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Homogenization *versus* homogenization-free method to measure muscle glycogen fractions

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Abstract. – OBJECTIVE: The glycogen is extracted from animal tissues with or without homogenization using cold perchloric acid. Three methods were compared for determination of glycogen in rat muscle at different physiological states.

MATERIALS AND METHODS: Two groups of five rats were kept at rest or 45 minutes muscular activity. The glycogen fractions were extracted and measured by using three methods.

RESULTS: The data of homogenization method shows that total glycogen decreased following 45 min physical activity and the change occurred entirely in acid soluble glycogen (ASG), while AIG did not change significantly. Similar results were obtained by using "total-glycogen-fractionation methods". The findings of "homogenization-free method" indicate that the acid insoluble fraction (AIG) was the main portion of muscle glycogen and the majority of changes occurred in AIG fraction.

CONCLUSIONS: The results of "homogenization method" are identical with "total glycogen fractionation", but differ with "homogenization-free" protocol. The ASG fraction is the major portion of muscle glycogen and is more metabolically active form.

Key Words:
Physical activity, Glycogen, Homogenization, Muscle.

Introduction

There are several methods to measure glycogen in animal tissues. The tissue is digested by hot alkaline[1], hot acid or cold acid-grinding[2,3]. Then, glycogen is extracted from the (supernatant of) tissue homogenate with ethanol[3]. Glycogen is labile in hot acid and undergoes hydrolysis, so it does not need to extract with ethanol in hot acid treatment[3]. Finally, glycogen is hydrolyzed to glucose and measured by using chemical or enzymatic methods[4,5].

The classical method was re-evaluated and optimized for assays of glycogen fractions[6]. In the classical 'homogenization method' the tissue is ground by cold perchloric acid (PCA). The extraction must be done at least twice to recover any acid soluble glycogen (ASG) quantitatively[6-9]. The last pellet is digested with hot alkaline to release acid insoluble fraction (AIG). Total glycogen could be calculated by summing the values of ASG and AIG or measured directly by hot alkaline[6].

In "homogenization-free method" the tissue that is submerged in cold PCA pressed by a plastic rod to the wall of the tube during 20 minutes[10,11]. The tissue suspension is then centrifuged; the supernatant is taken as ASG and the pellet is extracted by hot alkaline to remove AIG. We also introduced a new method in which total glycogen is extracted and divided directly to the fractions of ASG and AIG[12]. By using this procedure, glycogen fractions are measured simultaneously. Recently, a new era of research is conducted to study the physiological role of glycogen fractions[8-13]. However, the results of the methods with and without of homogenization are completely different[8,9]. Therefore, it is essential to compare the results of different methods.

Materials and Methods

Running Wheel Physical Stress Test

The male rats (200-220 g) were starved for 12 h overnight. One group of five rats was kept at rest and another group was put for 45 min in standard animal running wheel with 0.5 m circumstance at the rate of 30 rpm.

Muscle Sampling

The iliopsoas thigh muscle was isolated from rats anesthetized with diethyl ether and washed rapidly three times with ice cold isotonic saline. The tissue incised into several parts on a filter paper and preserved between aluminum foil at −70°C immediately.

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Measurement of Total Glycogen

100 mg of tissue was weighed, transferred quantitatively to 200 µL 30% KOH and heated in boiling water bath for 10 min with regular mixing. After cooling, ethanol was added at a final concentration of 55%, vortexed and centrifuged 10 min at 1700 ×g. The supernatant was decanted off and the pellet re-suspended in 1 mL of distilled water and 100 µL was analyzed for total glycogen in triplicate.

Homogenization Method

The optimized method has reported in reference-6. In brief, 100 mg tissue was weighed, ground with cold PCA and centrifuged. The supernatant containing ASG was decanted and the pellet was re-extracted for further one step with 1 mL fresh PCA to extract any acid soluble glycogen. To extract AIG, 200 µL of 30% KOH was added to the last pellet and heated in boiling water bath for 10 min. After cooling, it was extracted with ethanol and centrifuged 10 min at 1700 ×g. The supernatant was decanted off and the pellet re-suspended in 1 mL of distilled water and 100 µL was analyzed for AIG.

Homogenization-free Method

The 100 mg tissue was weighed, transferred into a tube and added 2 mL ice cold 10% PCA[10,11]. The sample was pressed with a glass rod occasionally during 20 min incubation in ice surrounding. The ground sample was centrifuged 10 min at 5000 rpm at 4°C. The supernatant containing ASG was decanted into another tube and the pellet was extracted for AIG as described above.

Total Glycogen Fractionation Method

30 µL PCA (70%) was added to the suspension of total glycogen and mixed[12]. The sample was centrifuged 5 min at 280×g. ASG was remained in the suspension while AIG was precipitated. The supernatant contains ASG was decanted into another tube. The pellet was resolved as AIG in 1 mL of distilled water with the help of 10 µL 30% of KOH.

Assay of Glycogen

100 mL of sample was used for the measurement of glycogen fractions by chemical method of phenol-sulfuric acid[14].

Statistical Analysis

The results are presented as the means ± SD of three inter-assays performed at least in five samples. The significant differences between samples and corresponding control were accessed by student's *t*-test. All p-values are two-tailed and differences were considered significant if *p*-values were ≤0.05.

Results

Glycogen Fractions and Muscular Activity

To test and compare three procedures, glycogen fractions were measured by three methods at rest and after 45 min muscle activity. The data of homogenization method shows that the ASG and AIG fractions are about 70% and 30% of total muscle glycogen at rest (Table I). The results also indicate that following 45 min muscle activity, total glycogen decreased and the change occurred entirely in ASG, while AIG did not change significantly. Similar results were obtained by using "total glycogen fractionation" procedure (Figure 1). The findings of "homogenization-free method" indicate that the majority of changes occurred in AIG fraction. Nevertheless, ASG also changed significantly.

Discussion

In the current study, the fractions of glycogen were measured in rat muscle at different physiological states by using three methods. The findings of "homogenization method" indicate that ASG is

Table I. The effect of muscular activity on glycogen fractions. The muscles of five rats were analyzed for glycogen fractions at rest and after 45 min activity. *, **And ***indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.

	Homogenization method		Homogenization – free method	
Glycogen fractions	Rest	Activity	Rest	Activity
Total	1.92 ± 0.09	1.20 ± 0.09***	1.92 ± 0.09	1.20 ± 0.08***
ASG	1.38 ± 0.06	0.7 ± 0.15**	0.68 ± 0.10	0.42 ± 0.08*
AIG	0.57 ± 0.05	0.48 ± 0.14	1.33 ± 0.05	0.66 ± 0.11**

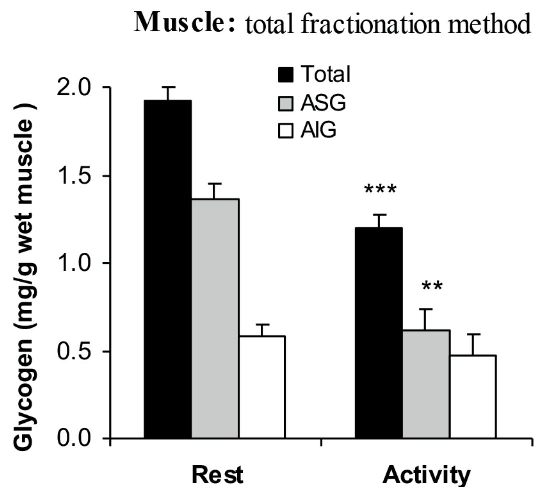

Figure 1. Muscle glycogen fractions following muscular activity. The descriptions are as the legend of Table I.

the major portion of rat muscle glycogen and is more metabolically active form. The results obtained by using the "homogenization-free method" were different as AIG was the main metabolic player. The results were the same for "classical homogenization" and "total-glycogen-fractionation methods" as there were no any significant differences between them.

The findings of the 'homogenization method' show that total glycogen decreased following 45 min activity and the response occurred wholly in ASG, while AIG changed insignificantly. The finding is clearly in accordance with the early experiments used the classical homogenization procedure[1-6], but is in contrast to the recent homogenization free protocol of Adamo and Graham[10,11]. This method is encountered with three main problems; high relative error in weighting, incomplete homogenization and precipitation of ASG with AIG and so overestimation of AIG. The high relative error could be seen as high CV% of their results[11] and is attributed to very small sample size 3-7 mg taken by biopsy. The current study did not encounter this problem because using of 100 mg tissue sample. We showed in the previous study that the yield of the recovery of ASG during successive extractions depends on the tissue concentration, and about 8% of ASG remained in the second pellet at the ratio of 50 mg tissue per 2 mL PCA. This value is related to the liver tissue and the current data indicates that there is about 10% ASG in the second pellet. In homogenization free protocol, the extraction has been done only once by a glass rod followed by unnecessarily high-speed centrifugation. Therefore, ASG has not extracted completely and some extracted ASG precipitates again causing a marked overestimation of AIG. As James et al[8] and Barnes et al[9] mentioned, earlier studies that used a homogenizatio n procedure have consistently reported more ASG than the recent studies without homogenization[1-6]. Indeed in the third protocol, some ASG contaminates AIG fraction and the changes that are seen in AIG is attributed to ASG fraction. In the homogenization-free method, if the second pellet is homogenized with further 1 mL PCA and centrifuged with mild extent, the result of two methods will be identical (results not shown).

The values of ASG, AIG and total glycogen and the responses to the muscular activity obtained by the Rasouli's protocol are the same as the classical method, but the procedure is more easy and precise. The procedure avoided several extraction-centrifugation steps. Hence, no any ASG is lost through successive extractions and less AIG is lost via autolysis. The time and extent of centrifugation have been chosen to be low in the fractionation step, so that no any ASG is co-precipitated with AIG[9,15].

Conclusions

The results of "homogenization method" are identical with "total glycogen fractionation", but differ with "homogenization-free" protocol. The ASG fraction is the major portion of rat muscle glycogen and is more metabolically active form.

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Conflict of interest

The authors declare no conflicts of interest.

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