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***In Vivo* Brown Adipose Tissue Detection and Characterization using Water-lipid Intermolecular Zero Quantum Coherences**

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Abstract

Brown Adipose Tissue (BAT) and White Adipose Tissue (WAT) depots are non-invasively characterized *in vitro* and *in vivo* in healthy and obese mice using intermolecular Zero-Quantum Coherence (iZQC) transitions between lipid and water spins. IZQCs enable selective detection of spatial correlation between water and lipid spins and thereby the hydration of fatty deposits with subvoxel resolution. At about a 100 micrometer distance scale, the major observed peaks are between water, methylene protons at 1.3ppm, and olefinic protons at 5.3 ppm. Our *in vitro* results show that the methylene-olefinic iZQC signal is strong both in BAT and in WAT, but that the water-methylene iZQC signal is characteristic only of BAT tissue. *In vivo*, the ratio of these peaks is substantially higher in lean or young mice than in old or obese mice.

Keywords

Brown adipose tissue; white adipose tissue; Intermolecular zero quantum coherences

Introduction

The physiological distribution and composition of adipose tissue is directly correlated with predisposition to obesity and its associated diseases. Adipose tissue is comprised of two functionally different types of fat: white and brown. White Adipose Tissue (WAT), considered to be “bad fat”, stores energy and causes obesity, while Brown Adipose Tissue (BAT), when activated, dissipates energy in the form of heat. Due to its capacity to burn calories, BAT is thought to have a large impact on long-term energy balance, and thereby represents an obvious target for obesity drugs (1).

Until recently, BAT was thought to be present in substantive quantities only in small rodents and infants, and was rarely seen in adult humans(1). In recent studies, BAT activity has been detected in adult humans through fluorodeoxyglucose-aided CT/PET scans (2–8). Metabolic activity of BAT tissues was detected after cold exposure in a plane extending from the anterior neck to the thorax, along the thoracic spine. It is more abundant in younger subjects, and least frequent in old or obese people, suggesting that BAT contributes to the regulation of energy expenditure and adiposity in humans as well as in rodents. However, ¹⁸FDG-PET can only be used to detect BAT in metabolically active tissue, and the use of the fairly high radiation doses associated with CT/PET scans limits the use of this technique.

NMR is a powerful non-invasive tool for biological research that yields a natural, tissue-dependent fingerprint of chemical composition, structure, and metabolism. Fat composition of both BAT and WAT have already been determined by ^1H and ^{13}C spectroscopy *in vitro*, in tissue extracted from normal and cold acclimatized animals(9). It has been shown that fat protons in BAT have a lower unsaturated component than in WAT, and that this component is further decreased by adrenergic stimulation (either through norepinephrine administration or cold acclimatization)(9). Extension of these methods to *in vivo* applications is challenging because the tissue composition analysis is compromised by the poor spectral resolution observed in fatty tissue, due largely to strong susceptibility gradients. Because of the current interest in lipid composition and metabolism, alternatives such as localized single voxel spectroscopy have been proposed for obtaining clean NMR spectra of lipid depots *in vivo* (10–13). *In vivo* data collected using localized single voxel spectroscopy show differences between WAT and BAT through the ratio of different lipid components(11). Unfortunately, these methods cannot be used to differentiate BAT and WAT when they are inter-mixed or mixed with other tissues, as is often the case. The presence of different tissues in the same voxel may preclude correct quantification of the different lipid components. Moreover, the tedious shimming procedures often necessary to reach acceptable spectral resolution, and the detection of signal from only one very small voxel at a time, make such analyses time consuming, especially for large volume samples.

BAT and WAT differ also in their hydrolipidic ratio (14–16), which seems to reflect their morphological difference. In BAT, the presence of multi-locular adipocytes, which contain large numbers of small lipid droplets surrounded by an abundance of intracellular water, makes it more hydrated than WAT, which has a mono-locular adipocyte with a single large lipid droplet that occupies almost the entire cell space. The water/fat ratio in fat depots is not only correlated to the morphological structure of the tissue, but also seems to be a good marker of BAT activity. It has been shown that in multi-locular fat cells, the relative amount of water and fat changes with age or as the animal is exposed to adrenergic stimulation. In adulthood, as BAT activity decreases, the tissue structure starts to change, as initially multi-locular tissue starts to become uni-locular (17,18) with a corresponding decrease in water content(16). On the other hand, under adrenergic stimulation, there is an increase in the hydrolipidic ratio which seems to reflect the depletion of lipids as they get used within the cell for heat production (19). This makes the cellular water/fat ratio a good biomarker to differentiate BAT from WAT, *in vitro* in tissue extracts, as well as *in vivo*.

The hydrolipidic ratio has been studied non-invasively with CSI-type techniques in small animals and has been mapped in different areas of the body with relatively high spatial resolution (14,15,20). The major limitation of this method, as with the single voxel spectroscopy methods, is that it cannot be used to detect the BAT depots that are mixed with WAT or that are situated around visceral organs. Measurements of the hydrolipidic ratio at a voxel resolution can be misleading when different types of cells are intermixed on a sub-voxel scale.

To overcome this problem, we use a spectroscopic method that allows us to probe the hydrolipidic ratio of displaced fat depots at the cellular level. This method probes the spatial correlation between fat and water spins at a cellular level by detecting the intermolecular zero quantum coherence (iZQC) transitions between water and fat spins at a distance (which we call the correlation distance) of about one hundred micrometers (21–24). This distance is smaller than that usually probed by conventional imaging methods, and above that conventionally probed by diffusion-based methods. At this scale, which is the cellular length scale (fat cells have a diameter that ranges from 50–200 μm), the spatial correlation between water and fat spins is very different for WAT and BAT tissue. WAT depots are comprised mainly of fat spins, while BAT tissues present a more or less equal distribution of water and

fat spins. This means that the iZQC signal between water and fat spins is expected to be maximal in BAT tissue, where water spins are equally mixed with fat spins, and minimal in WAT and muscle tissue, where one spin species is much more abundant than the other. The iZQC signal is proportional, to a first approximation, to the product of the concentrations of the two spins at the selected correlation distance (24). Therefore, in voxels where the two spins are present with the same concentration, the iZQC signal is maximal only when the two spins are homogeneously inter-mixed, and minimal when the two spins are separated beyond the selected correlation distance. This makes iZQC-based contrast ideal for BAT detection when the tissue is infiltrated into or mixed with other tissues, as often is the case. This idea is used here to detect the *in vivo* prevalence of either BAT or WAT tissue in the fat depots of different mouse strains. Since this method is intrinsically insensitive to local magnetic field inhomogeneities at length scales exceeding the selected correlation distance(25), it allows us to analyze large volumes where fat depots are scattered over different regions, without the need for localization, shimming, or water suppression.

Methods

All measurements are performed on a 7 T small animal magnetic resonance tomographic imager with a 30 cm bore (BioSpec® 70/30; Bruker BioSpin MRI GmbH, Ettlingen, Germany) equipped with an imaging gradient coil system (gradient strength, 400 mT/m) with a ^1H transmit-receive coil of either 35 mm (normal and nude mice) or 72 mm (obese models) inner diameter.

Lipid-water iZQC spectra

All iZQC spectra are acquired using the sequence shown in Figure 1. This sequence is customized to selectively detect methylene protons ($-\text{CH}_2-$) at 1.3 ppm, which are spatially coupled with other methylene protons, with olefinic methylene protons ($-\text{CH}=\text{CH}-$) at 5.3 ppm, and with water at 4.7 ppm. Unwanted water-water coherences, as well as uncoupled water and fat components are dephased by a combination of gradient pulses, selective RF excitations, and RF phase cycling.

In the sequence, the first 90° pulse (Hermite, 1 ms duration) excites all coherence orders, among which are the zero-quantum (iZQC) and double-quantum (iDQC) coherences. The frequency selective 180° pulse (Gaussian, 3.458 ms) applied at 1.3 ppm does not affect same spin coherences, but cross-converts mixed spin iDQCs with iZQC coherences. A final 90° broad-band pulse (Hemite, 1 ms), converts iZQC and iDQC coherences to detectable single-quantum (iSQC) antiphase magnetization. The use of a broad band pulse allows for the detection of both ($F1=\omega_I-\omega_S$, $F2=\omega_I$) and ($F1=\omega_S-\omega_I$, $F2=\omega_S$) for which the correlation distance is given by $\pi/[\gamma^*(g1-g2)*T]$ and $\pi/[\gamma^*(g1+g2)*T]$ respectively, as well as for the detection of methylene-methylene iZQC coherences, for which the correlation distance is given by $\pi/[\gamma^*(g1-g2)*T]$.

After the mixing pulse, a slice selective refocusing pulse (Hermite, 1 ms) is applied to detect signal from a specific axial slice centered either on the scapula or on the abdomen (slice thickness = 20 mm, 40 mm, respectively). A crusher module (90 degree RF pulse surrounded by pulsed field gradients of different strengths oriented along the magic angle) at the beginning of the sequence dephases any signal from stimulated echoes. After all the coherences are excited by the first 90° broad-band pulse to specifically select the iDQC $\rightarrow$ iZQC $\rightarrow$ iSQC coherence transfer pathway, we use a simultaneous phase cycle of the first excitation pulse (x, -x, y, -y) and the receiver (x, x, -x, -x), and a gradient combination of $g1:g2:g3 = 10.5:-4.2:21$ Gauss/cm, with a 1 ms duration. This gradient combination is used to select a correlation distance comparable to the size of adipose cells, specifically a

correlation distance of 80 and 186 μm for the ($F1=\omega_I-\omega_S$, $F2=\omega_I$) and ($F1=\omega_S-\omega_I$, $F2=\omega_S$) coherences, respectively.

For the acquisition of the 2D iZQC spectrum, the t_1 delay is stepped in increments of 0.333ms. Each t_1 point is collected with a repetition time (TR) of 3500 ms, an echo time (TE) of 14.9 ms, and a number of repetitions (NEX) of 4. The projection of this 2D iZQC spectrum along the indirectly detected dimension $F1$ yields the 1D-iZQC spectrum, where each resonance frequency line represents a correlation between spins on different molecules.

Animal preparation

All animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Nude mice (two 6 months old and one 2 years old, nu/nu Charles River), lean mice (two 4 weeks old, two 6 months old and two 8 months old, C57BL/6J from Jackson Lab), dietary-induced obese mice (one 6 months old, DIO from Jackson Lab), and ob/ob C57BL/6J mice (two 4 weeks old, and one 6 months old, Jackson Lab) were used in this study. All animals were housed at a constant temperature (20–24°C) in 12-h light/dark cycles and fed standard mouse chow *ad libitum*. For the experiments, animals were induced with Nembutal (60mg/kg) in a single intraperitoneal injection before the study. During the study the animals were free breathing with the rate monitored using MR-compatible animal monitoring equipment from SA Instruments (Stony Brook, NY). Body temperature was monitored with a rectal temperature probe and regulated and maintained using forced, heated air. At the completion of the study, the animals were euthanized through an overdose of pentobarbital.

Tissue extracts

BAT and WAT were extracted from lean and obese C57BL/6J mice after euthanization. BAT was carefully extracted from the interscapular region of lean mice, while WAT was extracted from the abdominal cavities of obese mice. Muscle tissue was extracted from lean animal thighs. After extraction, the samples were immediately put into vials, scanned, and discarded at the end of the study.

Results

Figure 2 shows standard 1D NMR (90°-acquire) spectra along-side the iZQC spectra of excised WAT, BAT, and muscle tissue samples. The standard NMR spectra show very small amounts of lipids in the muscle tissue, high lipid content in WAT, and almost a 50:50 water lipid ratio in the BAT. In the iZQC spectrum, the peaks appear along $F1$ at the expected resonance frequency difference between the selected methylene peak ($P2$) and the other spin (water or olefinic spin) participating in the transition. Aside from the methylene-methylene iZQC peak at $F2=F1=0\text{Hz}$ ($P2-P2$), which is derived from same spin double quantum methylene-methylene coherences that have been transferred along the Z axis by the selective inversion pulse, an olefinic-methylene peak ($P9-P2$) is present in the BAT and WAT spectra but noticeably absent in the spectra of excised muscle. In addition to these peaks in the BAT spectrum, an extra water-methylene peak (water- $P2$) can be observed. This peak is present in the BAT spectra and absent in the WAT and muscle tissue spectra. Unlike in WAT and BAT spectra, the muscle spectra contain only a small signal contamination which is observed in correspondence with the N-methyl protons of creatine at 3.0 ppm, while both the water-methylene and olefinic-methylene peaks are completely absent.

Figure 3 shows the 2D spectrum acquired from the abdomen of a diet induced obese mouse *post mortem*, with the sequence in figure 1, and the corresponding iZQC spectrum ($F1$ projection). Although the selected voxel comprises the entire mouse abdomen (40 mm

selected slice), the achieved resolution in the iZQC spectrum is comparable to that obtained using localized spectroscopy with water suppression in (11). Compared to the standard SQC spectrum, which shows the water and unresolved lipid peaks, the iZQC spectrum shows well resolved lipid peaks and the absence of a water-methylene peak. The same kind of resolution is obtained *in vivo* in different mouse strains (Figure 4). By comparing these spectra, we can see a remarkable difference in the ratio between the water-methylene peak (water-P2 peak) and the olefinic-methylene iZQC peak (P9-P2 peak). In obese mice, the predominant peak is the olefinic-methylene peak, while the water-methylene peak seems to be completely absent. This peak is, on the other hand, the predominant peak in young lean mice ($\sim 13 \pm 3$ in 4 weeks old lean mice), and is smaller in obese or old animals ($\sim 3 \pm 1$ in 6 months old nude animals, 2 ± 1 in 8 months old lean animals, and 0.7 ± 0.2 in 2 years old nude animals) (Figure 5). A systematic difference is also typically seen for the same animal between the spectra acquired by selecting the scapular region and that acquired by selecting the lower abdomen (Figure 6). In lean animals, the water-methylene and olefinic-methylene peak ratio is usually higher in the scapular region ($\sim 2.5 \pm 1$ in 6 months lean animals) and lower in the abdomen ($\sim 0.4 \pm 0.1$ in 6 months lean animals). The difference is usually larger in healthy lean animals and reduced in DIO animals fed with standard chow (0.6 ± 0.2 in the interscapular region and 0.3 ± 0.1 in the abdominal region of 6 months old DIO mice).

Discussion

We observe a systematically large difference in the water-methylene and the olefinic-methylene peak ratio in the iZQC spectrum of BAT and WAT extracts. Unlike the olefinic-methylene peak, the water-methylene peak seems to be characteristic only of BAT, while it seems to be completely absent in the spectra of WAT and muscle tissue extracts. This signal seems to follow the difference in the cellular structure of these tissues. In BAT, the presence of numerous small intracellular lipid droplets that are surrounded by an abundance of intracellular water (20,26) lead to a more equal distribution of water and fat spins and to a large water-methylene iZQC peak, whereas in WAT the presence of a large single lipid droplet in the adipocytes leads to the predominance of lipid molecules over water molecules and to a predominance of the olefinic-methylene peak. The iZQC spectrum in this case is in agreement with what we would predict to be the water-lipid peak by looking at the standard 1D spectrum of Figure 2. This spectrum shows the presence of water and lipid spins in almost a 1:1 ratio only for BAT, while WAT and muscle tissue spectra are characterized by the presence of only one type of spin, either lipid or water. The iZQC signal is indeed proportional, to a first approximation in a homogenous solution, to the product of the two spin concentrations, and therefore is enhanced when the two spins are present in similar concentrations, and is considerably diminished when one spin is much more abundant than the other, as in WAT or muscle tissue.

In vivo, the situation seems to be very different. In this case, although standard NMR spectra of obese mice show the presence of water and lipid spins in almost a 1:1 ratio, the iZQC spectra show almost no water-methylene peak. In lean mice, on the other hand, although the standard NMR spectra show very small lipid-water ratios, the water-methylene peak is the dominant peak in the iZQC spectra. This apparent incongruence clearly suggests that we are detecting something different from the mere product of the overall water and fat concentrations in the selected voxel. We are indeed detecting not the spatial correlation between these spins at the voxel level but at the cellular level. In obese mice, despite the fact that water and lipid spins are present overall in an almost a 1:1 ratio, at the cellular level this is no longer the case. Most of the water signal in obese animals does not come from the water in lipid depots but from the water in visceral organs. The water content in the lipid depots of obese animals is very low since these depots are mainly made by white adipocytes (27). This means that when we probe the spatial correlation between fat and water spins at

the cellular level with the iZQC sequence, we will find a very small spatial correlation, which will lead to a strong reduction of the water-methylene peak (i.e. water-fat peak) with respect to the olefinic-methylene peak (i.e. the fat-fat peak). In contrast, in lean animals, although the standard 1D spectrum reveals an unbalanced water-fat ratio, the water-fat peak is usually the predominant peak. This is because in lean animals, the few lipid depots consist mainly of BAT, which unlike WAT is highly hydrated and presents a higher spatial correlation between water and fat spins at the selected correlation distance.

The highest water-fat, fat-fat peak ratio was found in nude and young lean mice. This seems to reflect the higher BAT/WAT ratio present in the fat depots of these animals. In the hairless mouse strain, the cold-induced thermogenesis mechanism of BAT is further enhanced, mainly due to a diminished insulating capacity of the hairless skin, with a consequent BAT hyperplasia(28), while in young mice, the presence of large BAT depots is needed for temperature homeostasis. We also noticed a difference in the water-lipid ratio between the DIO mice and the lean or nude mice. The DIO mice are highly susceptible to developing obesity when fed with a high-fat diet. In our case these mice were fed a high fat diet until 3 months old, followed by standard chow. Therefore, these mice did not develop, but rather retained a weight more or less similar to the lean controls. However, when compared to the normal and nude mice, they present a reduced water-lipid peak and an increased lipid-lipid peak, reflecting the low content of BAT in their lipid depots with respect to the nude or healthy mice, as confirmed by post mortem dissections.

A smaller difference was also seen, in the same mouse, when different areas were analyzed. A larger water/lipid iZQC peak was consistently found in the spectra from the scapular region and a lower peak was found in the spectra from the abdominal area. This seems to be consistent with the fact that fat depots in the scapular region consist mainly of BAT, while WAT seems to be predominant in the fat depots of the abdominal area, especially in old or obese mice.

In all of these experiments, we analyzed very large volumes, but the detected signals gave information only about the lipid depots of the selected volume. In lean animals, this comprises mainly the visceral fat which surrounds internal organs and that is currently inaccessible by standard NMR and MRI methods. Because the iZQC signal detects resonance frequency differences between the spins, it is intrinsically insensitive to magnetic field inhomogeneities on a length scale larger than the selected correlation distance. This allowed us to acquire high resolution spectra that yielded information about the overall lipid composition of the various depots in the body, despite their delocalization. The comparison between different iZQC spectra was simplified because the iZQC resonance frequency lines are only proportional to the chemical shift difference between the spins and are therefore unaffected by variations in the resonance frequency offset. This allowed for an easy comparison between the different mouse strains and a clean differentiation based on their water-fat/fat-fat peak ratio.

Although the iZQC signal intensity is about 10% of the original SQ signal, it is enough to localize BAT depots in the body. The water-fat iZQC signal comes indeed from only the BAT depots, which are more or less localized in the body.

We also noticed that the water-fat/fat-fat iZQC peak ratio was strongly dependent on the mouse strain and age, but with a small variability (see standard deviation on the water-fat/fat-fat peak ratio) among members of the same litter. These differences are strongly correlated to the high BAT/WAT ratio differences seen between different mice strains and mice ages. This is because an increase in BAT produces an increase in the water-fat iZQC peak, while an increase in WAT produces a strong increase in the fat-fat peak and a

corresponding reduction of the water-fat/fat-fat peak ratio. Previous papers have shown(29,30) that the BAT/WAT ratio is highly variable with the strain and age of animals, due to their differing needs for nonshivering thermogenesis through-out their lives. BAT is predominant in newborns, and this is consistent with the strong need for nonshivering thermogenesis shortly after birth, since the normal animal surrounding temperatures (~23°C) are well below mammalian body temperature (37°C). During this stage, the BAT/WAT ratio is at its highest and the water-fat/fat-fat peak ratio is also at its highest. After a few days, as the animal begins to develop fur, its need for non shivering thermogenesis decreases and BAT does not develop further, unless the animal is exposed to cold. At thermoneutral conditions, as the animal ages other, mainly WAT depots start to develop, and the BAT/WAT ratio begins to decrease, resulting in a reduction in the water-fat/fat-fat peak ratio. Since this decrease is usually smaller in nude animals, which exhibit a BAT thermogenesis need throughout life, while it is very high in DIO or genetically obese mice that develop abnormally large WAT depots, a large difference in water-fat/fat-fat peak ratio can be found in adult animals of different species.

Conclusions

A water-methylene peak is detected in the iZQC spectrum of BAT. This peak is completely absent in the spectra of WAT and muscle tissue and can therefore be regarded as a marker for BAT tissue. This approach is insensitive to magnetic field inhomogeneities, and allows for an overall *in vivo* analysis of fat depots displaced in different regions. More specifically, this method can be used to detect the larger BAT depots (>60%) surrounding visceral organs, which are impossible to localize using standard NMR techniques.

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Figure 1.

Modified iZQC pulse sequence used to detect the spatial coupling of the fat spins with the water spins. A first 90 degree pulse is used to excite both double and zero quantum coherences between water and fat spins. An inversion pulse is used to interconvert iZQC with iDQC. The broad band mixing pulse allows the simultaneous detection of fat-fat and water-fat spin coherences. A crusher module at the beginning of the sequence prevents the refocusing of stimulated echoes.

Figure 2.

Comparison of standard 1D and iZQC spectra acquired from excised tissue samples.

a. Standard 1D spectra (90-acquisition) of excised BAT, WAT and muscle tissues samples.

b–d. 2D iZQC spectra of excised BAT (b), WAT (c) and muscle tissue (d) acquired with the sequence in Figure 1. e. Projection along the indirectly detected dimension F1 of the 2D iZQC spectra from BAT, WAT, and muscle shown in figure b, c and d.

The methylene lipid peak was chosen on resonance in all the experiments. The standard 1D spectra in a) show almost a 1:1 ratio between the water and fat spins for BAT, a predominance of fat spins in WAT, and of water spins in muscle tissue. The 1D iZQC spectra in e) show the presence of an olefinic-methylene peak only for BAT and WAT, and the presence of a water-methylene peak only for BAT. The muscle spectrum, on the other hand, shows only a small contamination from the creatine peak.

Figure 3.

Standard 1D and 2D iZQC spectra acquired *post mortem* on an obese mouse. a. View of the selected slice covering the entire abdomen. b. Standard 1D (90-acquisition) spectrum that shows the abundance of both water and fat spins in the selected slice. c. 2D iZQC spectrum acquired with the sequence in Figure 1 from the 40mm slice. d. High resolution iZQC spectrum obtained by projecting the 2D spectrum in c along the F1 dimension. Different lipid components are easily identified despite the large size of the selected voxel. Peaks are labeled as in ref. (11).

Figure 4.

2D iZQC spectra and 1D projections of the 2D iZQC spectra acquired from the interscapular area of different mouse strains: four week old lean mouse (a), four week old obese mouse (b), six month old nude mouse (c), two year old nude mouse (d). The water-fat iZQC peak visible in the 1D iZQC projections is the dominant peak in young and nude mice and is highly diminished in obese mice.

Figure 5.

1D projections of the 2D iZQC spectra acquired from the interscapular area of different mice showing the large difference in the water-fat/fat-fat peak ratio. a. Comparison between the 1D iZQC projections of a 4 week old lean mouse and an obese mouse of the same age. b. Comparison between the 1D iZQC projections of lean mice of different ages. The water-fat / fat-fat peak ratio is higher in young and lean mice but decreases as the animal becomes old or develops obesity.

Figure 6.

Comparison of the iZQC 1D spectra acquired from the scapular region (20mm thick slice) and from the abdomen (40mm thick slice) of a lean mouse (a) and of a DIO mice (b). In both mice the water-fat/fat-fat peak ratio is higher in the lipid depots localized around the neck and lower in the lipid depots of the abdomen.