arachidonoyl-CoA. The pH optimum for the reaction was between 7.4 and 10, and the reaction did not require Ca^{2+} (data not shown). A kinetic analysis was conducted by measuring acyltransferase activity in the microsomal fraction derived from vector- and mLPAAT3-transfected CHO-K1 cells, using increasing concentrations (1.5–100 μM) of [1-^{14}C]arachidonoyl-CoA in the presence of 50 μM palmitoyl-LPA, or using increasing concentrations (6.25-100 μM) of palmitoyl-LPA in the presence of 25 μM [1-^{14}C]arachidonoyl-CoA (Supplemental Fig. 2A and B). The Km values of mLPAAT3 were 15.9 μM for arachidonoyl-CoA and 26.3 μM for palmitoyl-LPA. The corresponding Vmax values were 50.4 and 21.8 nmol/min/mg.

The role of the highly conserved motifs NHX_4D and EGTR on enzyme activity

We constructed three single point mutants of mLPAAT3 (H96A, D101A, and E176A, arrows in Fig. 1B). Both LPAAT and LPIAT activities of mLPAAT3 were completely suppressed by these mutations (Fig. 5A). Expression of wild type, H96A, D101A, and E176A mutants was confirmed by Western blot analysis (Fig. 5B). These results indicate that the motifs are critical for the enzymatic activity and that both enzymatic activities (LPAAT and LPIAT) reside on a single protein.

Age-dependent mLPAAT3 expression and LPAAT activity in the testis

Total RNA of the testis was prepared from C57BL/6J mice at various ages. Interestingly, mLPAAT3 mRNA level was upregulated significantly until 15 week, then increased only slightly (Fig. 6A). In another independent experiment, after 15 weeks, mLPAAT3 expression level decreases slightly (Data not shown). The trend of this age-dependent mLPAAT3 change was observed in protein level as well by Western blot analysis (Fig. 6B). Additionally, LPAAT activity of the testis at 2, 8, 45 weeks was checked using palmitoyl-LPA as an acceptor and arachidonoyl-CoA as a donor. We observed that LPAAT activity increased in an age-dependent fashion (Fig. 6C).

The effect of sex hormones on mLPAAT3 expression

Since the level of sex hormones changes with age (41-47), we investigated whether age-dependent mLPAAT3 expression is derived from the induction by sex hormones. In testicular cell line TM4 cells, mLPAAT3 was upregulated significantly with β-estradiol treatment for 24 h (Fig. 6D).

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The substrate selectivity of mLPAAT1

We examined the acyl-CoA selectivity of mLPAAT1 using palmitoyl-LPA as an acceptor. mLPAAT1 demonstrated LPAAT activity using palmitoyl-LPA as an acceptor and palmitoyl-CoA, oleoyl-CoA , and arachidonoyl-CoA as a donor (Supplemental Fig. 3). Thus, at least mLPAAT1 seems to be different from mLPAAT3 in acyl-CoA preference.

DISCUSSION

Here we have presented the first detailed characterization of mLPAAT3. mLPAAT3, previously known as mAGPAT3 is a 43.3 kDa protein with four putative transmembrane domains and is localized in ER. Interestingly, mLPAAT3 has a clear preference for arachidonoyl-CoA as a donor in the synthesis of PA through the *de novo* pathway. Additionally, the enzyme exhibited LPIAT activity using arachidonoyl-CoA as a donor, suggesting that mLPAAT3 can function in the remodeling pathway as well. Prior to this report, mouse acyl-CoA:lysocardiolipin acyltransferase (ALCAT1 or LCLAT1) and mLPCAT1 are the known enzymes that could synthesize phospholipids both by the *de novo* and remodeling pathways (25,57). LPT1 (otherwise known as ALE1 or SLC4) in yeast (58 - 61) and human MBOAT2 (62) are acyltransferases, which can synthesize phospholipids by both pathways in other species. As enzymes with LPIAT activity, LPT1 (ALE1 or SLC4) (58 - 61) and MBOA7 (MBOAT7 or LPIAT1) (35, 62) had been described. The highly conserved motifs NHX_4D and EGTR are important for LPAAT and LPIAT activities of mLPAAT3, since point mutations in either of these motifs completely suppressed both activities.

At least seven mouse AGPAT family members exist, but their biological functions remain to be determined. At this point, even their biochemical characteristics are not well defined. Among them, LPAAT activities of mLPAAT1 and mLPAAT2 have been relatively well documented. mLPAAT1 exhibits LPAAT activity using oleoyl-LPA as an acceptor and palmitoyl-CoA, oleoyl-CoA, and arachidonoyl-CoA as donors (21). We also demonstrated that mLPAAT1 exhibits LPAAT activity using palmitoyl-LPA as an acceptor, and prefers palmitoyl-CoA, oleoyl-CoA, and arachidonoyl-CoA as donors (Supplemental Fig. 3). mLPAAT2 has LPAAT activity using oleoyl-LPA as an acceptor and oleoyl-CoA as a donor (37). The human homologue hLPAAT1 shows the highest activity with linoleoyl-LPA, palmitoyl-LPA, and myristoyl-LPA as acceptors and linoleoyl-CoA and palmitoyl-CoA as donors (40). hLPAAT2 exhibits LPAAT activity with palmitoyl LPA as an acceptor and myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, and arachidonyl-CoA as donors (34). Furthermore, mutations in hLPAAT2 cause congenital generalized lipodystrophy (41). To elucidate the biological roles of AGPAT family members, it will be particularly important to investigate their tissue distributions and biochemical characteristics.

We found that mLPAAT3 possessed a strong LPAAT activity using arachidonoyl-CoA and is highly expressed in the testis (Fig. 2A and 2B). This is particularly interesting because: (i) PA has a low arachidonic acid content at the *sn*-2 position in most

tissues (11-13) and (ii) CDP-diacylglycerol synthase 1 is highly localized to the testis and converts preferably 1-stearoyl-2-arachidonoyl-PA as a substrate to CDP-diacylglycerol, a precursor of glycerophospholipids including PI (42, 43). Since mLPAAT3 exhibits LPAAT activity using 1-stearoyl LPA and arachidonoyl-CoA, the enzyme may be functionally coupled with CDP-diacylglycerol synthase 1 to generate PI through the *de novo* pathway (Fig. 7A). Considering that mLPAAT3 also exhibited LPIAT activity with arachidonoyl-CoA, it can possibly help to produce PI effectively through both the *de novo* and remodeling pathways in the testis (Fig. 7A). Previously identified LPIAT (mouse MBOA7) is expressed ubiquitously including in the testis (Fig. 2C). Therefore, MBOA7 and CDP-diacylglycerol synthase 1 may be functionally coupled to produce PI effectively as well (Fig. 7B). There might still exist other enzymes to assist to produce PI in the testis. Limitation of our study is that the biochemical characterization of mLPAAT3 was done using over-expression system because the purification of this enzyme is extremely difficult due to its multiple transmembrane domains.

Additionally, mLPAAT3 expression in the testis is enhanced significantly in an age-dependent manner while relatively young, and then keeps a steady level (Fig. 6A). β-Estradiol induces mLPAAT3 in TM4 cells (Fig. 6C). In males, β-estradiol is produced by aromatization of testosterone. In human, β-estradiol increases initially throughout sex maturation (50), and then either slightly increases (51), remains steady (52, 53), or even slightly decreases (54-56) with age depending on studies. The reason for these differences of β-estradiol levels in various studies are unclear, but may be due to a wide range of β-estradiol level in the elderly. Considering this induction of β-estradiol in human is similar to that of mLPAAT3, β-estradiol may be a main regulator of mLPAAT3 expression.

In conclusion, we have isolated an enzyme, mLPAAT3, which catalyzes PA and PI production in the *de novo* and remodeling pathways, respectively. mLPAAT3 is highly expressed in the testis and exhibits a clear preference for arachidonoyl-CoA. In conjunction with CDP-diacylglycerol synthase 1, this enzyme may play an important role in PI production in the testis (Fig. 7A). Additionally, mLPAAT3 expression increases age-dependently while relatively young, and β-estradiol seems to be its important regulator. Further studies are needed to elucidate the roles of mLPAAT3 *in vivo*, in particular in the relationship with β-estradiol.

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REFERANCES

1. MacDonald,JI., Sprecher,H. 1991 Phospholipid fatty acids remodeling in mammalian cells. *Biochim. Biophys. Acta.* 1084: 105-121
2. Wood,R., Harlow,RD. 1969 Structural analyses of rat liver phosphoglycerides. *Arch. Biochim. Biophys.* 135: 272-281
3. Schlame,M., Brody,S., Hosetler,KY. 1993 Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. *Eur. J. Biochem.* 212: 727-735
4. Yamashita,A., Sugiura,T., Waku,K. 1997 Acyltransferases and transacylases involved in fatty acids remodeling of phospholipids and metabolism of bioactive lipids in mammalian cells. *J. Biochem.* 122:1-16
5. Kennedy,E.P. 1961 Biosynthesis of complex lipids. *Fed.Proc.* 20: 934-940
6. Shindou,H., Shimizu,T. Acyl-CoA:lysophospholipid acyltransferases. *J. Biol. Chem.* (in press)
7. Shindou,H., Hishikawa,D., Harayama,T., Yuki,K., Shimizu,T. Recent progress on Acyl-CoA: lysophospholipid acyltransferase research. *J. Lipid Res.* (in press)
8. Lands,W.E. 1960 Metabolism of glycerolipids. II. The enzymatic acylation of lysolecithin. *J.Biol.Chem.* 235: 2233-2237
9. Lands,W.E., and Merkl,I. 1963 Metabolism of glycerolipids. III. Reactivity of various acyl esters of coenzyme A with alpha-acylglycerophosphorylcholine, and positional specificities in lecithin synthesis. *J.Biol.Chem.* 238: 898-904
10. Merkl,I., and Lands,W.E. 1963 Metabolism of glycerolipids. IV. Synthesis of phosphatidylethanolamine. *J.Biol.Chem.* 238: 905-906
11. Possmayer,F., Scherphof,G.L., Dubbelman,T.M., van Golde,L.M., and van Deenen,L.L. 1969 Positional specificity of saturated and unsaturated fatty acids in phosphatidic acid from rat liver. *Biochim. Biophys. Acta.* 176: 95-110
12. Akesson,B., Elovson,J., Arvidson,G. 1970 Initial incorporation into rat liver glycerolipids of intraportally injected (3H) glycerol. *Biochim. Biophys. Acta.* 210: 15-27

13. Baker,R.R., Thompson,W. 1972 Positional distribution and turnover of fatty acids in phosphatidic acid, phosphinositides, phosphatidylcholine and phosphatidylethanolamine in rat brain *in vivo*. *Biochim. Biophys. Acta.* 270: 489-503

14. Holub,B.J., Kulsis,A. 1971 Structural and metabolic interrelationships among glycerophosphatides of rat liver *in vivo*. *Can. J. Biochem.* 49: 1347-1356

15. Shindou,H., Hishikawa,D., Nakanishi,H., Harayama,T., Ishii,S., Taguchi,R., and Shimizu,T. 2007 A single enzyme catalyzes both platelet-activating factor production and membrane biogenesis of inflammatory cells. Cloning and characterization of acetyl-CoA:Lyso-PAF acetyltransferase. *J. Biol. Chem.* 282: 6532-9

16. Nakanish,H., Shindou,H., Hishikawa,D., Harayama,T.,Ogasawara,R., Suwabe,A., Taguchi,R., and Shimizu,T. 2006 Cloning and characterization of mouse lung-type acyl-CoA:lysophosphatidylcholine acyltransferase 1 (LPCAT1). Expression in alveolar type II cells and possible involvement in surfactant production. *J. Biol. Chem.* 281: 20140-20147

17. Hishikawa,D., Shindou,H., Kobayashi,S., Nakanishi,H., Taguchi,R., and Shimizu,T. 2008 Discovery of a lysophospholipid acyltransferase family essential for membrane asymmetry and diversity. *Proc. Natl. Acad. Sci. USA.* 105: 2830-2835

18. Cases,S., Stone,SJ., Zhou,P., Yen,E.,Tow,B., Lardizabal,KD., Voelker,T., Farese,RV Jr. 2001 Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. *J. Biol. Chem.* 276: 38870-38876

19. Yen,CL.,Stone,SJ., Cases,S., Zhou,P., and Farese RV Jr. 2002 Identification of a gene encoding MGAT1, a monoacylglycerol acyltransferase. *Proc. Natl. Acad. Sci. USA.* 99: 8512-8517

20. Shin,DH.,Paulaauskis,JD.,Moustaid,N., and Sul,HS 1991 Transcriptional regulation of p90 with sequence homology to Escherichia coli glycerol-3-phosphate acyltransferase. *J. Biol. Chem.* 266: 23834-23839

21. Kume,K., and Shimizu,T. 1997 cDNA cloning and expression of murine 1-acyl-sn-glycerol-3-phosphate acyltransferase. *Biochem.Biophys.Res.Commun.* 237: 663-666

22. West,J., Tompkins,CK., Balantac,N., Nudelman,E., Meengs,B., White,T., Bursten,S., Coleman,J., Kumar,A., Singer,JW., Leung,DW. 1997 Cloning and expression of two human lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. *DNA Cell Biol.* 16: 691-701

23. Stamps,AC., Elmore,MA., Hill,ME., Kelly,K., Makda,AA., Finnen,MJ. 1997 A human cDNA sequence with homology to non-mammalian lysophosphatidic acid acyltransferases. *Biochem. J.* 326: 455-461

24. Yang,Y., Cao,J., Shi,Y. 2004 Identification and characterization of a gene encoding human LPGAT1, an endoplasmic reticulum-associated lysophosphatidylglycerol acyltransferase. *J. Biol. Chem.* 279: 55866-55874

25. Cao,J., Liu,Y., Lockwood,J., Burn,P., Shi,Y. 2004 A novel cardiolipin-remodeling pathway revealed by a gene encoding an endoplasmic reticulum-associated acyl-CoA: lysocardiolipin acyltransferase (ALCAT1) in mouse *J. Biol. Chem.* 279: 31727-31734

26. Cao,J., Shan,D., Revett,T., Li,D., Wu,L., Tobin,JF., Gimeno,RE. 2008 Molecular identification of a novel mammalian brain isoform of acyl-CoA: lysophospholipid acyltransferase with prominent ethanolamine lysophospholipid acylating activity, LPEAT2 *J. Biol. Chem.* 283: 19049-19057

27. Zhao,Y., Chen,YQ., Bonacci,TM., Bredt,DS., Li,S., Bensch,WR., Moller,DE., Kowala,M., Konrad,RJ., Cao,G. 2008 Identification and characterization of a major liver lysophosphatidylcholine acyltransferase. *J. Biol. Chem.* 283; 8258-8265

28. Harada,N., Hara,S., Yoshida,M., Zenitani,T., Mawatari,K., Nakano,M., Takahashi,A., Hosaka,T., Yoshimoto,K., Nakaya,Y. 2007 Molecular cloning of a murine glycerol-3-phosphate acyltransferase-like protein 1 (xGPAT1) *Mol. Cell Biochem.* 297: 41-51

29. Wang,S., Lee,DP., Gond,N., Schwebrock,NM., Mashek,DG., Gonzalez-Baro,MR., Stapleton,C, Li,LO., Lewin,TM., Coleman,RA. 2007 Cloning and functional characterization of a novel mitochondrial N-ethylmaleimide-sensitive glycerol-3-phosphate acyltransferase (GPAT2). *Arch. Biochem. Biophys.* 465: 347-358

30. Cao,J., Li,JL., Li,D., Tobin,JF., Gimeno,RE. 2006 Molecular identification of microsomal acyl-CoA: glycerol-3-phosphate acyltransferase, a key enzyme in de novo triacylglycerol synthesis. *Proc. Natl. Acad. Sci. USA.* 103: 19695-19700

31. Yet, S. F., Lee,S., Hahm,YT., Sul,HS. 1993. Expression and Identification of P90 as the Murine Mitochondrial Glycerol-3-Phosphate Acyltransferase. *Biochemistry.* 32: 9486-9491

32. Chen, YQ., Kuo,MS., Li,S., Bui,HH., Peake,DA., Sanders,PE., Thibodeaux,SJ., Chu,S., Qian,YW., Zhao,Y., Bredt,DS., Moller,DE., Konrad,RJ., Beigneux,AP., Young,SG., Cao,G. 2008. Agpat6 Is a Novel Microsomal Glycerol-3-Phosphate Acyltransferase. *J*

Biol Chem. 283: 10048-10057

33. Nagle,CA., Vergnes,L., Dejong,H., Wang,S., Lewin,TM.,Reue,K., and Coleman,RA. 2008 Identification of a novel sn-glycerol-3-phosphate acyltransferase isoform, GPAT4, as the enzyme deficient in Agpat6-/- mice. *J. Lipid Res.* 49: 823-831
34. Eberhardt,C., Gray,PW., and Tjoelker,LW. 1997 Human lysophosphatidic acid acyltransferase. cDNA cloning, expression , and localization to chromosome 9q34.3. *J. Biol. Chem.* 272: 20299-20305
35. Lee,HC., Inoue,T., Imae,R., Kono,N., Shirae,S., Matsuda,S., Gengyo-Ando,K., Mitani,S., Arai,H. 2008 Caenorhabditis elegans mboa-7, a member of the MBOAT family is required for selective incorporation of polyunsaturated fatty acids into phosphatidylinositol. *Mol. Biol. Cell* 19: 1174-1184
36. Dircks,L., and Sul,H.S. 1999 Acyltransferases of de novo glycerophospholipid biosynthesis. *Prog. Lipid Res.* 38: 461-479
37. Lu,B., Jiang,YJ., Zhou,Y., Xu,FY., Hatch,GM., Choy,PC. 2005 Cloning and characterization of murine 1-acyl-sn-glycerol 3-phosphate acyltransferases and their regulation by PPARalpha in murine heart. *Biochem. J.* 385: 469-77
38. Lewin, TM.,Wang,P., and Coleman,RA. 1999 Analysis of amino acid motifs diagnostic for the sn-glycerol-3-phosphate acyltransferase reaction. *Biochemistry* 38: 5764-577
39. Harayama,T., Shindou,H., Ogasawara,R., Suwabe,A., and Shimizu,T. 2008 Identification of a novel noninflammatory biosynthetic pathway of platelet-activating factor. *J. Biol. Chem.* 283:11097-11106
40. Yamashita,A., Nakanishi,H., Suzuki,H., Kamata,R., Tanaka,K., Waku,K., and Sugiura,T. 2007 Topology of acyltransferase motifs and substrate specificity and accessibility in 1-acyl-sn-glycero-3-phosphate acyltransferase 1. *Biochim. Biophys. Acta.* 1771: 1202-1215
41. Agarwal,AK., Arioglu,E., De Almeida,S., Akkoc,N., Taylor,SI., Bowcock,AM., Barnes,RI., and Garg,A. 2002 AGPAT2 is mutated in congenital generalized lipodystrophy linked to chromosome 9q34. *Nat. Genet.* 31: 21-23
42. Saito,S., Goto,K., Tonosaki,A., and Kondo, H. 1997 Gene cloning and characterization of CDP-diaylglycerol synthase from rat brain. *J. Biol. Chem.* 272: 9503-9509
43. Inglis-Broadgate,SL., Ocaka,L., Banerjee,R., Gaasenbeek,M., Chapple,JP., Cheetham,ME., Clark,BJ., Hunt,DM., Halford,S. 2005 Isolation and characterization of murine Cds (CDP-diacylglycerol synthase) 1 and 2. *Gene* 356: 19-31

44. Niwa,H., Yamashita,K., Miyazaki,J. 1991 Efficient selection for high-expression transfectants with a novel eukaryotic vector. 108: 193-199

45. Bradford,M.M. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254

46. Bligh,E.G., and Dyer,W.J. 1959 A rapid method of total lipid extraction and purification. *Can.J.Biochem.Physiol.* 37: 911-917

47. Thompson,JD., Higgins,DG., Gibson,TJ. 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680

48. Arai,M., Mitsuke,H., Ikeda,M., Xia,J.-X., Kikuchi,T., Satake,M., and Shimizu,T. 2004 ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. *Nucleic Acids Res.* 32: 390-393

49. Shikano S., Li,M. 2003 Membrane receptor trafficking : evidence of proximal and distal zones conferred by two independent endoplasmic reticulum localized signals. *Proc. Natl. Acad. Sci. USA.* 100: 5783-5788

50. Andersson,AM., Juul,A., Petersen,JH., Muller,J., Groome,NP., Skakkebaek,NE. 1997 Serum inhibin B in healthy pubertal and adolescent boys : relation to age, stage of puberty, and follicle-stimulating hormone, luteinizing hormone, testosterone, and estradiol levels. *J. Clin. Endocrinol. Metab.* 82: 3976-3981

51. Bjørnerem,A., Straume,B., Midtby,M., Fønnebø,V., Sundsfjord,J., Svartberg,J., Acharya,G., Oian,P., Bertsen,GK. 2004 Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a population: the Tromsø Study. *J. Clin. Endocrinol. Metab.* 89: 6039-6047

52. Muller,M., den Tonkelaar,I., Thijssen,JH., Grobbee,DE., van der Schouw,YT. 2003 Endogenous sex hormones in men aged 40-80 years. Eur. J. Endocrinol.149: 583-589

53. Bélanger,A., Candas,B., Dupont,A., Cusan,L., Diamond,P., Gomez,JL., Labrie,F. 1994 Changes in serum concentrations of conjugated and unconjugated steroids in 40-80 year old men. *J. Clin. Endocrinol. Metab.* 79: 1086-1090

54. Ferrni,R., Barret-Conner,E. 1998 Sex hormones and age: a cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men. *Am. J. Epidemiol.*147: 750-754

55. Orwoll,E., Lambert,LC., Marshall,LM., Phipps,K., Blank,J., Barret-Conner,E., Cauley,J., Ensrud,K., Cummings,S. 2006 Testosterone and estradiol among older men. *J. Clin. Endocrinol. Metab.* 91: 1336-1344

56. van den Beld,AW., de Jong,FH., Grobbee,DE., Pols,HA., Lamberts,SW. 2000 Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *J. Clin. Endocrinol. Metab.* 85: 3276-3286

57. Chen,X., Hyatt,BA., Mucenski,ML., Mason,RJ., amd Shannon,JM. 2006 Identification and characterization of a lysophosphatidylcholine acyltransferase in alveolar type II cells. *Proc Natl Acad Sci USA.* 103:11724-11729

58. Jain,S., Stanford,N., Bhagwat,N., Seiler,N., Costanzo,M., Boone,C., Oelkers,P. 2007 Identification of a novel lysophospholipid acyltransferase in *Saccharomyces cerevisiae J. Biol. Chem.* 282, 30562-30569

59. Benghezai,M, Roubaty,C., Veepuri,V., Knudsen,J., Conzelmann,A. 2007 SLC1 and SLC4 encode partially redundant acyl-coenzyme A 1-acylglycerol-3-phosphate O-acyltransferases of budding yeast *J. Biol. Chem.* 282, 30845-30855

60. Tamaki,H., Shimada,A., Ito,Y., Ohya,M., Takase,J., Miyashita,M., Miyagawa,H., Nozaki,H., Nakayama,R., Kumagai,H. 2007 LPT1 encodes a membrane-bound O-acyltransferase involved in the acylation of lysophopholipids in the yeast *Saccharomyces cerevisiae J. Biol. Chem.* 282, 34288-34298

61. Riekhof,WR., Wu,J., Gijón,MA., Zarini,S., Murphy,RC., Voelker,DR. 2007 Lysophosphatidylcholine metabolism in *Saccharomyces cerevisiae J. Biol. Chem.* 282, 36853-36861

62. Gijón,MA., Riekhof,WR, Zarini,S., Murphy,RC., Voelker,DR. 2008 Lysophospholipid acyltransferases and arachidonate recycling in human neutrophils *J. Biol. Chem.* 283, 30235-30245

FOOTNOTES

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Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB377215 (mouse)

Figure legends

Figure 1. Phylogenetic tree of AGPAT family members and alignment of mLPAAT1, 2, and 3

(A) A phylogenetic tree was drawn by using ClustalW, DDBJ (http://www.clustalw.ddbj.nig.ac.jp/top-j.html). Sequences of mouse acyltransferases are available in DDBJ/EMBL/GenBank databases. mLPAAT3 is circled. (B) mLPAAT1, mLPAAT2 and mLPAAT3 sequences were aligned using Genetic-Mac software. Conserved putative catalytic motif NHX_4D and binding motif EGTR are underlined. Mutated amino acids are indicated by arrows (See Fig. 5). The accession numbers are shown as follows: GPAT1 (NP_032175), GPAT2 (NP_001074558), GPAT3 (NP_766303), GPAT4 (NP_061213), LPAAT1 (NP_061350), LPAAT2 (NP_080488), LPAAT3 (AB377215), LPGAT1 (NP_758470), ALCAT1 (acyl-CoA:lysocardiolipin acyltransferase 1, also called as LCLAT1) (Q3UN02), LPCAT1 (BAE94687), LysoPAFAT/LPCAT2 (BAF47695), LPAATδ (NP_080920), LPAATε (NP_081068), LPAATη (NP_997089), and AT Like 1B (NP_081875).

Figure 2. Expression profile of mLPAAT3 and mMBOA7 (LPIAT1) in mice

Expression levels of mLPAAT3 mRNA (A) and mMBOA7 (LPIAT1) mRNA (C) in 21 tissues from C57BL/6J mice were analyzed using quantitative real-time PCR. (A) mLPAAT3 was expressed predominantly in the testis, whereas (C) mMBOA7 (LPIAT1) was ubiquitously expressed. Similar results were obtained in a separate independent experiment. (B) Expression of mLPAAT3 was analyzed in protein level by Western blot using anti-mLPAAT3 antiserum. 3μg of 100,000 x *g* pellets from various tissues were loaded in each lane. Br, Lu, Li, Sp, Ki, and Te stand for brain, lung, liver, spleen, kidney, and testis, respectively. mLPAAT3 was highly expressed in the testis. High expression was noted in the liver and kidney as well. The results are representative of three independent experiments.

Figure 3. Subcellular localization of FLAG-mLPAAT3 in CHO-K1 cells and endogenous mLPAAT3 in TM4 cells

(A) At 48 h post-transfection, proteins from CHO-K1 cells were subjected to Western blot using anti-M2 FLAG antibody. Expression of FLAG-mLPAAT3 was confirmed by Western blot. Homogenates of pCXN2.1 vector or FLAG-mLPAAT3-pCXN2.1 transfected CHO-K1 cells were separated by differential centrifugation using an ER extraction kit (Sigma) as described in Materials and Methods. 2 μg of 9,000 x *g* pellets (indicated as Mito), 100,000 x *g*

pellets (ER) were loaded in the lanes indicated. Molecular sizes are indicated on the left in kDa. Results are representative of two independent experiments. (B) As a marker of mitochondria, anti cytochrome c oxidase antibody was used. Molecular sizes are indicated on the left in kDa. Results are representative of two independent experiments. (C) Subcellular localization of endogenous mLPAAT3 in testicular cell line TM4 cells was confirmed by Western blot. 1.5μg of 9,000 x *g* pellets (Mito) and 100,000 x *g* pellets (ER) were loaded in the lanes indicated. mLPAAT3 was detected using rabbit anti-mLPAAT3 antiserum. Results are representative of two independent experiments. (D) As a marker of mitochondria, anti cytochrome c oxidase antibody was used. Molecular sizes are indicated on the left in kDa. Results are representative of two independent experiments.

Figure 4. Substrate selectivity of mLPAAT3

(A) Lysophospholipid preferences of mLPAAT3 were determined. Acyltransferase activity was examined using 2 μg protein (100,000 x *g* pellet), 25 μM [1-^{14}C]arachidonoyl-CoA (33,000 dpm), and 50 μM lysophospholipids. Data are shown as mean + S.D. of triplicate measurements. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. * represents $P < 0.05$. (B) The preference of mLPAAT3 for various LPA acceptors was examined using oleoyl-CoA or arachidonoyl-CoA as a donor. Acyltransferase activity was examined using 2 μg protein, 25 μM [1-^{14}C]oleoyl-CoA (33,000 dpm), and 50 μM lysophospholipids. Data are shown as mean + S.D. of triplicate measurements. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. Only for stearoyl-LPA group, t test was used for analysis. * represents $P < 0.05$. (C) The acyl-CoA selectivity of mLPAAT3 was examined using palmitoyl-LPA as an acceptor. Acyltransferase activity was examined using 2 μg protein, 25 μM acyl-CoAs (33,000 dpm), and 50 μM palmitoyl-LPA, with the exception that 100 μM acetyl-CoA (111,000 dpm, 185MBq / mmol) was used. Data are shown as mean + S.D. of triplicate measurements. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. * represents $P < 0.05$. (D) The acyl-CoA selectivity of mLPAAT3 was examined for LPIAT activity. Acyltransferase activity was examined using 2 μg protein, 25 μM arachidonoyl-CoA (33,000 dpm), and 50 μM LPI. The concentration of acetyl-CoA used was 100 μM (111,000 dpm, 185MBq / mmol). Data are shown as mean + S.D. of triplicate measurements. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. * represents $P < 0.05$. In (A-D),

results are representative of two independent experiments.

Figure 5. The role of highly conserved motifs NHX_4D and EGTR in LPLAT activity of mLPAAT3

We constructed H96A, D101A, and E176A mutants of mLPAAT3 by mutating single amino acids in highly conserved motifs NHX_4D or EGTR (Fig. 1B). (A) The acyltransferase activity of mLPAAT3 wild-type and three mutants was measured using 2 μg protein (100,000 x *g* pellet), 50 μM palmitoyl-LPA, or LPI as an acceptor and 25 μM arachidonoyl-CoA (33,000 dpm) as a donor. Data represent mean + S.D. of triplicate samples measurements. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. * represents $P < 0.05$. The results are representative of two independent experiments. (B) Expression of mLPAAT3 wild-type and three mutants was confirmed by Western blot analysis. The results are representative of two independent experiments.

Figure 6. Age-dependent expression of mLPAAT3 in the testis and mLPAAT3 induction in testicular cell line

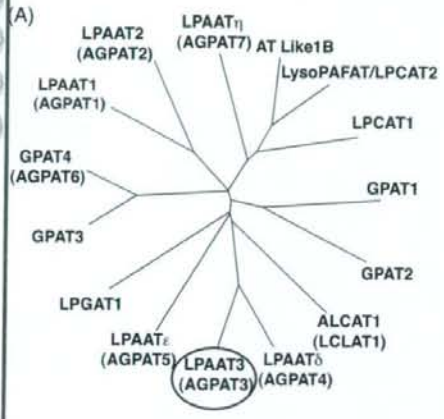
(A) mLPAAT3 mRNA expression in the testis at various ages was compared using real-time Q-PCR. mLPAAT3 mRNA expression is enhanced significantly in an age-dependent manner until 15 week of age. The results are representative of two independent experiments. (B) mLPAAT3 protein expression in the testis at different ages was compared by Western blot using anti-mLPAAT3 antiserum. 4μg each of 100,000 x *g* pellets at different ages were loaded. Results are representative of two independent experiments. (C) LPAAT activity of 2, 8, 45 weeks testis was examined using 1 μg protein (100,000 x g pellet), 25 μM arachidonoyl-CoA (33,000 dpm), and 50 μM palmitoyl LPA. Data represent mean + S.D. of triplicate samples measurements. The results are representative of two independent experiments. (D) Testicular cell line TM4 cells were treated with either mock, 100nM β-estradiol, DHT, or testosterone for 24h. mLPAAT3 mRNA level was compared using real-time Q-PCR. β-estradiol induced mLPAAT3 significantly. Data represent mean + S.D. of three independent experiments. Statistical significance was analyzed using ANOVA with Tukey *post hoc* pairwise comparisons. * represents $p < 0.05$.

Figure 7. Proposed model for PI production in the testis

At least, mLPAAT3 and MBOA7 (LPIAT1) may be an important enzyme to produce PI in the testis. (A) Both mLPAAT3 (Fig. 2A) and CDP-diacylglycerol synthase 1 (41, 42) are highly expressed in the testis. CDP-diacylglycerol synthase 1 prefers 1-stearoyl-2-arachidonoyl PA as a substrate and mLPAAT3 shows LPAAT activity using stearoyl-LPA as an acceptor and arachidonoyl-CoA as a donor. Since 1-stearoyl-2-arachidonoyl species are major components of PI, we hypothesized that in the testis, mLPAAT3 helps to produce PI effectively through both the *de novo* and remodeling pathways. (B) Also in the testis, CDP-diacylglycerol synthase 1 (41, 42) and MBOA7 (LPIAT1) (Fig. 2C) may generate PI effectively through the *de novo* and remodeling pathways, respectively. In (A) and (B), the *de novo* and remodeling pathways are enclosed in dotted lines.

Figure1

(A)



(B)

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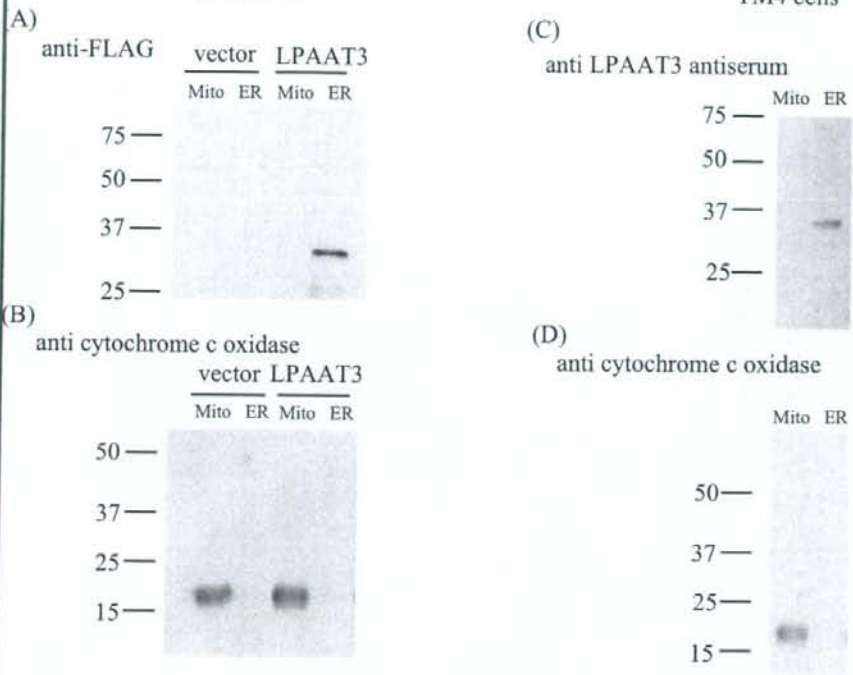
Figure 2

Figure 3



ure 4

Figure 5