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In vivo assessment of hepatic triglycerides in murine non-alcoholic fatty liver disease using magnetic resonance spectroscopy

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ABSTRACT

In vivo ^{1}H magnetic resonance spectroscopy (MRS) was used to examine the progression of fatty liver in two murine models of progressive hepatic steatosis: leptin-deficient obese (*ob/ob*) mice and mice maintained on a diet deficient in methionine and choline (MCDD). *Ob/ob* mice displayed high levels of intracellular hepatic triglycerides as early as 9 weeks after birth, as observed with MRS and histopathology. Single voxel spectra of *ob/ob* liver displayed strong resonances arising from saturated (1.3 ppm) and unsaturated (2.8 and 5.3 ppm) fatty acyl chains that could be resolved in the absence of water suppression. Hepatic inflammation, induced by lipopolysaccharide administration, led to a significant increase in unsaturated and polyunsaturated fatty acyl chain resonances ($P<0.05$), indicating a change in the composition of hepatic triglycerides in lipid droplets. Mice maintained on the MCDD displayed histological evidence of hepatic steatosis as early as two weeks, progressing to macrovesicular steatohepatitis at 10 weeks. The histological changes were accompanied by significant increases in saturated and unsaturated fatty acyl chain resonances and a significant decrease in the lipid/(water + lipid) ratio ($P<0.05$). These results indicate that *in vivo* ^{1}H MRS may be a suitable method to monitor the progression of steatohepatitis.

1. Introduction

Hepatic steatosis (the accumulation of lipid droplets within hepatocytes) has become an increasingly common condition in the United States. Current estimates indicate that hepatic steatosis is present in 20% of the general population [1–3] with higher prevalence rates reported among the obese and individuals with metabolic syndrome (hypertriglyceridemia, low levels of high-density lipoproteins, hypertension and insulin resistance) [4]. An accurate non-invasive diagnosis of steatosis is important as it is now recognized that a progressive form of non-alcoholic fatty liver disease (NAFLD) exists that can ultimately result in end stage liver disease [2,5]. This progressive form of fatty liver disease is known as non-alcoholic steatohepatitis (NASH) [6]. The overall prevalence of NASH is estimated to be approximately 20% among the obese and 3% for the lean population [1–3]. Furthermore, current estimates indicate that 20–30% of individuals with NASH go on to develop cirrhosis [7–10]. These facts highlight the need to be able to detect steatosis and accurately distinguish between uncomplicated NAFLD and NASH, however this is a difficult clinical task. Furthermore, there is an increasing need to develop non-invasive biomarkers for detection and staging of this disease [11]. Current clinical methods of diagnosing this condition include the liver biopsy and abdominal imaging. The liver biopsy is the gold standard with which to accurately assess the severity of steatosis and to diagnose NASH [11,12]. This invasive technique, however, has a number of inherent problems, foremost of which is its risk of complications and potential for sampling errors. Steatosis can also be non-invasively diagnosed with ultrasound, computed tomography or magnetic resonance imaging. However, these modalities only provide an estimate on the quantity of fat infiltration within the liver and are incapable of differentiating between benign hepatic steatosis and the more progressive form of fatty liver disease [13–15].

In vivo magnetic resonance spectroscopy (MRS) has the potential to provide information about both the quantity and composition of fat within the liver *in situ*. Since MR techniques are sensitive only to molecules with a high degree of rotational molecular motion, the lipid resonances observed arise only from the triglycerides in intracellular fat droplets; lipids in membrane bilayer conformation are not detectable using high-resolution NMR techniques [16]. To date, most

Abbreviations: CHESS, Chemical Shift Selective (water suppression); FOV, field of view; i.p., intraperitoneal; LPS, lipopolysaccharide; MCDD, methionine–choline deficient diet; MRS, magnetic resonance spectroscopy; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMR, nuclear magnetic resonance; *ob/ob*, leptin-deficient obese mice; PLA2, phospholipase A2; PRESS, point resolved spectroscopy; NT, Number of transients; TE, echo time; TM, mixing time; TR, repetition time; TNF, tumor necrosis factor

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in vivo ^{1}H MRS studies of liver in humans [17–20] and animals [21,22] have been limited by low spectral resolution. However, the implementation of spectroscopy at fields of 4.7 T and higher, as well as improved localization sequences and the introduction of respiratory gating have led to marked improvement in the quality of animal MR spectra of liver and it is now possible to obtain more detailed information about lipid composition [23–25].

A number of animal models are available for the study of hepatic steatosis. The leptin-deficient obese/obese (*ob*/*ob*) mouse is a widely used model for studies of NAFLD [26]. Similar to the human condition, these mice exhibit severe hepatic steatosis in the setting of obesity and metabolic syndrome. In addition the *ob*/*ob* mice are prone to hepatic injury induced by endotoxin exposure [27–30]. Doses of lipopolysaccharide (LPS) that are relatively innocuous for lean mice elicit an acute hepatic necroinflammatory response in *ob*/*ob* mice [29,30]. A chronic murine model of NASH can also be produced by placing mice on a methionine/choline deficient diet (MCDD). Methionine and choline deficiencies produces pronounced fatty liver and steatohepatitis in mice over ten weeks [31,32].

In this study localized *in vivo* ^{1}H MRS was utilized to assess the content and composition of hepatic triglycerides in these two murine models of NAFLD. We implemented the PRESS (point resolved spectroscopy) localization sequence with respiratory gating to obtain *in vivo* spectra with sufficient spectral resolution to observe resonances from unsaturated and polyunsaturated fatty acids in steatotic liver triglycerides. These advances allow determination of changes in the content and composition of liver triglycerides in a non-invasive manner. Our objectives were to identify potential non-invasive biomarkers during the onset and progression of steatohepatitis that could be used for monitoring the progression of this disease.

2. Materials and methods

2.1. Animal procurement and care

All protocols for animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Nine-week-old male *ob*/*ob* C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a temperature and light controlled animal facility. All animals were permitted *ad libitum* access to food and water. Obese mice were maintained on standard laboratory rodent chow (Lab Diet, Rodent Diet 5001). An additional cohort of lean male C57BL/6J mice were placed on a diet deficient in methionine/choline (ICN Biochemicals, Cleveland, OH).

In a preliminary study of obese mice, baseline ^{1}H MRS examinations were performed on nine-week-old male *ob*/*ob* C57BL/6J mice ($n=6$). Subsequent to the MR experiments, the animals were sacrificed and the liver excised and fixed in phosphate-buffered formalin for histology. In a subsequent study, a cohort of *ob*/*ob* mice received 2.0 mg/kg of LPS intraperitoneally (*E. coli* 0111:B4 endotoxin; Sigma Aldrich, St. Louis, MO) and 24 h following LPS administration ^{1}H MRS examinations were performed ($n=5$). The control group ($n=6$) received no LPS injection. A second group of controls were included in this study, consisting of *ob*/*ob* mice that received intraperitoneal (i.p.) injections of saline with subsequent fasting for 24 h ($n=5$). Following the 24-hour time point MR exams were performed, thereafter animals were sacrificed and liver tissue samples collected for histology.

For the MCDD studies, nine-week old male C57BL/6J mice were placed on the methionine/choline deficient diet for a period of ten weeks. During this period serial MRS examinations were performed on mice at 2, 6 and 10 weeks. Following MR examinations, a subset of mice were sacrificed ($n=7$, 8 and 6 for 2, 6 and 10 weeks respectively) and liver samples were collected for examination by histology. An additional group of male mice ($n=3$) maintained on standard laboratory rodent chow served as baseline controls.

2.2. In vivo ^{1}H MRS

All MRS examinations were performed on a 4.7 T Varian Inova imaging system equipped with a 40 cm horizontal bore magnet. Anesthesia was induced using ketamine/acepromazine (50/5 mg/kg i.p.) and maintained with isoflurane (1%). A fiber optic mouse temperature probe and pneumatic pillow (SA Inc., Stony Brook, NY) were used to monitor/regulate core temperature and perform triggered respiratory gating. Animals were placed prone on a 15 mm diameter receive only surface coil such that the liver lay directly above the sensitive volume of the coil. The mouse/surface coil was then placed in a 50-mm diameter transmit birdcage volume coil system. Respiratory triggered multi-slice gradient echo images were acquired in the axial plane with a repetition time (TR) = 300 ms, echo time (TE) = 5.0 ms, slice thickness of 1 mm, field of view (FOV) 5 cm × 5 cm and a matrix size of 256 × 128. Utilizing the axial images, a 32-mm^3 (4 × 4 × 2 mm) voxel was prescribed within the liver in an area free of large hepatic vessels and surrounding visceral fat. Localized shimming was performed until a line width of less than 50 Hz was achieved for the water resonance. Respiratory-gated localized proton MRS was accomplished using a PRESS pulse sequence (TR = 3 s, TE = 12 ms, TE2 = 11 ms, NT = 256, spectral width 2000 Hz, 2048 data points). Localized spectra were acquired with and without CHESS (chemical shift selective) water suppression. Between one and three spectral voxels were collected per subject. Similar spectral profiles and lipid metabolite measurements were obtained between the voxels within each subject. This is consistent with the general observation that fatty liver disease is a diffuse and homogeneous condition.

Spectral data processing was performed using NUTS software (Acorn NMR Inc., Livermore CA). The free induction decays underwent 0.5 Hz exponential line-broadening prior to Fourier transformation, and the resulting spectra were processed with manual phase and baseline correction. Peaks were registered relative to the lipid methyl resonance (0.90 ppm), which served as an internal chemical shift reference. Peak assignments were based on published data and the spectra of authentic compounds [33,34]. Resonance intensities were calculated by Lorentzian/Gaussian curve-fitting. No correction for T_1 was made to the resonance intensities, since for a TR of 3 s, all resonances will be fully relaxed except for the water and terminal methyl groups which would be greater than 90% relaxed. Literature reports of lipid methylene and methine T_1s in normal and steatotic rat liver range between 0.39 and 0.60 s [35,36]. The T_1s terminal methyl group and the water resonance are longer at 1.16 and 1.2 s respectively [35,36]. Unsaturation indices were calculated according to Zancanaro et al. [37] and are defined in Table 1. Peak area ratios were scaled to the intensity of the terminal methyl group at 0.9 ppm and to the relative number of protons in each group contributing to the resonance. The ratio of the resonance at 1.3 ppm to the methyl resonance at 0.9 ppm (CH_2/CH_3 ratio) was used as an estimate of total saturated fatty acids. The 2.8/0.9 ppm resonance area ratio is called the polyunsaturated bond index since the 2.8 ppm resonance arises from the bis-allylic

Table 1
Indices of fatty acid composition from analysis of ^{1}H MR spectra.

	Fatty acid component	Peak area ratio	Index
Lipid quantity	$[(-\mathbf{CH_2}-)_n/[\mathbf{H_2O} + (-\mathbf{CH_2}-)_n]$	1.3/(1.3 + 4.7) ppm	Lipid/(water + lipid) Ratio
Saturated component	$3(-\mathbf{CH_2}-)/2(-\mathbf{CH_3})$	1.3/0.9 ppm	CH_2/CH_3 ratio
Unsaturated components	$3(-\mathbf{CH_2}-CH=CH-\mathbf{CH_2})/4(-\mathbf{CH_3})$	2.0/0.9 ppm	Total unsaturated fatty acid index
	$3(-CH=CH-\mathbf{CH_2}-CH=CH-)/2(-\mathbf{CH_3})$	2.8/0.9 ppm	Polyunsaturated bond index
	$3(-\mathbf{CH}=\mathbf{CH}-)/2(-\mathbf{CH_3})$	5.3/0.9 ppm	Total unsaturated bond index