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RESEARCH REPORT

A Modified Enzymatic Method for Measurement of Glycogen Content in Glycogen Storage Disease Type IV

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Abstract Deficiency of glycogen branching enzyme in glycogen storage disease type IV (GSD IV) results in accumulation of less-branched and poorly soluble polysaccharides (polyglucosan bodies) in multiple tissues. Standard enzymatic method, when used to quantify glycogen content in GSD IV tissues, causes significant loss of the polysaccharides during preparation of tissue lysates. We report a modified method including an extra boiling step to dissolve the insoluble glycogen, ultimately preserving the glycogen content in tissue homogenates from GSD IV mice. Muscle tissues from wild-type, GSD II and GSD IV mice and GSD III dogs were homogenized in cold water, and homogenate of each tissue was divided into two parts. One part was immediately clarified by centrifugation at 4°C (STD-prep); the other part was boiled for 5 min then centrifuged (Boil-prep) at room temperature. When glycogen was quantified enzymatically in tissue lysates, no significant differences were found between the STD-prep and the Boil-prep for wild-type, GSD II and GSD III muscles. In contrast, glycogen content for GSD IV muscle in the STD-prep was only 11% of that in the Boil-prep, similar to wild-type values. Similar results were observed in other tissues of GSD IV mice and fibroblast cells from a GSD IV patient. This study provides important information for improving disease diagnosis, monitoring disease progression, and evaluating treatment outcomes in both clinical and preclinical clinical settings for GSD IV. This report should be used as an updated protocol in clinical diagnostic laboratories.

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Introduction

In animal cells, glycogen synthesis is primarily catalyzed by two enzymes, glycogen synthase (GS, EC 2.4.1.11), which adds glucose residues to a linear chain, and glycogen branching enzyme (GBE, EC 2.4.1.18), which adds branches to the growing glycogen molecule. Although the majority of glycogen is degraded in the cytoplasm by the combined action of glycogen phosphorylase and glycogen debranching enzyme (GDE, EC 2.4.1.25/EC 3.2.1.33), a small percentage of glycogen is transported to and hydrolyzed in lysosomes by acid α-glucosidase (GAA, EC 3.2.1.20) (Huijing 1975; Chen et al. 2009).

Glycogen storage diseases (GSDs) are a group of inherited disorders caused by deficiency of a certain enzyme involved in glycogen synthesis or degradation. While the accumulation of glycogen in liver and muscle tissues is the common consequence of these diseases, the molecular structure and property of glycogen varies between specific GSDs. For example, deficiency of GAA in GSD II causes accumulation of glycogen with normal structure in the lysosomes. In GSD III, loss of GDE enzyme activity hinders further breakdown of glycogen from branching points, resulting in the accumulation of abnormal glycogen with short outer chains (Chen et al. 2009). In GSD IV, deficiency of GBE leads to the production of less-branched and poorly soluble polysaccharides (polyglucosan bodies, PB) in all body tissues (Brown and Brown 1966; Fernandes and Huijing 1968; Mercier and Whelan 1970).

Biochemical quantification of glycogen content is critical for disease diagnosis, disease progression monitoring, and therapeutic outcomes evaluation in both clinical and preclinical settings. An enzymatic method based on homogenization of tissues in cold water followed by *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) digestion has become widely used for measuring glycogen content in tissue (Huijing 1970; Van Hove et al. 1996; Kikuchi et al. 1998; Raben et al. 2003). In the past decade, our team has had success using this method to quantify glycogen in various tissues from experimental animals with GSD type I, II, or III (Sun et al. 2005, 2007; Koeberl et al. 2006; Yi et al. 2012, 2014). Recently, in our work with a mouse model of GSD IV, we found that the measured tissue glycogen contents were at extremely low levels, which contradicts with the observation that strongly PAS-positive PB were present in these tissues. Considering the low solubility of the PB in GSD IV, we speculated that the majority of glycogen was lost during the lysate preparation. Here we describe a modified enzymatic method for glycogen quantification in GSD IV.

Materials and Methods

Animal Tissues

Muscle tissues were obtained from 3-month-old GAA knockout (GSD II) mice (Raben et al. 1998) and from 4-month-old GSD IIIa dogs (Yi et al. 2012). GSD IV (*Gbe1*$^{ys/ys}$) mice (Akman et al. 2015) were euthanized at age of 3 months following overnight fasting for collection of tissues. Muscle tissues from 3-month-old wild-type (C57BL/6) mice were used as controls. Fresh tissues were fixed in 10% neutral buffered formalin for PAS staining or frozen in −80°C freezer until use (Yi et al. 2012). All animal experiments were approved by the Institutional Animal Care & Use Committee at Duke University and were in accordance with the National Institutes of Health guidelines.

Tissue Lysate Preparation

Frozen tissues (50–100 mg) were homogenized in ice-cold deionized water (20 ml water/g tissue) and sonicated three times for 15 s with 30-s intervals between pulses, using a Misonix XL2020 ultrasonicator (Yi et al. 2012). Homogenate of each tissue was divided into two parts and processed separately: one part was immediately clarified by centrifugation at 4°C (STD-prep); the other part was boiled for 5 min then centrifuged at room temperature (Boil-prep).

Cell Culture and Cell Lysates

Fibroblasts derived from skin biopsies of a patient with GSD II and one with GSD IV were harvested after 3 days in culture in 10-cm plates (Van Hove et al. 1996). The cell pellet from each plate was resuspended in 300 μl cold water and sonicated three times. The STD-prep and Boil-prep cell lysates were then prepared as described above. Protein concentration of the STD-prep was determined using BCA method.

Glycogen Content Measurement

Glycogen contents in the tissue and cell lysates (both the STD-prep and the Boil-prep) were assayed as described (Van Hove et al. 1996; Yi et al. 2012).

Statistical Analysis of Glycogen Content

The significance of differences between the STD-prep and Boil-prep of the same group of samples was assessed using two-tailed, paired student *T*-test. Mean ± standard deviation were shown.

Results

Glycogen Staining and Quantitation in Skeletal Muscles from Wild-Type and GSD Animals

PAS staining of glycogen revealed no visible PAS-positive materials in wild-type (Wt) mice. In GSD II mice, glycogen-filled lysosomes of various sizes were scattered throughout the tissue; in GSD III dogs, filamentous glycogen aggregates and large pools of glycogen were seen; in GSD IV mice, granular glycogen particles were observed in most myocytes (Fig. 1a).

When glycogen was quantified in tissue lysates, no significant differences were found between the STD-prep and the Boil-prep for wild-type (Wt), GSD II and GSD III muscles (Fig. 1b). In contrast, the GSD IV muscle showed a very low level of glycogen in the STD-prep lysates (3.17 ± 1.15 μmol glucose/g tissue), similar to that of wild-type muscle, while the Boil-prep showed a markedly higher level (34.5 ± 12.7), indicating significant loss of glycogen in the STD-prep lysates (Fig. 1b).

Glycogen Staining and Quantitation in Other Tissues from GSD IV Mice

PAS staining of glycogen was also performed on other tissues of GSD IV mice at age 3 months. As shown in

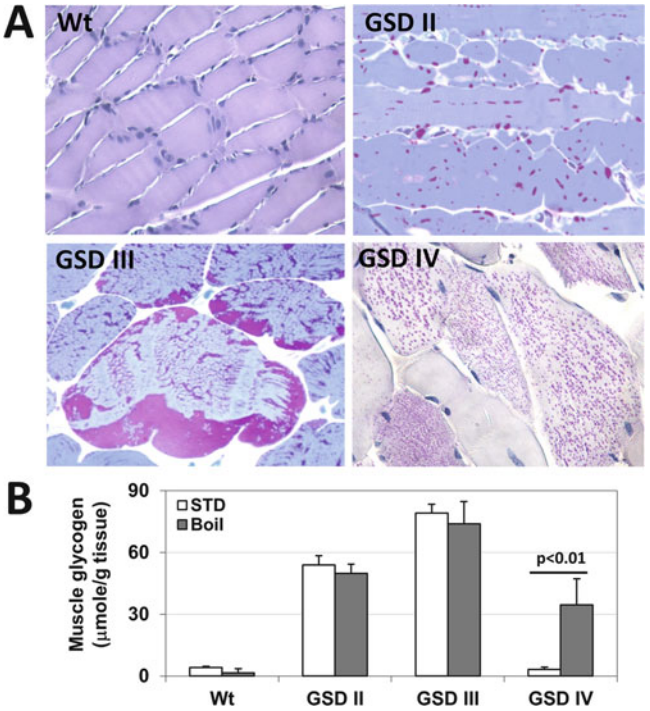

Fig. 1 Glycogen in skeletal muscles from wild-type (Wt) and GSD animals. (**a**) Representative PAS staining of muscle (gastrocnemius) sections form Wt mice, GSD II mice, GSD IIIa dogs, and GSD IV mice (magnification 400×). (**b**) Comparison of the STD-prep and the Boil-prep methods for quantitation of glycogen in muscles from animals in (**a**). n=5 for mice, n=4 for dogs

Fig. 2a, most hepatocytes were loaded with glycogen (fasted); the diaphragm has similar glycogen accumulation pattern as the gastrocnemius muscle; clusters of glycogen particles were occasionally found in the heart; PAS-positive granules were clearly present in the brain (cerebrum). Glycogen quantitation showed significantly lower glycogen contents in the STD-preps than in the Boil-preps for all the tissues (Fig. 2b). Glycogen content in the STD-prep was 28% of that in the Boil-prep for the liver (fasted) and was 21% for the heart and 8% for both the brain and diaphragm (Fig. 2b).

Glycogen Quantitation in Fibroblasts from Patients with GSD II and IV

In cultured human patient skin fibroblasts, the STD-prep of the GSD IV cells presented 50% less glycogen than the Boil-prep; the Boil-prep of GSD II cells presented 10% more glycogen than the STD-prep (Fig. 3).

Discussion

Mutations in the *Gbe1* gene cause a complete or partial loss of GBE activity in GSD IV, which leads to an increase in the ratio of GS to GBE, a critical determinant of PB formation during the process of glycogen synthesis (Raben et al. 2001; Pederson et al. 2003; Kakhlon et al. 2013). The Y329S is the most common mutation found in Jewish families of Ashkenazi ancestry with adult onset GSD IV, also referred to as adult polyglucosan body disease (Lossos et al. 1998; Mochel et al. 2012). Recently we obtained a new mouse model of GSD IV ($Gbe1^{ys/y}$ mice) carrying the knock-in Y329S mutation (Akman et al. 2015). The residual enzyme activity in the affected mice was approximately 10–19% of wild-type value in skeletal and cardiac muscles, 30% in the brain, and less than 1% in liver (data not shown). PAS staining showed significant PB accumulation in all these tissues.

In a standard enzymatic method for glycogen quantitation, tissue homogenization in cold water or buffer followed by an immediate centrifugation has been a widely used procedure for its simplicity, sensitivity, and ability to analyze other metabolites and enzyme activities in the same homogenate (Huijing 1970; Murat and Serfaty 1974; Van Hove et al. 1996). But this procedure is not suitable for GSD IV glycogen measurement due to the heavy loss of insoluble glycogen during sample preparation. In this study, we described a modified method that includes an extra boiling step prior to centrifugation of tissue homogenates to dissolve the insoluble glycogen in GSD IV (Mercier and Whelan 1970). To determine the length of boiling time needed for complete glycogen dissolution, we quantified glycogen after boiling the homogenates (150–300 μl) 3, 5,

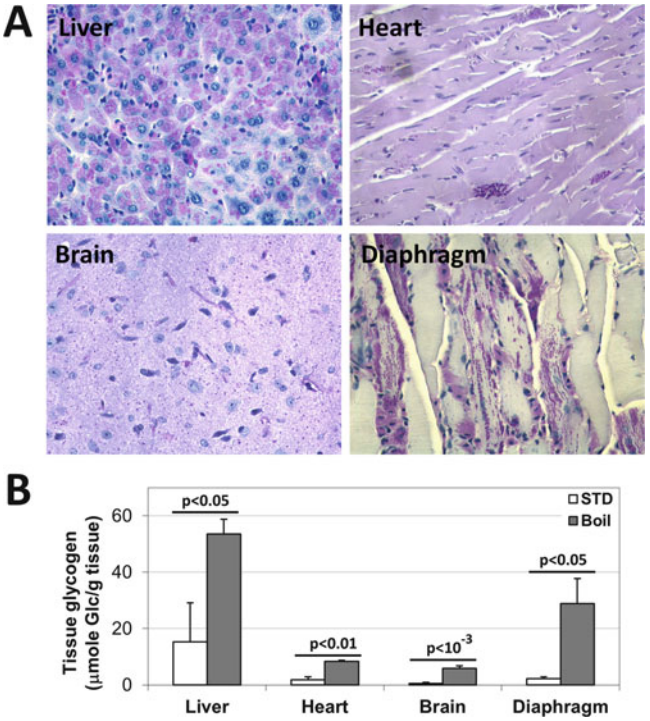

Fig. 2 Glycogen in other tissues from the GSD IV mice. (**a**) PAS staining shows glycogen deposits of various degrees in the liver, diaphragm, heart, and brain (cerebrum) of the GSD IV mice (magnification 400×). (**b**) Comparison of the STD-prep and the Boil-prep methods for quantitation of glycogen in these tissues. n=5 mice

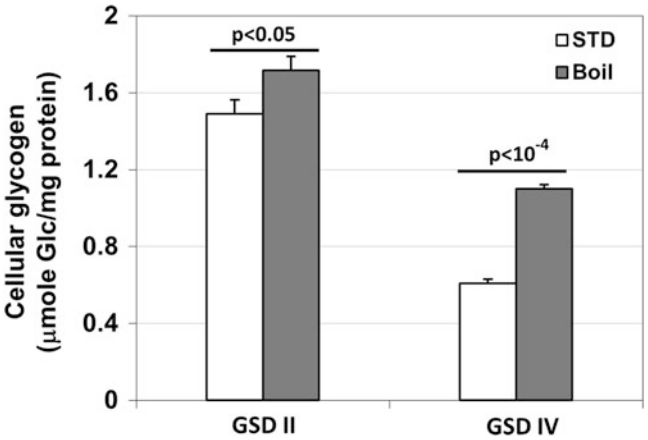

Fig. 3 Comparison of the STD-prep and the Boil-prep methods for quantitation of glycogen in cultured skin fibroblasts from a patient with GSD II and one with GSD IV. n=4 plates for each patient

10, and 15 min and saw no difference among all the time points (data not shown). This method is likely also applicable to Lafora disease, a related polyglucosan body disease caused by mutations in EPM2A or EPM2B (Minassian 2001), but this needs to be verified by experiments. Another more tedious and less sensitive method involving boiling tissue homogenate in KOH followed by ethanol precipitation of glycogen prior to the amyloglucosidase digestion is also suitable for determining glycogen content in GSD IV, but this procedure requires larger size of tissues, which limits its clinical application (Koeberl et al. 1990; Suzuki et al. 2001; Pederson et al. 2004).

This study provides an improved protocol for quantifying the insoluble glycogen in GSD IV without the need of glycogen isolation prior to the enzyme digestion. More importantly, the modified method allows determination of glycogen content in very small biopsy samples, which is extremely useful for clinical diagnostic laboratories. Vali-

dation with sufficient numbers of patient samples and normal controls will be necessary before applying this method to clinical diagnosis.

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Synopsis of the Article

An improved method for quantitation of insoluble glycogen in glycogen storage disease type IV is described.

Compliance with Ethics Guidelines

Conflict of Interest

Haiqing Yi, Quan Zhang, Chunyu Yang, Priya S. Kishnani, and Baodong Sun declare that they have no conflict of interest.

Informed Consent

This article does not contain any studies with human subjects performed by any of the authors. The research protocol with human GSD patients' cells in this study was approved and declared by Duke IRB as research not involving human subjects.

Animal Rights

All institutional and national guidelines for the care and use of laboratory animals were followed.

Details of the Contributions of Individual Authors

Haiqing Yi – conducted the majority of the experiments, reported the work, and prepared manuscript

Quan Zhang – conducted some of the experiments

Chunyu Yang – conducted some of the experiments

Priya S. Kishnani – reviewed and edited the manuscript

Baodong Sun – planned and supervised this study and wrote the manuscript

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