol (LPI). On the contrary, MBOAT7 showed weak enzymatic activity for transferring saturated and unsaturated fatty acids, regardless the lipid substrate. Missense mutations in the putative catalytic residues (N321A, H356A, N321A + H356A) result in a loss of O-acyltransferase activity.

Thus, MBOAT7 catalyzes the transfer of PUFAs to lipid acceptors. MBOAT7 shows the highest affinity for LPI, and missense mutations at the MBOAT7 putative catalytic dyad inhibit the O-acyltransferase activity of the protein. Our findings support the hypothesis that the association between the *MBOAT7 rs641738* variant and the increased risk of NAFLD is mediated by changes in the hepatic phosphatidylinositol acyl-chain remodeling. Taken together, the increased knowledge of the enzymatic activity of MBOAT7 gives insights into the understanding on the basis of FLD.

1. Introduction

Human membrane bound O-acyltransferase domain-containing 7 (MBOAT7), also known as lysophosphatidylinositol acyltransferase 1 (LPIAT1), belongs to the membrane-bound O-acyl-transferase (MBOAT) family. The MBOAT family comprises mostly enzymes involved in the remodeling of lipids [1,2] esterifying free fatty acids to the hydroxyl group of their lipid substrates. Notably, the acyl-chain composition of phospholipids strongly influences their properties and the affinity of enzymes for their phospholipid substrates [3]. Homozygosity for severe loss of function genetic variants in *MBOAT7* results in human neurodevelopment disorders and intellectual disability, suggesting a critical role for arachidonic acid-containing phosphatidylinositol (PI) remodeling in the development of human brain [4–7]. Consistently, *Mboat7*-deficient mice (*Mboat7*$^{-/-}$) showed severe developmental brain defects [8], atrophy of the cerebral cortex and hippocampus, abnormal cortical lamination and a higher number of apoptotic cells in the cortex [9].

A genetic variant *rs641738* in the locus containing *MBOAT7* associates with the entire spectrum of fatty liver disease (FLD) [10,11] increasing the risk for non-alcoholic steatohepatitis (NASH), alcohol-related cirrhosis, and liver fibrosis in patients infected with viral hepatitis [12–18]. In obese individuals, hepatic *MBOAT7* mRNA decreased from normal liver to steatohepatitis, independently of diabetes, inflammation and *MBOAT7* genotype [19]. Carriers of the *rs641738*

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https://doi.org/10.1016/j.bbalip.2021.158891
Received 1 October 2020; Received in revised form 13 January 2021; Accepted 23 January 2021
Available online 27 January 2021

variant show reduced *MBOAT7* gene expression level, reduced protein synthesis, and lower levels of arachidonoyl-PI (20:4-PI) [13]. Recently, hepatic MBOAT7 levels have been shown to be reduced in murine models of fatty liver, and by hyper-insulinemia [19]. Moreover, hepatocyte specific *Mboat7* inactivation is associated with spontaneous increased in hepatic triglyceride content fueled by an increased PI turnover [20]. Similarly to humans, hepatocyte specific *Mboat7* inactivation results in a decreased hepatic PUFAs-containing PI concentration in the brain [8].

MBOAT7 is a 472 amino acids-long O-acyl transferase and is highly expressed in the liver and in the brain. The enzyme is an integral membrane protein anchored to endomembranes [21], such as endoplasmic reticulum (ER), lipid droplets (LD), and mitochondria-associated membranes (MAM) [13]. The catalytic site of the protein is currently unknown. However, homology modelling analyses suggest the presence of a putative catalytic dyad composed of a conserved asparagine (Asn-321) and a preserved histidine (His-356) [22].

MBOAT7 catalyzes the acylation of the *sn-2* position of lysophospholipids [23], providing the acyl-chain remodeling of phospholipids via a post-synthetic process known as Lands' cycle [24,25]. The Lands' cycle is composed by orchestrated reactions of hydrolysis, acyl-CoA synthesis, and re-acylation, which regulate the remodeling and the availability of phospholipids [24–26]. In a RNA interference-based genetic screen using *C. elegans*, mboa-7 has been cloned as an acyltransferase that selectively incorporates arachidonic acid (AA) into PI [22]. Interestingly, human neutrophils express *MBOAT7* mRNA and neutrophils microsomes incorporate arachidonoyl chains into PI [23]. According to these results, MBOAT7 is implicated in the arachidonate recycling of PI. However, the experimental data obtained so far have been carried out with purified microsomal membranes that do not allow a quantitative characterization of the enzymatic activity and affinity of the target protein. Moreover, contaminations with other enzymes cannot be excluded in these systems.

In this manuscript, we established a protocol to produce pure MBOAT7 protein in *Pichia pastoris* [27], a yeast system allowing purification of eukaryotic membrane proteins in high yields [28]. Then, we characterized the enzymatic activity, the substrate specificity and the exact localization of the catalytic dyad of MBOAT7.

2. Materials and methods

2.1. Human MBOAT7 cloning

The *P. pastoris* wild type strain (X-33) and the pPICZB vector were purchased from Invitrogen (Carlsbad, USA). Restriction enzymes and cloning reagents were purchased from New England BioLabs (Massachusetts, USA). The human *MBOAT7* wild type gene was codon optimized for *P. pastoris* by GeneArt Gene Synthesis (Thermo Fisher Scientific), and amplified using the following primers: the forward primer *Eco*RI-*MBOAT7*: ATA CCG GAA TTC **AAA ATG** TCC CCA GAA GAG TGG A, and the reverse primer *Xba*I-*MBOAT7*: A TAC TAG TCT AGA TTA GTG GTG GTG GTG ATG ATG TT, coding a C-terminal HIS_6-tag. Restriction sites are underlined, and the Kozak consensus sequence is shown in bold. The amplified cDNA was inserted in the *Eco*RI/*Xba*I sites in the pPICZB vector, and the resulting construct was verified by sequencing (Eurofins Genomics, Ebersberg, Germany). The MBOAT7 H356A, MBOAT7 N321A and MBOAT7 N321A_H356A mutant forms optimized for *P. pastoris* were synthesized by GeneArt Synthesis (Thermo Fisher Scientific). The amplified cDNAs were inserted in the pPICZB vector.

2.2. High-zeocin screening

The resulting constructs were linearized using *Pme*I restriction enzyme (Fermentas, MA, USA) and transformed into *P. pastoris* wild type strain X-33 by electroporation [27]. To select multi-copy transformants, 52 colonies were tested for growth on Yeast Extract Peptone Dextrose Sorbitol (YPDS) containing 2000 μg/mL of zeocin [29] [30]. Small scale production of each protein form was performed as previously described [31].

2.3. Large-scale production of the recombinant MBOAT7

P. pastoris cells expressing MBOAT7 wild type or mutants were precultured in 100 mL buffered glycerol-complex medium (BMGY), reaching an optical density (OD_{600}) of approximately 4. Then, 3 L fermentors, having an Initial Fermentation Volume (IFV) of 1.5 L basal salt medium [32], and containing 6.53 mL of PTM1 trace salts [33], were inoculated using the 100 mL pre-culture. After 24 h, the glycerol was consumed, and the cells were fed with 250 mL of 50% glycerol (*v*/v) to increase biomass. The MBOAT7 protein production was induced by co-feeding the culture with methanol 50% (300 mL) and sorbitol 60% (200 mL) for 40 h. Cells were harvested and frozen at −20 °C.

2.4. P. pastoris membranes preparation

P. pastoris cells were resuspended in Buffer A [50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] at a concentration of 0.2 g/mL. Cells were broken using a BeadBeater homogenizer (BioSpec, Bartlesville, UK) and a 0.5 mm glass beads, on ice (10 cycles of 30 s). Unbroken cells were collected by centrifugation (5000 rpm, 40 min, 4 °C). Subsequently, the cell membrane fraction was collected from the supernatant by ultracentrifugation (42,000 rpm, 1 h, 4 °C). The pellet, containing the membrane fraction, was resuspended in Buffer B (50 mM Tris HCl pH 7.4, 150 mM NaCl, 20% glycerol), and homogenized by an electric homogenizer at a concentration of approximately 300 mg/mL.

2.5. Solubilization screen of MBOAT7 from P. pastoris membranes

Cell membranes were incubated with 2% Fos-Choline-12 (a harsh, zwitterionic detergent), 2% Triton X-100 (a mild, non-ionic detergent), or 2% SDS (a harsh, denaturant ionic detergent) for 2 h at 4 °C. Samples were subjected to ultracentrifugation (42,000 rpm, 45 min, 4 °C). The supernatants containing the proteins dislodged from the membranes were collected and subjected to immunoblotting analysis.

2.6. Immunoblotting analysis

Protein samples were mixed with Laemmli buffer containing 2-mercaptoethanol, and boiled for 5 min at 95 °C. Samples were size-separated by SDS-PAGE (10% acrylamide gel), and transferred onto nitrocellulose membranes (0.4 A, 1 h). Membranes were incubated with mouse anti-Histidine (Clontech Laboratories, Cat. #631212) for 1 h, washed twice with 0.2% tris-buffered saline containing 0.2% tween (0.2% TBS-T) for 10 min, incubated with HRP-conjugated anti-mouse (GE Healthcare, Cat. #LNA9310V) for 1 h, then washed 3 times with 0.2% TBS-T for 10 min. Membranes were incubated with chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA) for 5 min, bands were visualized by Chemidoc XRS System (Bio-Rad, Hercules, CA) and quantified using Image Lab Software (Bio-Rad).

2.7. Purification of MBOAT7 wild type and mutants

The membranes were solubilized using Buffer B with 2% Fos-choline-12 and 0.2% Sodium deoxycholate, as previously described [31]. The solubilized membranes were subjected to ultracentrifugation (42,000 rpm, 45 min, 4 °C). The supernatant containing the proteins of interest was collected and incubated, by gentle agitation (overnight, 4 °C), with 3 mL Ni-NTA resin pre-equilibrated using 40 mL of equilibration buffer (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20% glycerol, 0.1% Fos-choline-12, 0.1% Sodium deoxycholate, 50 mM Imidazole). The day after, the

resin was packed into polypropylen column (Qiagen, Hilden, Germany), and washed with 40 mL of washing buffer (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20% glycerol, 0.1% Fos-choline-12, 0.1% Sodium deoxycholate, 50 mM Imidazole). Firstly, the MBOAT7 proteins were eluted using 3 mL of elution buffer 200 (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20% glycerol, 0.1% Fos-choline-12, 0.1% Sodium deoxycholate, 200 mM Imidazole), and secondarily with 3 mL of elution buffer 400 (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20% glycerol, 0.1% Fos-choline-12, 0.1% Sodium deoxycholate, 400 mM Imidazole). The six fractions (1 mL each) were concentrated to 2 mL using Vivaspin columns (Sartorius Stedim Biotech, Goettingen, Germany), and desalted using a PD-10 desalting columns (GE Healthcare, Chicago, IL). To remove imidazole, the MBOAT7 proteins were eluted with 3.5 mL of desalting buffer (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 20% glycerol, 0.1% Fos Choline-12, 0.1% Sodium deoxycholate). The purified proteins were stored at −80 °C.

2.8. MBOAT7 O-acyltransferase activity assay using lysophospholipids as acyl-acceptors

Purified MBOAT7 wild type (5 μg) was incubated with: 1) 50 μM of each of the five selected lysophospholipids (lysophosphatidylinositol (LPI), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), or lysophosphatidic acid (LPA) (Avanti Lipids Polar, Inc., Alabaster, Alabama); 2) 15 μM of each of the five different acyl-CoA esters (stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), arachidonoyl-CoA (20:4-CoA), or eicosapentaenoyl-CoA (20:5-CoA)) (Sigma-Aldrich); and 3) 5 μM of each of the five free, radiolabeled [^{14}C]acyl-CoA esters (stearoyl[1-^{14}C] CoA, oleoyl[1-^{14}C]CoA, linoleoyl[1-^{14}C]CoA, arachidonoyl[1-^{14}C]CoA, or eicosapentaenoyl[1-^{14}C]CoA) (American Radiolabeled Chemicals, St. Louis, MO). The reaction mix was incubated for 1 h at 37 °C. The enzymatic reaction was stopped by adding 1 mL of chloroform:methanol (2:1, vol/vol) and 0,4 mL of acidified solution (orthophosphoric acid 2%). After centrifugation (3500 *g*, 15 min, 4 °C), the lower organic phase, containing lipids, was collected and dried under a gentle nitrogen stream. Lipids were reconstituted in chloroform, and separated by one-dimensional thin layer chromatography (TLC) using TLC silica gel plates (Merck-Millipore, Darmstadt, Germany). Human liver PI (Avanti Lipids Polar, Inc.) was used as marker. Chloroform:methanol:ammonium hydroxide:water (43:38:5:7, vol/vol) was used as mobile phase. Bands were visualized by iodine vapor staining. The area of TLC silica gel corresponding to the radiolabeled phospholipids released as consequence of the MBOAT7 activity were cut out, and the rate of radiolabeled products in each area was measured by liquid scintillation counting.

Purified MBOAT7 wild type, MBOAT7 H356A, MBOAT7 N321A or MBOAT7 N321A_H356A (5 μg) were incubated with 50 μM of LPI, 5 μM of radiolabeled [^{14}C]-20:4-CoA or [^{14}C]-20:5-CoA, and with 15 μM of the corresponding free, unlabeled 20:4-CoA or 20:5-CoA, in presence or absence of thimerosal 200 μM (SigmaAldrich). The samples underwent the experimental steps described above.

2.9. MBOAT7 O-acyltransferase activity assay using LPI with different sn-1 acyl-composition as acyl-acceptors

Purified MBOAT7 wild type (5 μg) was incubated with 50 μM of 16:0-LPI or 18:0-LPI, 15 μM of free, unlabeled 20:4-CoA, and with 5 μM of radiolabeled 20:4-CoA. The samples underwent the experimental steps described above.

2.10. Determination of the MBOAT7 enzymatic kinetics

Purified MBOAT7 wild type (5 μg) was incubated with five different concentrations (10 μM, 20 μM, 50 μM, 120 μM and 150 μM) of each of the five selected lysophospolipids (LPI, LPS, LPA, LPE, LPC), with five different concentrations (1 μM, 2 μM, 5 μM, 12 μM and 15 μM) of free, radiolabeled [^{14}C]-20:4-CoA or [^{14}C]-20:5-CoA, and with five different concentrations (3 μM, 6 μM, 15 μM, 36 μM and 45 μM) of the corresponding free, unlabeled 20:4-CoA or 20:5-CoA. The samples underwent the experimental steps described above. The kinetics curves, V_{max}, K_m and K_{cat} were defined by the Michaelis-Menten model using GraphPad Prism 7 (San Diego, CA).

3. Results

3.1. Large-scale production, solubilization and purification of the MBOAT7 proteins

The optimized *MBOAT7* wild type gene and the three mutants were sub-cloned into the pPICZB vector. The constructs were linearized using the *Pme*I restriction enzyme, and *P. pastoris* wild type strain X-33 cells were transformed. Three clones were selected and used for a small-scale production test. The clones with the highest protein production level were used for a large-scale production. *P. pastoris* cells were grown under tightly controlled conditions, resulting in a total cell mass of approximately 400 g. The MBOAT7 protein production was obtained using a Methanol/Sorbitol co-feeding. Broken cells were subjected to ultracentrifugation and immunoblotting. MBOAT7 was detected in the crude extract and was particularly enriched in the membrane fraction (Fig. 1A). The membrane fraction was incubated with 2% Fos-Choline-12 (a harsh zwitterionic detergent), 2% Triton X-100 (a mild non-ionic detergent), or 2% SDS (a harsh, ionic, denaturant detergent, used as a positive control) to identify the best detergent able to dislodge MBOAT7 from the membrane without denaturing the protein. MBOAT7 was effectively removed from the membrane by 2% Fos-Choline-12 (Supplementary Fig. 1). Fos-Choline-12 was therefore used as detergent for the next steps of solubilization and purification of MBOAT7. The solubilized proteins were purified by Ni-affinity chromatography, resulting in highly pure samples. The elution of the purified proteins was evaluated by Coomassie-stained SDS-PAGE by running six eluted fractions after MBOAT7 purification (Fig. 1B), and by Immunoblotting analysis (Fig. 1C). Most of the protein was found in fractions 3–5 after elution (1 mL each) in 200 mM imidazole and 400 mM imidazole. MBOAT7 protein migrated predominantly as one large monomeric band of 40 KDa molecular weight. The final protein yield after purification was estimated to be 4 mg per liter of cell culture.

3.2. Purified MBOAT7 favors PUFAs as acyl-donors

MBOAT7 catalyzes O-acyltransferase activity transferring free acyl-donors, such as fatty acids, to free acyl-acceptors. Purified MBOAT7 wild type was incubated with each of the following five free, radiolabeled [^{14}C]acyl-CoA esters, 18:0-CoA, 18:1-CoA, 18:2-CoA, 20:4-CoA, 20:5-CoA, and with each of the following five lysophospholipids (LPI, LPS, LPA, LPE, or LPC). Purified MBOAT7 preferentially transferred free 20:4-CoA and 20:5-CoA to the *sn-2* position of lysophospholipids, whilst showed weak O-acyltransferase activity transferring 18:0-CoA, 18:1-CoA and 18:2-CoA (Fig. 2).

3.3. The O-acyltransferase activity of MBOAT7 is not affected by the sn-1 composition of lysophospholipids

MBOAT7 promotes the transferring of 20:4-CoA and 20:5-CoA to the *sn-2* position of phospholipids containing 18:0 at *sn-1*. To test whether 18:0 at *sn-1* is required for this activity, purified enzyme was mixed with free, radiolabeled and unlabeled 20:4-CoA, and with 16:0-LPI or 18:0-LPI. MBOAT7 transferred free 20:4-CoA to 16:0-LPI or 18:0-LPI releasing labeled 16:0–20:4 PI or 18:0–20:4 PI, indifferently (Supplementary Fig. 2). These results suggest that the *sn-1* acyl-chain composition of lysophospholipids does not affect the MBOAT7 affinity for the acyl-acceptor.

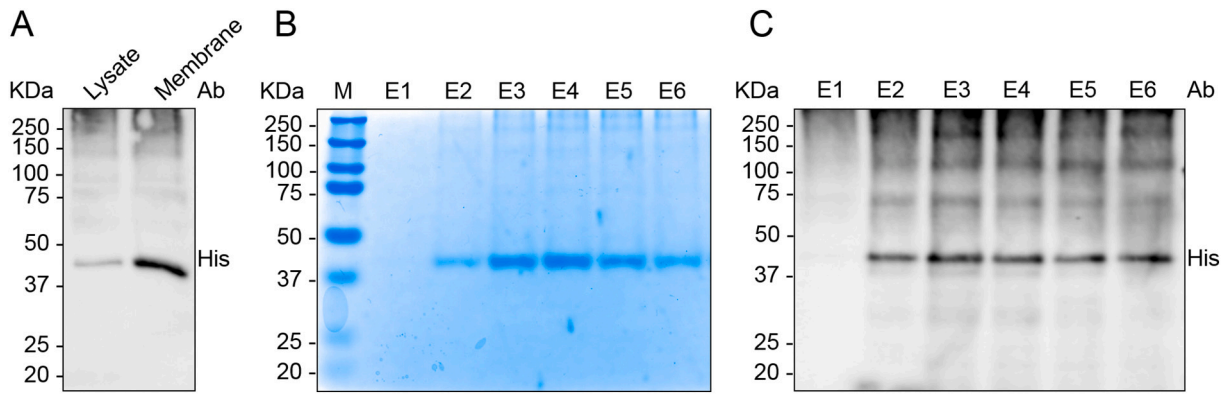

Fig. 1. MBOAT7 was particularly enriched in the membrane fraction of *P. pastoris* cells. (**A**) Immunoblot analysis shows the MBOAT7 enrichment in membranes of *P. pastoris*. (**B**) Coomassie-stained SDS-PAGE and (**C**) Immunoblot analysis of six eluted fractions after MBOAT7 purification by Ni-chromatography. E1–E3: 200 mM imidazole; E4–E6: 400 mM imidazole. Abbreviations: His, histidine; E, eluted fraction.

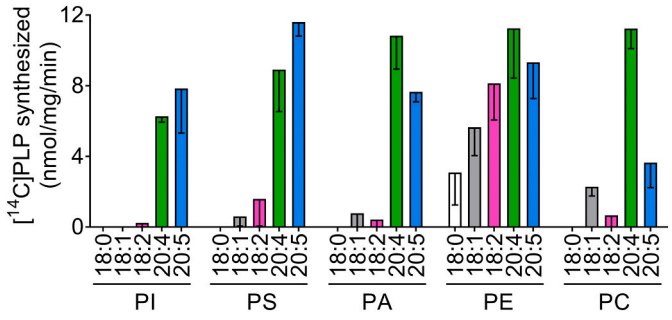

Fig. 2. Purified MBOAT7 preferentially transfers free, radiolabeled arachidonoyl[1-^{14}C]CoA or eicosapentaenoyl[1-^{14}C]CoA to lysophospholipids, releasing radiolabeled [^{14}C]phospholipids. Free, radiolabeled [^{14}C]acyl-CoA esters (18:0-CoA, 18:1-CoA, 18:2-CoA, 20:4-CoA, or 20:5-CoA) were used as acyl donors; lysophospholipids (LPI, LPS, LPA, LPE or LPC) were used as acyl acceptors. The rate of radiolabeled products released as a consequence of the MBOAT7 enzymatic activity (X-axis) was measured by liquid scintillation counting. Bars represent the mean ± standard deviation of $n = 3$ independent experiments carried out in triplicate. Abbreviations: 18:0, stearoyl-CoA; 18:1, oleoyl-CoA; 18:2, linoleoyl-CoA; 20:4, arachidonoyl-CoA; 20:5, eicosapentaenoyl-CoA; PI, phosphatidylinositol; PS, phosphatydylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PLP, phospholipid.

3.4. MBOAT7 shows the highest catalytic efficiency transferring 20:4 and 20:5 to LPI

MBOAT7 favors 20:4-CoA and 20:5-CoA as acyl-donors to synthesize newly remodeled phospholipids. To measure the enzymatic activity of MBOAT7, purified wild type protein was incubated with 20:4-CoA or 20:5-CoA, and with each of the five selected lysophospholipids, and the enzymatic kinetics was assessed. MBOAT7 showed the highest V_{max} for transferring [^{14}C]-20:4-CoA to LPE, and the lowest K_m for transferring [^{14}C]-20:5-CoA to LPI (Fig. 3, Table 1). The highest MBOAT7 catalytic efficiency (K_{cat}/K_m) was detected incubating MBOAT7 with LPI as acyl-acceptor and 20:4-CoA or 20:5-CoA as acyl-donors, or with LPE as acyl-acceptor and 20:4-CoA as acyl-donor (Table 1).

3.5. Missense mutations at the MBOAT7 catalytic site cause a loss of O-acyltransferase activity in absence of thimerosal

Purified MBOAT7 proteins carrying missense mutations in position 321, or 356, or 321 and 356, showed a loss of O-acyltransferase activity (Fig. 4 and Supplementary Fig. 3). Moreover, acyltransferase activity of the four MBOAT7 proteins was tested in presence or absence of the organomercury compound thimerosal showing a specific MBOAT7 inhibition. These results suggest that the Asn-321 and the His-356 residues are crucial for the O-acyltransferase activity of MBOAT7. Missense mutations in the putative catalytic site of MBOAT7 cause a strong loss of function of the enzyme, compared to the MBOAT7 wild type (Fig. 4 and Supplementary Fig. 3).

4. Discussion

The main finding of this work is that MBOAT7 contains a catalytic dyad (Asn321-His356) resulting in the transfer of 20:4 and 20:5 to LPI. Our findings support the hypothesis that the association between the *MBOAT7 rs641738* variant and the increased risk of NAFLD is mediated by changes in the hepatic PI acyl-chain remodeling.

MBOAT7 is a 472 amino acids-long enzyme that belongs to the membrane-bound O-acyl-transferase (MBOAT) family [2], which is composed by enzymes involved in lipid metabolism [34,35]. MBOAT7 is an integral membrane protein with 6 transmembrane domains [21], which has been located in endomembranes rich in phospholipids, such as ER, LP, and MAM [13]. MBOAT7 transfers free acyl-donors, such as PUFAs, to free lipid acyl-acceptors, such as lysophospholipids, remodeling the acyl-chain composition of phospholipids via the Lands' cycle [24]. PUFAs, such as 20:4 and 20:5, are generally esterified at the *sn-2* position of the glycerol backbone of phospholipids in mammals. MUFAs, such as oleic acid, are in general esterified at the *sn-1* or *sn-2* position, whereas saturated fatty acids, such as stearic acid, are commonly esterified at the *sn-1* position [36]. The re-acylation step is catalyzed by members of the MBOAT family, such as MBOAT5 [37] and MBOAT7 [23]. The acyl-chain composition of phospholipids strongly influences the properties of cellular membranes, vesicle trafficking, and the affinity of enzymes for lipid substrates [25,26].

The human *MBOAT7* gene was codon optimized for *P. pastoris*, amplified and inserted into the pPICZB vector. In order to investigate the ability of MBOAT7 to incorporate free fatty acids with different degrees of saturation, into lipid acyl-acceptors, we produced the human MBOAT7 protein in large-scale in *P. pastoris* cells. The five lysophospholipids used as acyl-acceptors were selected based on to the role and relative amount of the corresponding phospholipid in mammals. Free fatty acids were selected based on plasma lipidomics of individuals stratified by the *MBOAT7 rs641738* variant [16]. Specifically, carriers of the *rs641738* variant have lower levels of 20:4-PI/total-PI and 20:5-PI/total-PI ratios, and have higher levels of 18:1-PI/total-PI and 18:2-PI/total-PI ratios in plasma [13].

We tested the enzymatic activity of MBOAT7 wild type using three PUFAs (18:2-CoA, 20:4-CoA, or 20:5-CoA), a monounsaturated fatty acid (MUFA) (18:1-CoA), or a saturated fatty acid (18:0-CoA) as acyl-donors, and 5 selected phospholipids as acyl-acceptors. MBOAT7

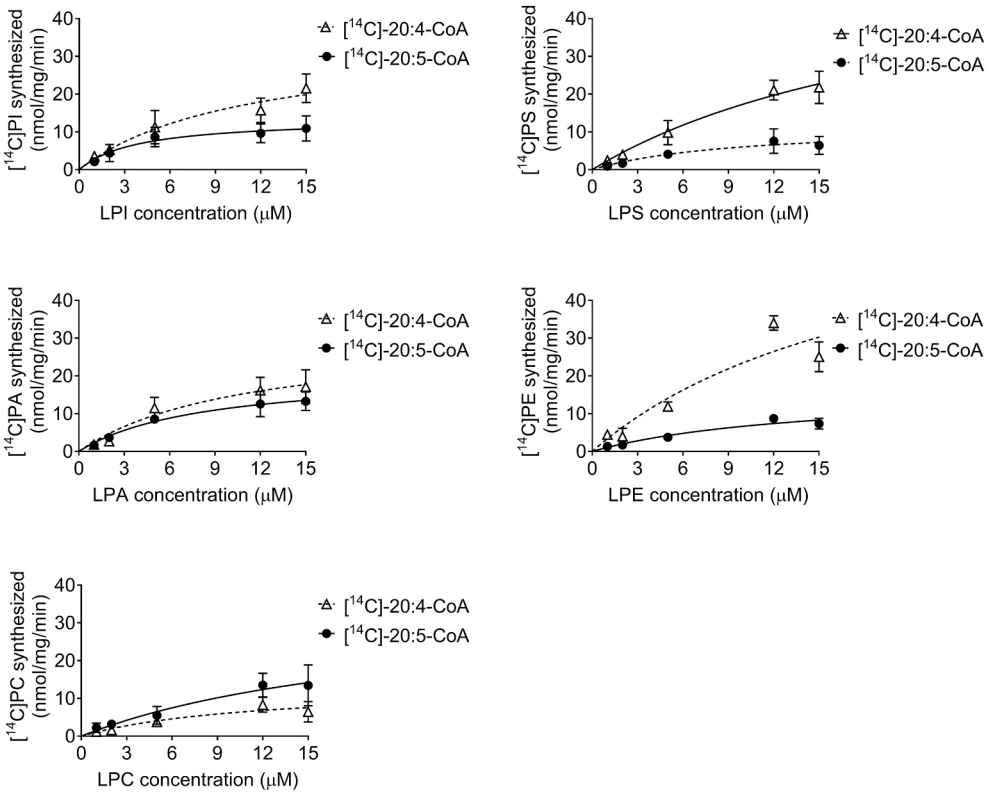

Fig. 3. Purified MBOAT7 showed the highest V_{max} transferring free [^{14}C]-20:4-CoA to LPE, and the lowest K_m transferring free [^{14}C]-20:5-CoA to LPI. Purified MBOAT7 was mixed with five different concentrations (1 μM, 2 μM, 5 μM, 12 μM and 15 μM) of each of the five selected lysophospolipids (LPI, LPS, LPA, LPE, LPC), with free, radiolabeled [^{14}C]-20:4-CoA or [^{14}C]-20:5-CoA, and with the corresponding free, unlabeled PUFA. The rate of radiolabeled product released (Y-axis) released as a consequence of the MBOAT7 enzymatic activity was measured by liquid scintillator counting. The kinetics curves were defined by the Michaelis-Menten model. The error bars represent the standard deviation of $n \geq 3$ independent experiments carried out in triplicate. Abbreviations: 20:4-CoA, arachidonoyl-CoA; 20:5-CoA, eicosapentaenoyl-CoA; LPI, lysophosphatidylinositol; PI, phospatidylinositol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; LPA, lysophosphatidic acid; PA, phosphatidic acid; LPS, lysophosphatydylserine; PS, phosphatydylserine.

Table 1
V_{max}, K_m and K_{cat} values for hMBOAT7 incubated with one of the lysophospholipids and either [^{14}C]-20:4-CoA or [^{14}C]-20:5-CoA.

Lysophospolipid acceptor	Fatty acid donor	V_{max} (nmol/mg/min)	K_m (μM)	K_{cat} (min^{-1})	K_{cat} / K_m (μM^{-1}*min^{-1})
LPI	[^{14}C]-20:4-CoA	34.4 ± 8	10.96 ± 4.99	6.87 ± 1.59	0.62
	[^{14}C]-20:5-CoA	13.57 ± 1.86	3.89 ± 1.55	2.71 ± 0.37	0.69
LPE	[^{14}C]-20:4-CoA	71.29 ± 31.93	20.34 ± 14.35	14.29 ± 6.39	0.70
	[^{14}C]-20:5-CoA	16.22 ± 4.89	14.34 ± 7.69	3.24 ± 0.98	0.22
LPA	[^{14}C]-20:4-CoA	30.68 ± 8.77	10.99 ± 6.16	6.13 ± 1.75	0.56
	[^{14}C]-20:5-CoA	21.04 ± 3.83	8.32 ± 3.31	4.21 ± 0.77	0.51
LPC	[^{14}C]-20:4-CoA	13.34 ± 5.18	11.48 ± 8.61	2.67 ± 1.04	0.23
	[^{14}C]-20:5-CoA	35.01 ± 23.21	22.04 ± 22.44	7.00 ± 4.64	0.32
LPS	[^{14}C]-20:4-CoA	59.77 ± 24.18	24.44 ± 14.72	11.95 ± 4.84	0.49
	[^{14}C]-20:5-CoA	11.96 ± 4.55	9.82 ± 7.66	2.39 ± 0.91	0.24

V_{max}, K_m and K_{cat} were calculated using the Michaelis-Menten model.

preferentially transferred 20:4-CoA and 20:5-CoA to LPI and to LPE, regardless the *sn-1* acyl composition of lysophopholipids. The affinity of MBOAT7 for 20:4 and 20:5 could be related to the folding of the chain from carbon number 5, due to the first double bond in both fatty acids. It is important to bear in mind that all the *in vitro* experiments using the purified proteins and substrates were performed in a non-physiological environment. The results obtained by using this approach need to be contextualized in a cellular environment in which the enzyme has a larger pool of lipid substrates available. In fact, in humans, lipidomic analyses by mass spectrometry of hepatic tissue shows a reduction of the PI in carriers of the *MBOAT7 rs641738*, with no differences in PE species [20]. Similarly, hepatic specific *Mboat7* knock out mice fed a chow diet results in a reduction in the liver 20:4-PI, with no changes in the PE [20,38]. Taken all this together, we conclude PI is the preferred acyl-acceptor of MBOAT7.

In our experiments, MBOAT7 shows the highest affinity (lowest K_m) transferring 20:5 to LPI, and the maximal enzymatic velocity (highest V_{max}) transferring free 20:4 to LPE. The highest MBOAT7 catalytic efficiencies were detected incubating the enzyme with 20:4 and LPI or LPE, and incubating 20:5 with LPI. However, a specific reduction in 20:4-PI, and not 20:5-PI was consistently observed in humans and mice. All this suggests that 20:4 is the preferential acyl-donor of MBOAT7.

Members of the MBOAT family show a preserved catalytic site composed of an asparagine (Asn) and a histidine (His) [2,22]. In the MBOAT7 protein, the catalytic dyad consists of the Asn-321 and the His-356. We produced three MBOAT7 protein mutants: one with a missense mutation in position 321 (MBOAT7 N321A); one in position 356 (MBOAT7 H356A); and one in both amino acid positions (MBOAT7 N321A_H356A). We tested the O-acyltransferase activity of the three MBOAT7 mutants. Mutations in these two amino acid residues lead to a loss of the enzymatic activity of the protein, confirming the importance of these residues for the activity of the enzyme. The specificity of the assay for the MBOAT7 acyltransferase activity was confirmed by the strong inhibition obtained in presence of thimerosal. These data demonstrate that the Asn-321 and His-356 are key amino acids for the enzymatic activity of MBOAT7, representing most likely the catalytic dyad of this protein. Missense mutations in this protein site lead to the loss of function of the protein that will presumably result in

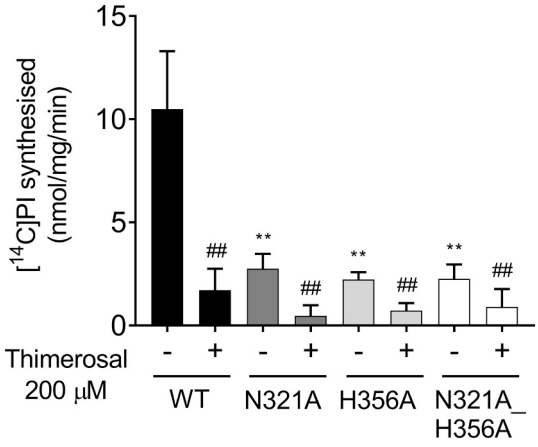

Fig. 4. Missense mutations at the putative catalytic site of MBOAT7 or thimerosal incubation lead to a loss of enzymatic activity. Purified MBOAT7, MBOAT7 H356A, MBOAT7 N321A or MBOAT7 N321A_H356A were incubated with free, radiolabeled [^{14}C]-20:4-CoA, and with LPI in presence or absence of thimerosal 200 μM. The rate of radiolabeled products released as a consequence of the enzymatic activity of the four MBOAT7 forms (Y-axis) was measured by liquid scintillation counting. Differences between the WT and mutants or thimerosal groups were analyzed using the non-parametric Mann-Whitney test. **, $p \leq 0.01$ vs WT without thimerosal; ##, $p \leq 0.01$ vs corresponding MBOAT7 protein without thimerosal. Bars represent the mean ± standard deviation of $n = 6$ independent experiments. Abbreviations: PI, phosphatidylinositol.

neurodevelopmental disorders if carried in homozygosity [4–6].

In conclusion, in this work we present the production protocol for human MBOAT7 enabling characterization of a key target in FDL, leading to an increased understanding of the factors causing the disease. In this study, we demonstrate that LPI and 20:4 are the preferred acyl acceptor and acyl donor of MBOAT7, respectively. We also identify the catalytic dyad (Asn-321_His-356) responsible for the protein enzymatic activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2021.158891.

CRediT authorship contribution statement

Andrea Caddeo: Investigation, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Kristina Hedfalk:** Conceptualization, Writing – review & editing. **Stefano Romeo:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Piero Pingitore:** Investigation, Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Swedish Research Council [Vetenskapsrådet (VR), 2016-01527] (SR), the Swedish Heart-Lung Foundation [20190274] (SR), the Swedish Federal Government funding under the Agreement on Medical Training and Medical Research (ALF) [716731] (SR), the Novo Nordisk Foundation Project Grants in Endocrinology and Metabolism [NNF19OC0056742], the Swedish Diabetes Foundation [DIA 2017-205] (SR), and Wallenberg Academy Fellows from the Knut and Alice Wallenberg Foundation [KAW 2017.0203] (SR).

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