Research Article

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Hepatic cholesteryl ester accumulation in lysosomal acid lipase deficiency: Non-invasive identification and treatment monitoring by magnetic resonance

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Background & Aims: Lysosomal Acid Lipase (LAL) deficiency is a rare metabolic storage disease, caused by a marked reduction in activity of LAL, which leads to accumulation of cholesteryl esters (CE) and triglycerides (TG) in lysosomes in many tissues. We used ^{1}H magnetic resonance (MR) spectroscopy to characterize the abnormalities in hepatic lipid content and composition in patients with LAL deficiency, and in *ex vivo* liver tissue from a LAL deficiency rat model. Secondly, we used MR spectroscopy to monitor the effects of an enzyme replacement therapy (ERT), sebelipase alfa (a recombinant human lysosomal acid lipase), on hepatic TG and CE content in the preclinical model.

Methods: Human studies employed cohorts of LAL-deficient patients and NAFLD subjects. Rat experimental groups comprised *ex vivo* liver samples of wild type, NAFLD, LAL-deficient, and LAL-deficient rats receiving 4 weeks of sebelipase alfa treatment. Hepatic ^{1}H MR spectroscopy was performed using 3T (human) and 7T (preclinical) MRI scanners to quantify hepatic cholesterol and triglyceride content.

Results: CE accumulation was identified in LAL deficiency in both human and preclinical studies. A significant decrease in hepatic CE was observed in LAL-deficient rats following treatment with sebelipase alfa.

Conclusions: We demonstrate an entirely non-invasive method to identify and quantify the hepatic lipid signature associated with a rare genetic cause of fatty liver. The approach provides a more favorable alternative to repeated biopsy sampling for diagnosis and disease progression / treatment monitoring of patients with LAL deficiency and other disorders characterised by increased free cholesterol and/or cholesteryl esters.

Keywords: Wolman disease; Cholesteryl ester storage disease; ^{1}H MR spectroscopy; ^{13}C MR spectroscopy; Liver fat; Lysosomal acid lipase; LIPA; LAL deficiency; Enzyme replacement therapy; Sebelipase alfa.

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Abbreviations: CESD, cholesteryl ester storage disease; LAL, lysosomal acid lipase; CE, cholesteryl ester; TG, triglyceride; ERT, enzyme replacement therapy; NAFLD, non-alcoholic fatty liver disease.

Introduction

Lysosomal Acid Lipase (LAL) deficiency is a rare autosomal recessive lysosomal storage disease for which there is no currently available effective treatment. The disease is caused by mutations of the *LIPA* gene encoding the LAL enzyme and deficiency of this enzyme leads to the accumulation of cholesteryl esters (CE) and triglycerides (TG) in a number of tissues. Although a single disease, LAL deficiency presents as a clinical continuum with two major phenotypes: the early onset phenotype is typically referred to as Wolman Disease and the late onset phenotype is frequently known as Cholesteryl Ester Storage Disease (CESD) [1]. Early onset LAL deficiency typically presents in the first 6 months of life and is the most rapidly fatal presentation. Growth failure associated with malabsorption, massive hepatosplenomegaly, rapidly progressive liver dysfunction, and anaemia are the predominant clinical features and key contributors to the early mortality. Survival beyond 1 year of age is highly unusual for these patients. Late onset LAL deficiency is an underappreciated cause of cirrhosis in both children and adults, and is more heterogeneous with respect to age of diagnosis. Elevation of serum transaminases, hepatomegaly, and dyslipidemia (high LDL, high triglycerides, and low HDL) are the important abnormalities. Disease complications include hepatic fibrosis with progression to cirrhosis and accelerated atherosclerosis [2–5]. Diagnosis of late onset disease requires a high index of clinical suspicion as the combination of elevated transaminases, fatty liver, and dyslipidemia is also seen in patients with the much more common diagnosis of metabolic syndrome. In contrast to steatosis in patients with metabolic syndrome, which is primarily due to increased accumulations of TG, steatosis in patients with LAL deficiency is associated with increases in both CE and TG.

Monitoring liver lipid accumulation in patients has traditionally involved analysis of biopsy samples. This approach is invasive, carries an associated risk to the patient, and samples only a small region of the organ and thus may be prone to sampling errors. Magnetic resonance techniques can provide an alternative to biopsy, offering a non-invasive, safe, and repeatable method to measure liver lipid content and composition. MRI and ^{1}H magnetic resonance (MR) spectroscopy methods are widely used in research studies to measure hepatic lipid content, providing either a measurement from a defined volume in the liver (via PRESS spectroscopy [6]) or an image of lipid distribution (via "Dixon method" imaging [7]). A good correlation has been observed between ^{1}H MR measures of lipid content and biopsy for steatosis [8,9]. ^{13}C MR spectroscopy can also be used, and has the advantage of providing more detailed information on the chemical composition of hepatic lipids than obtained in ^{1}H spectroscopy [10]. In this study, we have employed ^{1}H spectroscopy to quantify and characterize the hepatic lipid signature associated with a rare genetic defect of lipid metabolism in a cohort of patients with LAL deficiency, and also utilized a biochemical assay and ^{13}C spectroscopy in the preclinical studies to confirm that the observed changes in ^{1}H spectra originate from cholesterol/cholesteryl ester accumulation. The previously described rat model of LAL deficiency develops liver abnormalities that closely resemble the changes seen in patients with both early and late onset LAL deficiency [11], showing CE and TG accumulation in the liver, leading to rapid development of fibrosis in this model and in other organs.

We hypothesized that the differences in lipid content and composition resulting from LAL deficiency could be detected non-invasively by MR spectroscopy, both in humans and in the preclinical model. We hypothesised that a signature of LAL deficiency could be identified (comprising detection of the increases in hepatic CE and TG content), and that the reduction in hepatic CE and TG content, following enzyme replacement therapy with sebelipase alfa (Synageva BioPharma Corp., Lexington, MA, USA), could be observed with these methods.

In addition to providing a method to monitor the efficacy of therapies that target CE accumulation, the identification and non-invasive quantification of hepatic CE content may allow for the identification of patients with fatty liver who have increased CE content and may warrant consideration for diagnostic testing for LAL deficiency. Furthermore, a non-invasive method that allows quantification of specific lipid species could help differentiate patients with LAL deficiency from patients with non-alcoholic fatty liver disease (NAFLD) due to metabolic syndrome. With the recent progression of sebelipase alfa into clinical studies for patients with LAL deficiency [12–14], such an approach would also allow the non-invasive assessment of the effects of enzyme replacement on lipid substrate accumulation in key tissues including the liver in this disease.

Materials and methods

Recruitment of human subjects

Study participants (n = 3) were patients with late onset LAL deficiency (also known as Cholesteryl Ester Storage Disease) enrolled into the LAL-2-NH01 substudy in the UK [15]. This is a multicenter, prospective, observational study of a subset of patients with late onset LAL to characterize clinical phenotype and disease progression. The patients' diagnosis was confirmed by a previous diagnostic test (i.e., documented decreased LAL activity relative to the normal range of the lab performing the assay or molecular genetic testing confirming mutations consistent with the diagnosis of LAL deficiency).

A comparator group of ^{1}H spectroscopy datasets from NAFLD patients was obtained from a previous ^{1}H MR spectroscopy study performed at Newcastle University, where patients with NAFLD (defined as >5% intrahepatic lipid on ^{1}H-MRS, n = 5) were recruited from hepatology clinics within the Newcastle upon Tyne Hospitals Foundation Trust.

Human magnetic resonance spectroscopy

MR data were acquired on a Philips 3T Achieva whole body scanner (Philips Medical Systems, Best, The Netherlands) using a Philips 6-channel cardiac coil for ^{1}H imaging and spectroscopy. ^{1}H spectroscopy comprised acquisition of PRESS-localised spectra at six echo times (TR = 2.8 s, TE = 36, 50, 75, 100, 125, and 150 ms, spectral width = 2 kHz, 2k data points) from a 3 × 3 × 3 cm voxel positioned in the liver to avoid large vessels.

Analysis of human MR spectroscopy data

Spectra were processed using the Java-based magnetic resonance user interface (jMRUI version 4.0) [16,17] using the AMARES non-linear least square fitting algorithm [18] to determine peak areas. Resonances at 4.7, 1.3, and 0.9 ppm in ^{1}H spectra were quantified, which corresponded principally to protons in water, to CH_2 protons, and to CH_3 protons, respectively. Initial attempts at determining T_2 of protons contributing to these peaks highlighted significant J-coupling effects in lipid proton resonances at longer echo times. Thus no attempt was made to correct for T_2 differences between the resonances, and reported data originate from the shortest echo time employed (36 ms).

Tissue fat fraction (v/v) calculations were performed using the widely-employed method described by Longo *et al.* [8]. Since this approach does not distinguish between cholesterol and triglyceride, and is based on the assumption that hepatic lipid has a normal (predominantly triglyceride) composition, a spectral modelling approach was also employed to provide a quantitative measure of hepatic cholesterol and fatty acid moiety content. The ratio of amplitudes of 1.3–0.9 ppm ^{1}H spectral resonances was used to determine the molar ratio of cholesteryl to fatty acid moieties for each subject, and the ratio of 4.7–1.3 ppm resonances employed to quantify lipid content relative to water content. The model assumed that the hepatic triglyceride composition, and thus its *in vivo* ^{1}H spectrum, was identical to that observed experimentally and described in model form by Hamilton *et al.* [19] (mean fatty acid chain length of 17.45 carbons, mean double bond content of 1.92 per fatty acid moiety, and mean number of methylene-interrupted double bonds of 0.32 per fatty acid moiety). The *in vivo* spectrum of cholesterol moieties was assumed to be identical to the National Institute of Advanced Industrial Science and Technology (Japan) database cholesterol ^{1}H spectrum [20]. Thus in the spectral model, triglyceride protons contribute to 1.3–0.9 ppm ^{1}H spectral resonances at a ratio of 8.0:1 (as determined by Hamilton *et al.* [19]) and cholesterol moieties contribute to these peaks at a ratio of 1:12.4. Molar ratio of fatty acid and cholesterol moieties was determined from the experimentally measured ratio of 1.3–0.9 ppm peak amplitudes for each subject and then absolute concentrations of these components calculated using the liver tissue water fraction, water and lipid proton density, and tissue density assumptions described by Longo *et al.* [8].

Preclinical models of LAL deficiency and NAFLD

The rat model of LAL deficiency is a Donryu rat strain with a spontaneous deletion at the 3′ end of the *LIPA* gene, leading to deletion of 29 C-terminal amino acids [21]. Rats were obtained through the National Bio Resource Project for the Rat in Japan and maintained by breeding of heterozygous rats with the generation of homozygous LAL deficient progeny. Genotyping was performed by PCR analysis of DNA isolated from tail-clippings. 20–100 ng of CHELEX-extracted DNA [22] was subjected to PCR using three primers: For1, 5′-cagcacagggcacacacataggca-3′; Rev1, 5′-ccacccttgtacagcataaaagac-3′; and For2, 5′-cagagacgcaggcacaataactcc-3′ for 33 cycles of: 45 s at 95 °C, 45 s at 66 °C, 1 min 30 s at 72 °C. Reactions were resolved on ethidium bromide-stained 1% agarose gels and alleles identified by amplicons size; LAL+, 578 bp; LAL−, 341 bp.

LAL deficient rats were housed at the Animal Resources Complex in the College of Veterinary Medicine, University of Georgia, Athens, GA, USA, under the supervision of the University of Georgia Institutional Animal Care and Use Committee. Rats were provided feed (Purina Lab Irradiated Rodent Chow #5053) and water *ad libitum*. The three experimental groups comprised wild type (LAL+/+), LAL deficient (LAL−/−) genotypes, and LAL deficient animals that had received enzyme replacement therapy with sebelipase alfa. Rats were euthanized by CO_2