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Intramyocardial triglyceride quantification by magnetic resonance spectroscopy: *in vivo* and *ex vivo* correlation in human subjects

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Abstract

Accumulation of triglycerides (TG) in heart tissue has been associated with changes in left ventricular function. Proton magnetic resonance spectroscopy (^1H -MRS) is currently the only non-invasive *in vivo* method to measure myocardial TG content. The primary aim of this study was to determine if these *in vivo* measurements are specific to myocardial TG in human subjects. Thus, *in vivo* ^1H -MRS measurements were conducted on orthotopic heart transplant patients ($n = 8$) immediately before they underwent routine biopsies and *ex vivo* measurements were made on the endomyocardial biopsy samples. The correlation coefficient between the two measurements was 0.97, with $p < 0.005$, demonstrating for the first time the specificity of the *in vivo* measurement in human heart. From accompanying reliability experiments, the standardized typical error for the *in vivo* ^1H -MRS method was estimated to be 7.0%, with a 95% confidence interval from 5.5 to 9.4%. These results suggest that ^1H -MRS provides a specific and reliable measurement of myocardial TG content and is suitable for routine studies.

Keywords

myocardial; triglycerides; spectroscopy

Introduction

Abnormalities in myocardial fatty acid metabolism are central to the pathogenesis of obesity and diabetic cardiomyopathies (1–4). Typically, fatty acid uptake exceeds oxidation, leading to the accumulation of intramyocellular triglyceride (TG) within the heart. The increased myocardial deposition of TG can have numerous detrimental effects on myocellular health including reduced insulin sensitivity, increased oxidative stress, stimulation of cell death pathways, and systolic and diastolic dysfunction (4–6). In humans, *in vitro* measurements of myocardial TG content have been shown to be directly correlative with the presence of obesity- and diabetes-related heart failure (7). Thus, quantitative and reliable techniques to monitor *in vivo* TG accumulation in heart are important for disease diagnosis and management.

Localized ^1H magnetic resonance spectroscopy (MRS) is currently the only non-invasive *in vivo* method to measure TG content in tissue (8–11). MRS measurements of myocardial TG levels have been shown to be an independent predictor of diastolic function in patients with diabetes and are responsive to changes in fatty acid delivery to the heart (12,13). The validity of the method in the human heart is based on non-localized MRS measurements from excised rodent heart that correlated with biochemical measurements (9,14). However, the specificity of the method in human heart is unproven. Accordingly, the primary goal of this study was to determine if a similar correlation exists between *in vivo* MRS-derived and *ex vivo* measurements in the human heart. To achieve this aim, we first determined the reliability of our MRS protocol with test-retest measurements. Then, we compared *in vivo* MRS measurements with *ex vivo* measurements from biopsy tissue from the same heart and proximate location. Chemical extracts for biochemical or mass spectrometry TG assays were not possible due to the small biopsy size (~2 mg). Therefore, we used magic angle spinning and high resolution MRS, which have been validated against biochemical measurements, to quantify TG in *ex vivo* samples (9,14,15).

Methods

These studies were HIPAA-compliant and approved by the Human Research Protection Office at Washington University School of Medicine and the study participants signed an approved informed consent document.

Study Subjects

Ten control subjects with a broad range of body mass indexes ($28 \pm 6 \text{ kg/m}^2$) and physiological conditions (normal, obese, diabetic, HIV) underwent multiple repeat MRS measurements in the same magnet session to determine the reproducibility of the method. The study was repeated 2 to 10 days later in 7 of these subjects. Nine heart transplant patients who were scheduled for routine endomyocardial biopsy for allograft rejection surveillance were also scanned to correlate *in vivo* and *ex vivo* measurements. These subjects had no clinical evidence of active rejection at the time of the study and were scanned immediately prior to the biopsy to eliminate any effects of inflammation and bleeding. To further test reproducibility, multiple measurements were performed on 6 of the transplant subjects during the same magnet session. Values reported in the *in/ex vivo* correlation are an average of these measurements. Standard contraindications to MRI and an age of less than 18 years were the only other exclusion criteria.

In vivo MRS

Human studies were performed on a Siemens 1.5T MAGNETOM Sonata (Siemens Medical Systems, Erlangen Germany), with the body coil used as the transmitter and an 8-element body array coil used for reception. Short and long axis true-FISP images for voxel positioning were obtained during free breathing with both ECG and two-dimensional prospective acquisition correction (2D-PACE) respiratory gating. The ECG gating was chosen to correlate with the end systolic period and the navigator had a 2 mm acceptance window near end expiration. The spectroscopy data was collected with a similarly gated point-resolved spectroscopy (PRESS) sequence with a $6 \times 20 \times 20 \text{ mm}$ volume of interest (VOI) placed in the intraventricular septum (16,17). To minimize outer volume contamination (OVC), a 90° SLR excite pulse with a bandwidth time product (BWTP) of 13.1 and MAO refocusing pulses with BWTPs of 6.0 were used (18,19). Additionally, the first and second MAOs were saddled by crushers with 80 and $150\times$ residual signal suppression, respectively, regardless of voxel size or orientation (20). The sequence also alternated the gradient polarity every scan and used a COG5(2,3,2:0) phase cycle. The specific MRS acquisition parameters that were used were: the relaxation delay was defined

by the length of respiratory cycle, ~4 sec; echo delay (TE) = 24 ms, averages = 120 in blocks of 60 with localization verification in between, spectral width = 2 kHz, 512 data points, and a transmitter frequency of 2.9 ppm. Siemens automatic shimming was preformed prior to acquisition under breath hold. In general, subjects were not removed from the magnet, just relocalized with minor voxel adjustments, for each group of 120 averages.

The *in vivo* data were processed and analyzed using the AMARES algorithm within the jMRUI 3.0 software package (21,22). Briefly, the water resonance was first used to estimate the line shape. Then, the lipid resonances were constrained with a similar shape and other prior knowledge (16). The *in vivo* TG content is presented as the ratio of the sum of the areas from the methyl and methylene resonances to the area from the H₂O resonance times 100 - an approximate percentage.

Endomyocardial Biopsy

Immediately after the MRS study, the heart transplant subjects underwent endomyocardial biopsy. An additional right ventricular septal endocardial biopsy (2.3 ± 1.3 mg) was obtained using standard clinical procedures. The biopsy sample was immediately frozen in liquid nitrogen and stored at -80°C for *ex vivo* TG analysis.

Ex Vivo Analysis

Magic angle spinning MRS (MAS) analysis was done at 400.5 MHz using a Tecmag (Houston, TX, USA) Apollo console and a Doty Scientific (Columbia, SC, USA) 4 mm double resonance probe. The high resolution MRS (HR) analysis of the biopsy samples was done at 599.5 MHz using a Varian (Palo Alto, CA, USA) Unity Inova console and a 5 mm, 3 axis gradients (not used), triple resonance probe. Both MAS and HR spectra were acquired using a spin-echo sequence with TR = 4 s and TE = 24 ms, with pulse widths verified using the H₂O signal of the samples. The minor variations in the 180° widths (0.1 μs) suggest that the receptivities of the spectrometers were relatively constant. Thermogravimetric analysis (TGA) was done with a Mettler Toledo (Columbia, MA, USA) 50T/A851^e TGA.

Biopsies were first blotted dry with a chem-wipe, weighed (TGA), put in a MAS rotor, and spun at ~4 kHz. Room temperature shims were not available. 64 acquisitions were acquired at room temperature. Samples were refrozen immediately after acquisition. All MAS acquisitions took place sequentially on the same day. Each sample was next put into an HR tube with ~300 mg of D₂O (occupying ~70% of the coil length), spun at 20 Hz, and shimmed. 128 acquisitions were acquired at 37°C . For HR, two groups of samples were run for which general spectrometer performance was accounted for with a common H₂O/D₂O standard.

The small biopsy size and resulting large surface to volume ratio made scaling the area of the TG resonance by either the H₂O resonance area or wet weight inconsistent. Thus, after the HR acquisition, the dry weight of the biopsy was determined via TGA analysis in a nitrogen atmosphere with the temperature increased to and left at 90°C until a constant weight was achieved, typically 2 to 4 hours. The *ex vivo* lipid concentration is presented as the area of the TG methylene resonance from 1.15 to 1.25 ppm, determined as reported previously, scaled by the dry weight of the sample (14). All of the MAS areas were additionally scaled by a common factor to approach the HR scale.

Statistical Analysis

Correlations were calculated by linear regression, with a *P* value less than 0.05 considered statistically significant. The intraclass correlation coefficient (no agreement, ICC), measurement or typical error and standardized typical error (STE), both expressed as

coefficients of variation (CV), along with corresponding 95% confidence intervals were calculated as described by Hopkins from the log transformed data (23,24). The Kendall tau rank correlation was calculated via Mathematica™. Assessment of reliability was also determined by the methods outlined by Bland and Altman, with the coefficient of reliability listed as twice the standard deviation of the measurement differences (25). In the above calculations, all permutations of repeated intrasubject measurements were considered. Results are expressed as mean $\pm$ standard deviation (SD) or value(confidence interval).

Results

Reproducibility

All *in vivo* spectra had a signal to noise ratio (SNR) > 5 , determined using peak height, for the TG resonance and the half-height full-width (HHFW) line width < 0.3 ppm for the water peak. All intrasubject retest ($1.3 \pm 0.6\%$ TG) permutations (1 vs 2, 1 vs 3, 2 vs 3, etc.) are shown in Figure 1, along with the corresponding Bland-Altman plot of the differences ($-0.029 \pm 0.09\%$). Two measurements produced differences outside of 0.15 to -0.21 (± 2 SD) and were removed, leaving 60 retest measurements from which all further results are based and whose 53 differences are characterized by $-0.029 \pm 0.07\%$. The removed measurements were both the final measurements from long sessions, one the third of the first subject and the other the fifth. The Kendall tau rank correlation between the intrasubject mean and SD is 0.16. The log-transformed data have an ICC of 0.99(0.98–1.0) and a typical error and STE CV of 5.1(4.0–7.0)%, and 7.0(5.5–9.4)%, respectively. The STE CV from the non-transformed data was 7.4(5.9–10.0)%.

In vivo vs *ex vivo*

Figure 2 shows example *in vivo* and *ex vivo* (MAS and HR) spectra taken from one subject and Figure 3 shows all of the *in vivo* and corresponding HR spectra. The *in vivo* myocardial TG content in transplant subjects was on average $0.4 \pm 0.2\%$. The correlations between the *in vivo* and *ex vivo* MAS ($r^2 = 0.83$) and HR ($r^2 = 0.97$) measurements are shown in Figure 4. Because the shims were inoperable, MAS was not used for the second group of samples and further discussion will refer to the HR results. The *ex vivo* analysis of the smallest biopsy sample (0.15 mg, dry) was 1/2 of that expected, deemed an outlier, and not included in the analysis.

Discussion

^1H MRS is an invaluable tool for studying *in vivo* TG changes; however, this use has never been correlated against *in vitro* human myocardial TG measurements. This study was undertaken to determine if ^1H MRS does specifically measure the TG concentration in human myocardium or if the measurement is contaminated by nearby TG and H_2O . For this correlation, we first characterized the reliability of the technique. With an STE $\approx 8\%$ and data from 8 transplant subjects, regression correlations greater than ~ 0.85 are statistically significant ($p = 0.01$, $p = 0.05$) (23,26). Thus, the correlation between measurements from *in vivo* ^1H MRS and *ex vivo* biopsies from the same heart shows that ^1H MRS gated by motion and ECG does specifically measure the relative TG concentration in beating human hearts.

There are several factors that could have affected the *in/ex vivo* correlation, of which tissue heterogeneity may be the most prominent. The *in vivo* MRS measurements were obtained from a large (2.4 cm^3) region in the intraventricular septum. In contrast, the biopsy samples were small ($\sim 2 \text{ mg}$) and from the septum wall. There is a paucity of data regarding a potential heterogeneous myocellular TG distribution in the human septum. The correlation

in our data suggests that MRS sampling of a large voxel in the intraventricular septum is similar to that of the septum wall and not contaminated by epicardial fat or blood.

The slope in the correlation was not expected to be unity for several reasons. First, the tissue relaxation is different due to the different magnetic field strengths. Accurately measuring and accounting for these differences was not possible due to time restrictions. However, considering that the type and rate of molecular motion for the TG molecules in small micelles, upon which relaxation depends, is vastly homogeneous throughout the population, the differences should also be homogeneous, and should have little impact on the correlation. Similarly, because of the general homogeneous nature of myocellular tissue water content, the different ratios reported for the *in vivo* and *ex vivo* measurements also primarily affect the slope.

We also observed a constant offset between the *in vivo* and *ex vivo* measurements (non-zero intercept). Physiologically, it is unlikely that the various sized biopsies would contain a constant fraction of endocardium with endothelial cells high in TG. A more likely explanation is that a small fraction of TG is lost in the deconvolution of the broader *in vivo* TG and H₂O resonances. This possibility is supported by the intercept trending to zero as only higher TG ratios are used in the regression. It should be emphasized that in our measurements, the SNR was consistently > 5 for the TG resonance, calculated via peak height, and the HHFW line width was 0.3 ppm for the water resonance, two criteria we consider *necessary* to achieve a STE ~7%.

The MRS approach used in this study and the estimated STE are both similar to those of previous studies (9,27,28). However, the sequence and protocol do employ some modifications that result in consistent spectral quality and, concerning specificity, OVC. Thus, the correlation and STE may only hold for approaches with similar spectral and localization integrity.

In summary, the *in vivo* MRS measurement for myocardial TG had excellent, statistically significant correlation with *ex vivo* TG measurements from biopsy samples taken from the same subjects. This correlation demonstrates that ¹H MRS specifically measures *in vivo* human myocardial TG. Furthermore, considering the measurement error, the technique should be a reliable tool for monitoring the effects interventions in metabolic or cardiac studies.

Acknowledgments

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Figure 1.

Reliability of the ECG and respiratory gated ^1H -MRS measurement of intramyocellular TG with all permutations of intrasubject measurements plotted. The line is a guide with unity slope. The inset shows the corresponding Bland-Altman plot, with the difference between two intrasubject measurements plotted against the subject mean. The dashed lines are $\pm 2 \cdot \text{SD}$ and the solid line is the mean of the differences.

Figure 2.

The figure shows the spectra obtained from the *in vivo* and *ex vivo* experiments of one transplant subject. The pertinent resonances in the figure are water (4.6 ppm), methylene (1.2 ppm), and methyl (0.9 ppm). The top spectrum is from *in vivo* ^1H MRS, with the light, coincident line the AMARES fit to the spectrum. The middle and bottom spectra are from *ex vivo* 400 MHz MAS and 600 MHz HR, respectively. The increased line-width in the MAS compared to the HR spectrum results from inoperable shims. The upper left image shows the location and size of the voxel in the intraventricular septum.

Figure 3.

The figure shows TG-region expansions from all of the *in vivo* ^1H -MRS (left) and *ex vivo* HR (right) transplant spectra. The *in vivo* spectra are scaled by their H_2O -resonance area and the *ex vivo* spectra are scaled by their dry weight and other spectrometer related factors as described in the text. For visual clarity, the H_2O resonance was removed from the *in vivo* spectra using the jMRUI SVD filter. Five and 20 Hz Gaussian line-broadening was applied to the *in vivo* and *ex vivo* spectra, respectively.

Figure 4.

In vivo ¹H-MRS responses versus the corresponding *ex vivo* MAS (, $r^2 = 0.88$, $p = 0.005$) and HR (, $r^2 = 0.97$, $p = 10^{-5}$) measurements from endomyocardial biopsies. The lines results from the regression analysis. The MAS results are not ideal primarily because the shims were inoperable, resulting in spectra with lower SN and resolution than the HR spectra (figure 2).