APPLICATIONS OF NMR SPECTROSCOPY TO STUDY MUSCLE GLYCOGEN METABOLISM IN MAN

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ABSTRACT

Prior to the advent of nuclear magnetic resonance (NMR) spectroscopy, human glucose metabolism was studied through tracer and tissue biopsy methodology. NMR spectroscopy now provides a noninvasive means to monitor metabolic flux and intracellular metabolite concentrations continuously. $^{13}$C NMR spectroscopy has shown that muscle glycogen synthesis accounts for the majority of insulin-stimulated muscle glucose uptake in normal volunteers and that defects in this process are chiefly responsible for insulin resistance in type 1 and type 2 diabetes mellitus, as well as in other insulin resistant states (obesity, insulin-resistant offspring of type 2 diabetic parents, elevation of plasma FFA concentrations). Furthermore, using $^{31}$P NMR spectroscopy to measure intracellular glucose-6-phosphate, it has been shown that defects in insulin-stimulated glucose transport/phosphorylation activity are primarily responsible for the insulin resistance in these states.
INTRODUCTION

In vivo nuclear magnetic resonance (NMR) spectroscopy allows continuous, noninvasive monitoring of tissue concentrations of metabolites and metabolic fluxes in humans. It is therefore a very powerful tool for clinical investigation. In this chapter we review the applications of NMR spectroscopy in examining the regulation of muscle glycogen metabolism, both under normal conditions and in patients with type 1 or type 2 diabetes mellitus or in various insulin-resistant states. Previous reviews have discussed several other aspects of in vivo NMR spectroscopy (1–8).

**Basic Principles of In Vivo NMR Spectroscopy**

**PHYSICAL AND TECHNICAL BASIS** Some atomic nuclei possess magnetic properties, i.e. magnetic moment or “spin” (9). Their magnetic moments are usually randomly oriented in the weak magnetic field (50 µTesla) of the earth. However, the presence of a strong, static magnetic field creates torque on the magnetic moment and causes the nuclei to precess around their own axes with a characteristic frequency so as to align with (or against) the magnetic field. In the presence of an oscillating magnetic field (radiofrequency field) at the precessing frequency of the nuclei, they absorb this energy and change the angle of precession. During the interval between the excitation waves, the nuclei swing back into their original alignment and emit energy, which is detected by a receiver coil. Under experimental conditions, the different radio frequencies from various nuclei/compounds can be translated into a display of peak intensities vs frequencies by using a mathematical transformation (Fourier analysis). The frequency of a peak is characteristic of a certain nucleus/compound and the area under that peak corresponds to the concentration of that nucleus/compound. The ability to distinguish between different molecules containing the same nucleus relies on the “chemical shift,” given in parts per million (ppm). The chemical shift can be explained by the shielding effects of electrons surrounding the nucleus. The nuclei of different molecules thereby experience an altered static magnetic field and in turn resonate at an altered frequency, i.e. chemical shift, which is typical for the respective molecule.

Increasing the field strength of the static magnetic field improves the signal-to-noise ratio and thereby the sensitivity of the technique. Studies in humans are routinely performed at 1.5–4.7 Tesla. To examine defined small volumes of tissue, surface coils are placed snugly over the region of interest to ensure homogenous tissue filling in that region. The pulse angle and shape can be selected so that signals from other tissues, such as the subcutaneous fat layer, are largely suppressed (10).

**NUCLEI USED FOR NMR SPECTROSCOPY** From the broad range of magnetic nuclei, most studies to date have utilized $^1H$, $^{31}P$, and $^{13}C$ to determine skeletal
muscle glucose and glycogen metabolism. The suitability of a nucleus for NMR spectroscopy depends on its relative magnetic sensitivity, the tissue concentration range of the studied metabolites, and the chemical shift range. The relative magnetic sensitivity of a nucleus is defined as the product of an inherent property (NMR sensitivity of the nucleus relative to that of an equal number of protons) and its natural abundance.

$^1$H NMR Spectroscopy Protons ($^1$H) have a natural abundance close to 100% and overall offer the highest sensitivity for NMR observation. However, the relatively low concentration of metabolites compared to the proton concentration in water, as well as the low chemical shift range (10 ppm), limits $^1$H’s use for NMR spectroscopy.

$^{31}$P NMR Spectroscopy $^{31}$P occurs 100% in nature and allows quantification of intramuscular concentrations of adenosine-triphosphate (ATP), adenosine-diphosphate (ADP), inorganic phosphate (P$_i$), phosphocreatine (PCr), and glucose-6-phosphate (G6P). One of the earliest applications of $^{31}$P NMR spectroscopy, the determination of intracellular pH, is based on the observation that the chemical shift of inorganic phosphate is sensitive to changes of pH near its pK$_a$, which is within the physiological range (11). The use of $^{31}$P NMR spectroscopy to monitor intracellular phosphorylated metabolites and pH has been reviewed (3, 6).

$^{13}$C NMR Spectroscopy In contrast to $^1$H and $^{31}$P, $^{13}$C has a natural abundance of 1.1% and therefore a relatively low sensitivity. Nevertheless, this nucleus can be used to study glycogen (10, 12–15) and triglyceride metabolism (16), since these molecules typically occur in concentrations over 50 mM. Furthermore, $^{13}$C-enriched isotopes can be used to enhance the sensitivity by almost 100-fold (Figure 1).

Validation of in Vivo NMR Spectroscopy Measurement of tissue glycogen content by $^{13}$C NMR spectroscopy has been validated for skeletal muscle and liver by comparison with muscle (17) and liver (18) biopsies in rabbits and skeletal muscle in humans (19). There was typically excellent agreement ($r$ 0.9) between classical chemical and NMR-spectroscopic determination of glycogen over a broad concentration range. NMR spectroscopy was found to be more precise than the biopsy measurements; coefficients of variation between multiple measurements in the same subject are 4.3% (3.4 mM for basal concentrations) in muscle compared with 9.3% using the biopsy technique (18). Measurement of muscular G6P concentrations by $^{31}$P NMR spectroscopy has also been validated by comparison with chemical assay of G6P done on rat muscle frozen in situ (20).
Advantages and Limitations of In Vivo NMR Spectroscopy

ADVANTAGES Prior to the advent of in vivo NMR spectroscopy, glucose metabolism was studied in humans by using tracer methodology and biopsies obtained from the tissues of interest. The following limitations obscure the results obtained with this approach:

1. Tracers can be transferred to other molecules and reenter the tested metabolic pathway, necessitating complex corrections (21). Moreover, the administration of radioactive isotopes to healthy volunteers is not permitted in some countries.

2. Biopsies are limited to only a few time points, so time-course studies in a particular metabolite concentration are not feasible. In addition, stress hormone release during biopsies might alter the concentration of the measured metabolites.

3. Freeze clamping of tissues, or the time lag between excision and freeze clamping, may result in overestimation of different metabolic effects. Artificially high concentrations of intracellular free ADP can be explained by rapid hydrolysis of high-energy phosphate (1) or by the fact that most of the ADP in intact tissue is tightly bound to proteins of myofilaments (22). In

Figure 1  Time course of $^{13}$C NMR spectra of muscle glycogen (C-1 glycogen peak at 100.4 ppm) of a nondiabetic subject during a hyperglycemic-hyperinsulinemic clamp study (with permission from Ref. 14).
addition, a delay of several seconds during tissue handling leads to raised intramuscular G6P concentrations due to increased glycogenolysis (23).

4. Enzyme activities do not necessarily reflect the actual flux through metabolic pathways.

The advantages of in vivo NMR spectroscopy include (a) the avoidance of invasive procedures, (b) the avoidance of radiolabeled compounds, (c) the ability to follow the time course of metabolic reactions and thereby to trace fluxes through metabolic pathways in vivo, and (d) clinical imaging of the region of interest to confirm positioning of the probe and check for potential anatomical or pathological variations.

LIMITATIONS In addition to the technical limitations inherent in the method or in the biochemical conditions of the tissue of interest, discussed above, there are practical limitations on the use of NMR spectroscopy in vivo. Subjects must be able to lie still within the magnet for prolonged periods. This is necessary because collecting data for a reliable $^{13}$C or $^{31}$P NMR spectrum typically requires 5 to 20 min. Careful screening of potential subjects is therefore of critical importance for the success of an in vivo NMR experiment. Furthermore, subjects with metal implants must be excluded for safety reasons because of potential movement of paramagnetic metal in the presence of the strong magnetic field.

STUDIES ON SKELETAL MUSCLE METABOLISM IN HUMANS

Physiology of Muscle Metabolism

As shown by arteriovenous balance studies, skeletal muscle represents the major tissue responsible for insulin-stimulated glucose uptake in the body (24). Although indirect approaches using respiratory exchange measurements suggested that the majority of glucose taken up is preferentially metabolized nonoxidatively (24, 25), no consistent increase in the intramuscular glycogen concentration was detected under hyperinsulinemic conditions (26).

$^{13}$C NMR spectroscopy, combined with intravenous infusion of [1-$^{13}$C]glucose to enhance the sensitivity of the method, revealed basal (fasting) glycogen levels in gastrocnemius muscle of 73±11 mM and glycogen synthesis rates of 0.18±0.04 mmol/(liter muscle•min) under hyperglycemic (~10 mM)–hyperinsulinemic (~400 pM) clamp conditions (14) (Figures 1, 2). From the glucose infusion rates and indirect calorimetry, muscle glycogen formation was calculated to account for ~90% of whole-body glucose metabolism and all nonoxidative glucose disposal.

Another study examined the time course of glycogen concentrations after ingestion of a mixed meal by nondiabetic volunteers (27). Plasma concentra-
tions of glucose rose to ~7.3 mM and insulin concentrations rose to ~900 pM within 30 min of meal ingestion, while concentrations of lactate and triglycerides peaked at 75 min and at 225 min, respectively. After a delay of ~90 min, glycogen concentrations in gastrocnemius muscle increased at a rate of ~0.1 mmol/(liter muscle·min) and reached a peak concentration of 100–7 mM between 4 h and 5 h after meal ingestion. The increment in glycogen (17±3 mM) at a mean muscle mass of 22±2 kg represented a mean glucose incorporation of 60±8 g, indicating that 26–35% of the absorbed carbohydrates were stored as muscle glycogen. In the late postprandial phase (from the glycogen peak at ~4 h until 7 h after the meal), net glycogenolysis occurred at a rate of ~0.062 mmol/(liter muscle·min) (27).

**Type 2 Diabetes Mellitus**

Insulin resistance is a primary factor in the pathogenesis of type 2 diabetes mellitus (24, 28–30) and may precede the manifestation of the disease by decades (31). Moreover, in the normoglycemic offspring of type 2 diabetic parents, the presence of insulin resistance is the best predictor for the later development of the disease (32). In order to assess whether defects in insulin-stimulated muscle glycogen synthesis contribute to decreased insulin-stim-
ulated whole-body glucose uptake, rates of muscle glycogen synthesis were measured with $^{13}$C NMR spectroscopy in type 2 diabetic subjects under hyperglycemic (~10 mM)–hyperinsulinemic (~400 pM) clamp conditions (14). Rates of glycogen synthesis were reduced by ~60% in type 2 diabetic patients compared with nondiabetic age-weight–matched volunteers [0.08±0.03 vs 0.18±0.04 mmol/(liter muscle•min)] (14) (Figure 2).

Although glycogen synthase activity was lower in muscle biopsies (33, 34) and glucose transport by GLUT4 was reduced in cultured adipocytes (35) from type 2 diabetic patients, the relative role of particular enzymatic reactions in the reduction of insulin-stimulated muscle glycogen synthesis was not clear. Because of its location between transport/phosphorylation and glycogen synthase enzymes along the pathway of muscle glycogen synthesis, the intracellular G6P concentration is sensitive to the relative activities of these enzymes and the rate of glycolysis (Figure 3). If glycogen synthase activity was selectively impaired in diabetic patients, the initial rate of glucose entering the G6P pool would be identical in nondiabetic and diabetic subjects under hyper-

![Figure 3](https://example.com/image.png)

**Figure 3** Scheme of the intracellular fate of glucose in skeletal muscle. Following glucose transport by GLUT-4 and phosphorylation by hexokinase-II (HK-II), glucose-6-phosphate (G6P) is utilized for glycogen synthesis via glucose-1-phosphate (G1P) and uridine-diphosphatidyl-glucose (UDPGlu) or converted to fructose-6-phosphate (F6P). Most of the F6P is utilized by the glycolytic pathway after phosphorylation to fructose-1,6-bisphosphate by phosphofructokinase (PFK), and pyruvate, which is oxidized to acetyl-coenzyme A (acetyl-CoA) by mitochondrial pyruvate-dehydrogenase (PDH). Potential sites of free fatty acid (FFA) action in skeletal muscle: Randle’s hypothesis postulates that FFA oxidation increases intracellular citrate, NADH/NAD, and acetyl-CoA/CoA, which inhibit PDH. Citrate will also inhibit PFK and thereby cause G6P to rise. Elevation of G6P will then allosterically block HK-II. Alternatively, FFA could directly inhibit glucose transport/phosphorylation.
glycemic-hyperinsulinemic conditions. But slower removal of G6P due to reduced glycogen synthase activity would result in a rise of intracellular G6P in diabetic patients. On the other hand, similar or decreased G6P concentrations would indicate that glucose transport/phosphorylation was defective in diabetic patients.

In order to further characterize the defect and determine the rate-controlling steps for muscle glycogen synthesis, intracellular muscle G6P concentrations were measured by $^{31}$P NMR spectroscopy in type 2 diabetes mellitus (36) (Figure 2). Basal G6P concentrations were found to be similar in both nondiabetic and diabetic humans (~0.13 mM), but steady-state G6P levels were lower in type 2 diabetic (0.17±0.02 mM) than in nondiabetic humans (0.24±0.02 mM) during hyperglycemic-hyperinsulinemic clamps (36) (Figure 2). When plasma insulin concentrations were increased ~20-fold in the type 2 diabetic patients, whole-body glucose uptake and G6P concentrations increased, becoming similar to those of nondiabetic humans. These results suggest that the primary defect responsible for reduced whole-body glucose uptake and glycogen synthesis is likely to be located at the glucose transport and/or phosphorylation steps. Allosteric effectors (Pi, ADP, ATP, pH) of hexokinase were also measured by $^{31}$P NMR spectroscopy, but no difference between diabetic and nondiabetic subjects was detected.

Lean Normoglycemic Offspring of Type 2 Diabetic Parents

It had been suggested that hyperglycemia might increase muscle glycogen stores and thereby limit glycogen synthesis (33), but basal muscle glycogen levels were found to be even lower in type 2 diabetic than in nondiabetic volunteers (39±6 vs 73±11 mM) (14). It is also possible that the defect in glycogen synthesis could be simply attributed to insulin resistance induced by hyperglycemia, indicating a phenomenon often referred to as glucose toxicity (37, 38). Chronically elevated glucose concentrations may give rise to intracellular G6P, which is predominantly utilized by glycogen synthesis and glycolysis (Figure 3), but of which ~1–3% will be converted to fructose-6-phosphate and glucosamine-6-phosphate by the hexosamine pathway. Glucosamine in turn may interfere with translocation of glucose transporters (39, 40). Studies in rats (41) and insulin-dependent humans (37) suggested that chronic hyperglycemia may result in impaired glycogen synthesis. In order to eliminate the possibility that the observed defects were due to glucose toxicity, lean, normoglycemic, insulin-resistant offspring of type 2 diabetic patients were studied under similar hyperglycemic (~11 mM) and hyperinsulinemic (~480 pM) conditions (42) (Figure 2). They were insulin-resistant, as reflected by a ~50% reduction of insulin-stimulated whole-body glucose metabolism and a ~70% reduction in the rate of muscle glycogen synthesis. Furthermore, the increase in intramuscular G6P concentrations in these patients was reduced by ~40%
(0.16±0.02 vs 0.25±0.01 mM) during the clamp, suggesting that impaired glucose transport/phosphorylation was also responsible for their decreased insulin sensitivity. To assess whether decreased activity of glycogen synthase might also contribute to reduced glycogen synthesis, the nondiabetic control subjects were examined again at the identical degree of hyperinsulinemia, but at fasting plasma-glucose concentrations (euglycemia). Under these conditions, whole-body and nonoxidative glucose uptake as well as glycogen synthesis were similar to those of the offspring of type 2 diabetic patients. If glycogen synthase activity was the rate-controlling step for glycogen synthesis in these patients, they would be expected to have higher G6P concentrations than the control subjects. However, muscle G6P concentrations were similar to that of the control subjects (0.17±0.01 mM). Taken together, these results indicate that impaired glucose transport/phosphorylation is already present in normoglycemic, insulin-resistant, lean offspring of type 2 diabetic patients and may be a primary factor in the pathogenesis of type 2 diabetes mellitus.

In order to examine the mechanism by which exercise training improves insulin responsiveness in these subjects, 10 lean, insulin-resistant, normoglycemic offspring of type 2 diabetic parents were studied under baseline conditions (a) after one bout of stair-climbing exercise performed for 45 min at 65% of maximal aerobic capacity and (b) after six weeks of the same exercise protocol performed four times a week, using combined 13C and 31P NMR spectroscopy during hyperglycemic-hyperinsulinemic clamps (43). At baseline, nonoxidative glucose metabolism and muscle glycogen synthetic rate were ~58% and ~63% lower, respectively, in the offspring than in control subjects. Muscle glycogen synthesis increased by ~69% after one exercise session and further increased by ~102% after six weeks of exercise training, bringing it close to that of control subjects before exercise training. However, insulin-stimulated glycogen synthesis increased by a similar percentage in the control subjects following exercise training. Incremental G6P concentrations during the clamp were lower in the offspring at baseline (0.04±0.01 vs 0.09±0.01 mM of control), but completely normalized after exercise training (0.12±0.02 vs 0.08±0.01 mM of control). These results suggest that exercise training overcomes the defect of glucose transport/phosphorylation but unmasks an additional distal abnormality in the glycogen synthetic pathway, possibly occurring at glycogen synthase.

Obesity and Free Fatty Acids

The mechanism of insulin resistance in obesity was also recently studied, using a similar approach (44) (Figure 2). Rates of insulin-stimulated muscle glycogen synthesis were decreased by ~70% (0.052±0.008 mmol/l·min) and the increment in intramuscular G6P was also severely blunted (0.044±0.011 mM). This indicates that, counter to previous suggestions (34, 45), flux through gly-
cogen synthase is not rate-controlling. Taken together, these studies suggest a common defect in insulin-stimulated muscle glucose transport/phosphorylation in both lean offspring of type 2 diabetic parents and obese, insulin-resistant populations.

It is notable that these populations also share other metabolic abnormalities. Under fasting conditions, plasma concentrations of free fatty acids are frequently increased in obese (46, 47) but also in nonobese, insulin-resistant offspring of Mexican-American (48) and Caucasian type 2 diabetic patients (49). Randle (50, 51) postulated that increased free fatty acid oxidation leads to inactivation of pyruvate dehydrogenase and subsequent elevation of cytosolic citrate, which would inhibit phosphofructokinase, the key enzyme of the glycolytic pathway (Figure 3). This would in turn result in accumulation of intracellular G6P, which would allosterically inhibit hexokinase and thereby decrease muscle glucose uptake.

To test this hypothesis, we measured muscle glycogen synthesis and G6P concentrations by simultaneous 13C/31P NMR spectroscopy during euglycemic (~5 mM)–hyperinsulinemic (~400 pM) clamps in nondiabetic humans infused with heparin/triglyceride emulsion (52). Elevation of plasma free fatty acids (~2 mM) resulted in a parallel decrease of nonoxidative glucose metabolism and net rates of glycogen synthesis. The decreases became apparent after 3 h and rates were ~50% lower at 6 h compared with low free-fatty-acid conditions (Figure 4). This was in agreement with previous findings using a similar experimental approach (53–55). Surprisingly, the reduction in glycogen synthesis was preceded by a fall of intramuscular G6P concentrations, starting at ~1.5 h. These results demonstrate that the classic Randle mechanism, which predicts a rise in intracellular G6P, is not operating in humans under these conditions. Moreover, the finding of decreased G6P in the presence of reduced muscle glycogen synthesis indicates that glucose transport/phosphorylation becomes rate-controlling during elevation of plasma free fatty acids.

Type 1 Diabetes Mellitus

It is well established that patients with poorly controlled type 1 diabetes mellitus are insulin-resistant, a condition that resolves upon normalization of their glycemic control (37, 38). To examine the mechanism of insulin resistance in these individuals, poorly controlled type 1 diabetic subjects (mean hemoglobin A1c ~13.6%) were studied using combined 13C and 31P NMR spectroscopy during hyperglycemic (~9 mM) hyperinsulinemia (~400 pM) for 6 h (56) (Figure 2). Reduction of whole-body nonoxidative glucose metabolism by ~40% and the decrease in net rates of skeletal muscle glycogen synthesis [0.11±0.02 vs 0.20±0.01 mmol/(liter muscle•min)] showed that these patients were insulin-resistant. The decrease of muscle glycogen synthesis might be due to reduced glucose transport/phosphorylation or decreased glycogen synthase.
Figure 4  Whole-body glucose infusion rate, glucose oxidation rate, and increase in calf muscle glycogen and in glucose-6-phosphate at low (closed circle) and at elevated (open circle) plasma free fatty acid concentrations (with permission from Ref. 52).
activity. Steady-state intramuscular G6P concentrations were also ~40% lower in the poorly controlled type 1 diabetic patients than in the nondiabetic control subjects during the hyperglycemic-hyperinsulinemic clamp (0.18±0.02 vs 0.25±0.01 mM in control subjects). Even when the control subjects were studied at lower plasma-glucose concentrations to match the reduced nonoxidative glucose metabolism of type 1 diabetic patients, intramuscular G6P concentrations were not higher in the poorly controlled type 1 diabetic patients, indicating that decreased glucose transport/phosphorylation is responsible for their insulin resistance. The reduction of glycogen synthesis might also result from increased glycogen turnover. However, employing a ^13_C-^12_C pulse-chase technique to determine the relative flux through glycogen synthase and phosphorylase, Cline et al (56) found a decrease in skeletal muscle glycogen turnover (~16% vs ~33%), along with reduced glycogen breakdown in the poorly controlled type 1 diabetic patients. These results are consistent with Marshall et al’s hypothesis that chronic hyperglycemia stimulates the hexosamine pathway, leading to desensitization of insulin-stimulated skeletal muscle glucose transport activity (39, 40).

SUMMARY

In summary, use of the NMR technique to measure intracellular glycogen content and G6P has shown that insulin resistance exhibits a common defect in insulin-stimulated muscle glycogen synthesis that can be mostly attributed to a defect in glucose transport or phosphorylation activity. This has held true whether the insulin resistance is caused by acquired abnormalities, e.g. chronic hyperglycemia in type 1 diabetes mellitus (56) or increased availability of free fatty acids (44, 52), or by genetic factors as in type 2 diabetic patients (14, 36) and their offspring (42, 43).


Literature Cited


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