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# Homogenization-dependent responses of acid-soluble and acid-insoluble glycogen to exercise and refeeding in human muscles

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**Abstract**

Muscle glycogen exists as acid-insoluble (AIG) and acid-soluble (ASG) forms, with AIG levels reported in most recent studies in humans to be the most responsive to exercise and refeeding. Because the muscle samples in these studies were not homogenized to extract glycogen, such homogenization-free protocols might have resulted in a suboptimal yield of ASG. Our goal, therefore, was to determine whether similar findings can be achieved using homogenized muscle samples by comparing the effect of exercise and refeeding on ASG and AIG levels. Eight male participants cycled for 60 minutes at 70% $\dot{V}O_{2peak}$ before ingesting 10.9 ± 0.6 g carbohydrate per kilogram body mass over 24 hours. Muscle biopsies were taken before exercise and after 0, 2, and 24 hours of recovery. Using a homogenization-dependent protocol to extract glycogen, 77% to 91% of it was extracted as ASG, compared with 11% to 24% with a homogenization-free protocol. In response to exercise, muscle glycogen levels fell from 366 ± 24 to 184 ± 46 mmol/kg dry weight and returned to 232 ± 32 and 503 ± 59 mmol/kg dry weight after 2 and 24 hours, respectively. Acid-soluble glycogen but not AIG accounted for all the changes in total glycogen during exercise and refeeding when extracted using a homogenization-dependent protocol, but AIG was the most responsive fraction when extracted using a homogenization-free protocol. In conclusion, the patterns of response of ASG and AIG levels to changes in glycogen concentrations in human muscles are highly dependent on the protocol used to acid-extract glycogen, with the physiologic significance of the many previous studies on AIG and ASG being in need of revision.


## 1. Introduction

Since early last century, glycogen in skeletal muscle has been known to exist as 2 distinct fractions on the basis of its solubility in acid, namely, acid-soluble (ASG) and acid-insoluble (AIG) glycogen [1]. In the 1950s, these 2 types of glycogen were the subject of several studies that found that ASG accounts for at least 40% of total glycogen at rest and that it is the most responsive fraction to changes in total glycogen concentration [2-7]. Afterward, in the early 1990s, Lomako and colleagues [8,9] reported that AIG particles are much smaller than ASG, with both being covalently bound to a 37-kd protein, glycogenin, the gene and promoter structure of which were subsequently characterized by our laboratory [10,11]. It is the high protein (glycogenin) to glucosyl ratio of the AIG particle that was then proposed to be responsible for its low acid solubility [8,9]. Just as importantly, Lomako and colleagues also provided some evidence that AIG was an intermediate along the synthesis of ASG and, for this reason, referred to these fractions as *proglycogen* and *macroglycogen*, respectively [9]. Not surprisingly, their findings were at the origin of a renewed interest in the physiology of ASG and AIG, with several recent studies examining the responses of these glycogen fractions to a range of physiologic conditions [12-30]. In contrast to the earlier findings of the 1950s, these recent studies and some earlier ones in humans have reported that AIG is in general not only the major fraction of glycogen, but also the most responsive to changes in total muscle glycogen levels, except when the amounts of stored glycogen are elevated [12,14,19,23,28,31-35].

It is important to stress that most recent studies on ASG and AIG have adopted glycogen extraction protocols that do not include a homogenization step [12,14,19,23,28,31-36]. For instance, in one of the most commonly used protocols, glycogen is extracted by pressing with a plastic rod some freeze-dried muscle samples submerged in perchloric acid

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(PCA). The extract is then centrifuged, with AIG and ASG found in the pellet and supernatant, respectively [34,36]. Another homogenization-free protocol uses powdered freeze-dried muscle tissues for the acid extraction of glycogen [15-17,32,33,35]. Recently, however, we provided some evidence that some of the glycogen extracted from rat muscles without a homogenization step may precipitate in the presence of acid not because of its acid insolubility per se, but because it is trapped by the remnants of undisrupted muscle myofibrils with which it coprecipitates, thus causing a marked overestimation in the proportion of AIG [21]. In this regard, it is interesting to note that the studies on ASG and AIG in the 1950s were performed on homogenized muscle extracts and have consistently reported higher proportions of ASG compared with AIG [2,3,5-7,37,38]. Moreover, recently, we compared for the first time the effect of homogenization-dependent and -independent protocols on the acid extraction of glycogen in rat muscles and showed that ASG rather than AIG is the most abundant glycogen species when extracted with a homogenization-dependent protocol [21]. However, it is important to note that ASG and AIG responses to changes in muscle glycogen concentrations were not compared between extraction protocols not only in that study [21], but also in all of the previous research on AIG and ASG.

Given that most of the studies that have examined the patterns of response of AIG and ASG to changes in muscle glycogen levels in humans have been based on homogenization-free acid extraction protocols, it is unclear to what extent the use of such protocols results in patterns of change in glycogen levels that are similar to those obtained using a homogenization-dependent protocol because, as mentioned above, such a direct comparison has never been performed before. Moreover, although we have compared the effect of these different acid extraction protocols on the proportion of ASG and AIG in rat muscles, one cannot assume that such a comparison in humans would yield similar results given that glycogen levels in human muscles are 3- to 6-folds higher than those in rats. Considering that most studies on the pattern of change in ASG and AIG levels in humans have been concerned with the breakdown and resynthesis of muscle glycogen in response to exercise and refeeding [12-15,19,20], our goal was to determine the extent to which a homogenization-dependent protocol results in findings similar to those obtained without homogenization by comparing the effect of exercise and refeeding on ASG and AIG levels using the same muscle samples. In so doing, this study reexamines the physiologic significance of the past studies on ASG and AIG.

## 2. Experimental procedures

### 2.1. Participants

Eight male participants from the student population of the University of Western Australia volunteered for the study. All were made fully aware of the experimental procedure before they gave full written consent in accordance with university ethics policy. The descriptive characteristics of the participants were as follows: age, 20.0 ± 2.4 years; weight, 84.7 ± 10.0 kg; $\dot{V}O_{2peak}$, 58.3 ± 11.2 mL/(kg min). All participants were healthy, recreationally active nonsmokers and were required to complete a physical activity readiness questionnaire [39] to ensure that they were not currently on medication or receiving treatment of any preexisting medical condition or injury. This study was approved by the Human Rights Committee of the University of Western Australia and conformed to the Declaration of Helsinki.

### 2.2. Exercise and refeeding protocol

Before testing, participants were subjected to a familiarization session with equipment and personnel. During this session, $\dot{V}O_{2peak}$ was measured and anthropometric data were collected. No earlier than 1 week after this familiarization session, participants were required to attend the laboratory for the experimental trial. On the day before testing, participants fasted overnight (minimum of 12 hours) and were also required to refrain from heavy physical activity, caffeine, and alcohol for the preceding 48 hours. On the day of testing, participants were asked to perform a 5-minute warm-up. A biopsy and blood samples were taken before cycling for 1 hour at a workload corresponding to 70% $\dot{V}O_{2peak}$. Immediately after exercise, a second biopsy and blood sample were taken. Each participant was then asked to consume for 2 hours the equivalent of 0.6 g of carbohydrate per kilogram of body mass per half hour by ingesting a 20% (wt/vol) maltodextrose solution (Polycose; Abbott Nutrition, Columbus, OH) every 30 minutes for 2 hours. This intake of carbohydrate was chosen on the basis that it has been shown to maximize the rate of muscle glycogen synthesis postexercise [40]. After this initial 2-hour intake of carbohydrates, a third biopsy and blood samples were taken; and the participants were sent home with a supply of carbohydrate and asked to restrict their food intake to that provided by us. While at home, they were required to ingest before the end of the day a total of 10 g of carbohydrate per kilogram of body mass, mainly in the form of maltodextrose. The participants were also required to keep a food record until the end of the experiment and to refrain from any physical activity, caffeine, or alcohol immediately after the testing session and for the following 24 hours. Twenty-four hours after the end of exercise protocol, participants returned to the laboratory for a fourth muscle biopsy.

All biopsies were taken from the midthigh level of the vastus lateralis from both legs (2 biopsies per leg) using an improved version [41] of the percutaneous needle biopsy technique developed by Bergström [42], with suction applied manually. Each muscle biopsy was immediately freeze-clamped in liquid nitrogen and stored at −80°C for the later enzymatic analysis of muscle glycogen content.

### 2.3. Acid extraction of muscle glycogen

Acid extraction was preformed as previously described by James and colleagues [21], with only minor changes. Briefly, a previously freeze-clamped muscle biopsy sample was broken into small pieces in a precooled mortar and then freeze-dried for 48 hours. Once dry, fat, blood, and any other visible nonmuscular connective tissue were removed from the muscle samples. Muscle samples were then placed in a preweighed 2-mL sample tube before being reweighed to determine tissue sample weights. Small pieces of freeze-dried muscle samples shredded to small strands (<1 mg each; 6 mg total) were mixed thoroughly, and part of those muscle samples were homogenized in the presence of ice-cooled 1.5 mol/L PCA (200 $\mu$L per 3 mg of sample) in a 2-mL sample tube using an IKA Labortechnik T-8 homogenizer (Staufen, Germany). Afterward, the homogenate was centrifuged at 5000 rpm for 10 minutes before the supernatant was removed; and the pellet was resuspended and rehomogenized with ice-cooled 1.5 mol/L PCA (100 $\mu$L per 3 mg of sample) in a 2-mL sample tube. After another centrifugation, the pellet was collected and supernatants were combined. Other samples of the muscles prepared above were extracted using the protocol outlined in Adamo and Graham [36] by being placed in a glass tube in the presence of 1.5 mol/L PCA. The muscle samples were pressed against the tube with a plastic rod and left to stand for 20 minutes, then centrifuged at 5000 rpm for 10 minutes before the supernatant was removed.

### 2.4. Glycogen determination

The supernatants obtained above were vortexed before a 100-$\mu$L sample was removed for the determination of ASG and a 200-$\mu$L sample for free-glucose analysis. Afterward, 2 mol/L hydrochloric acid was added to the pellet and supernatant samples. Both samples were vortexed, and tube weights were recorded. The tubes were then placed in a 90°C water bath for 2 hours to hydrolyze glycogen into glucose, with the tubes being vortexed after 1 hour to aid digestion. After incubation, the samples were vortexed; and a 400-$\mu$L aliquot was removed and neutralized by the addition of 2 mol/L potassium carbonate. The resulting extracts were assayed for glucosyl units and corrected for free glucose. For the determination of total muscle glycogen, 1 aliquot of uncentrifuged 1.5-mol/L PCA muscle extracts prepared as described above was incubated in the presence of 2 mol/L hydrochloric acid to hydrolyze glycogen; and another aliquot was used for the assay of free glucose. The resulting hydrolyzed extracts were assayed for glucosyl units and corrected for free glucose. Finally, in some samples, glycogen levels measured after acid hydrolysis were compared with glycogen determined enzymatically as described in Adamo and Graham [36].

### 2.5. Expression of results and treatment and analysis of data

All of the glycogen results are expressed as millimole glucosyl units per kilogram dry weight tissue. The results obtained from the exercise/refeeding experiment were analyzed using a 2-way analysis of variance with repeated measures with time and treatments as independent variables, followed by a Fisher least significant difference post hoc test. All analyses were performed using SPSS (Chicago, IL) version 12, and all data are presented as mean ± standard error of the mean.

## 3. Results

### 3.1. Glycogen yield of homogenization-dependent and -independent protocols

There were a positive linear relationship (Fig. 1, $r = 0.95$) and no significant difference between total glycogen determined using our homogenization-dependent protocol and that determined using a well-established homogenization-independent protocol [36], the latter of which being a protocol that has already been extensively validated against other glycogen assay methods, including those based on enzymatic digestion of glycogen [34,36]. The coefficient of variations for ASG, AIG, and total glycogen determined as described here were 4.5%, 4.0%, and 4.1%, respectively, and within the published range [36].

The proportion of ASG extracted by the homogenization-free extraction protocol adopted here was significantly lower than that achieved by our homogenization-dependent protocol (Figs. 3 and 4, $P < .05$). Although all glycogen determinations were corrected for free glucose levels, these levels were less than 1% of total glycogen as reported previously [34,43], with, for instance, resting free glucose levels of only 1.7 ± 0.6 and 2.4 ± 0.6 mmol/kg dry weight for

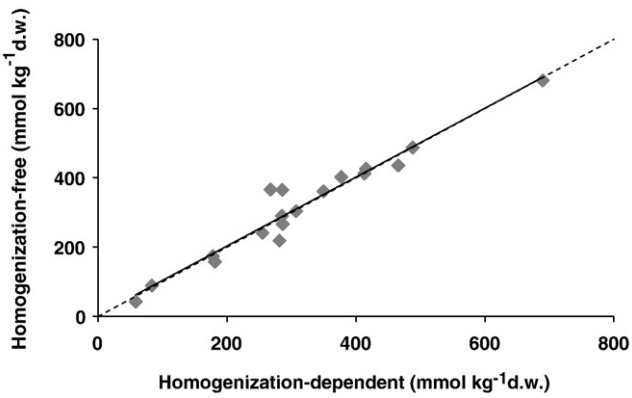


Fig. 1. Comparison of total glycogen in human muscle determined using a homogenization-free protocol and a homogenization-dependent protocol. The values shown are expressed in millimoles glucosyl units per kilogram dry tissue weight. Solid line, linear regression analysis: $y = 0.994 \times + 4.276$, $r = 0.971$, n = 18; dashed line, line of identity with slope = 1.

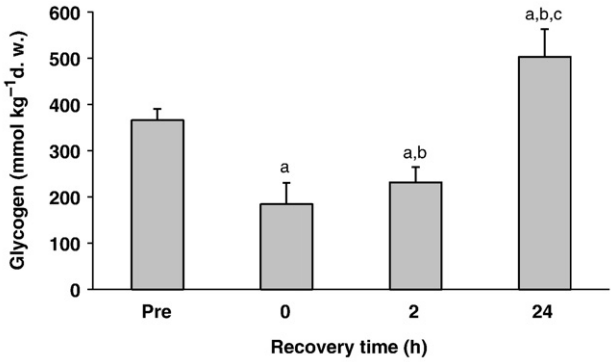


Fig. 2. Pattern of response of total muscle glycogen to exercise and recovery. The values shown represent means ± SEM (n = 8) and are expressed in millimoles glucosyl units per kilogram dry muscle. a, significantly different from preexercise ($P < .05$); b, significantly different from 0 hour ($P < .05$); c, significantly different from 2 hours ($P < .05$).

the homogenization-free and homogenization-dependent extraction protocols, respectively.

### 3.2. Effect of exercise and refeeding on ASG and AIG levels in human muscles

In response to 1 hour of exercise at an average power output of 203.7 ± 12.9 W, total glycogen levels decreased significantly (Fig. 2, $P < .05$). During the first 2 hours of recovery, glycogen concentrations increased significantly ($P < .05$), yet remained less than preexercise levels ($P < .05$). After 24 hours of recovery during which the participants ingested the equivalent of 10.9 ± 0.6 g/kg of carbohydrate, total glycogen reached levels significantly higher than those before exercise (Fig. 2; $P < .05$).

The responses of AIG and ASG to exercise were significantly different between the homogenization-free protocol and the homogenization-dependent protocols ($P < .05$). In response to exercise, there was a fall in both ASG and AIG concentrations extracted without a homogenization

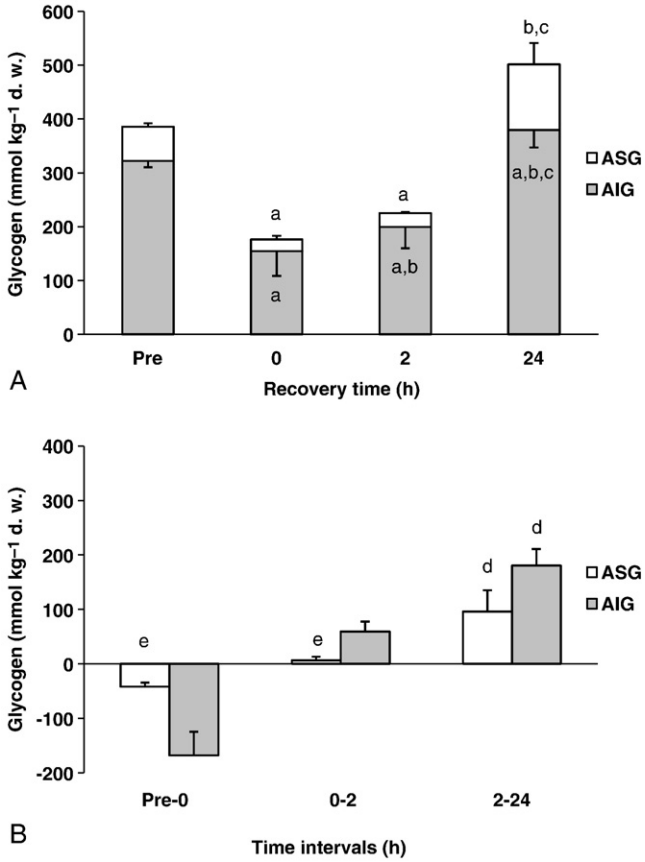


Fig. 3. Effect of exercise and recovery on (A) the pattern of response of ASG and AIG using a homogenization-free protocol and (B) changes in concentrations of ASG and AIG. The values shown represent means ± SEM (n = 8) and are expressed in millimoles glucosyl units per kilogram dry tissue weight. a, significantly different from preexercise ($P < .05$); b, significantly different from 0 hour ($P < .05$); c, significantly different from 2 hours ($P < .05$); d, significantly different from corresponding glycogen fraction at 0 to 2 hours ($P < .05$); e, significantly different from AIG of same time interval ($P < .05$).

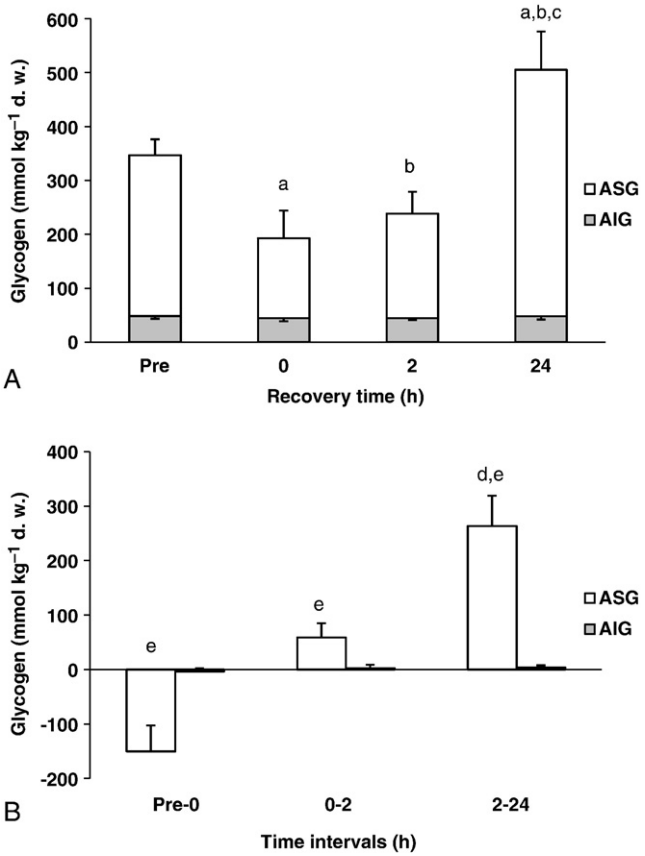


Fig. 4. Effect of exercise and recovery on (A) the pattern of response of ASG and AIG using a homogenization-dependent protocol and (B) changes in concentrations of ASG and AIG. The values shown represent means ± SEM (n = 8) and are expressed in millimoles glucosyl units per kilogram dry tissue weight. a, significantly different from preexercise ($P < .05$); b, significantly different from 0 hour ($P < .05$); c, significantly different from 2 hours ($P < .05$); d, significantly different from ASG at 0 to 2 hours ($P < .05$); e, significantly different from AIG of same time interval ($P < .05$).

step ($P < .05$), with AIG accounting for most of the fall in total glycogen (Fig. 3, $P < .05$). In contrast, ASG extracted using a homogenization-dependent extraction protocol accounted for the entire fall in total glycogen during the exercise bout ($P < .05$), whereas the AIG fraction remained at stable and low levels (Fig. 4, $P < .05$).

During the first 2 hours of recovery, the responses of AIG and ASG to refeeding were different between the 2 extraction protocols. The AIG determined using the homogenization-free extraction protocol accounted for the entire increase in total glycogen during the first 2 hours of recovery ($P < .05$), whereas ASG remained at stable and low levels (Fig. 3, $P > .05$). In contrast, using the homogenization-dependent extraction protocol, AIG remained at low and stable levels during the first 2 hours of recovery ($P > .05$), whereas the change in ASG levels accounted for the increase in total glycogen levels (Fig. 4, $P < .05$).

During the 2- to 24-hour recovery period, the responses of AIG and ASG were also affected by the protocol of glycogen extraction. The AIG and ASG determined using the homogenization-free extraction protocol contributed significantly to the increases in total glycogen (Fig. 3, $P < .05$). In contrast, using the homogenization-dependent protocol to extract glycogen, the rise in ASG concentration accounted for all the increase in total glycogen concentrations ($P < .05$), whereas the levels of AIG remained unchanged and were not significantly different from either pre- or postexercise levels (Fig. 4, $P > .05$).

## 4. Discussion

In recent years, there has been a considerable volume of research aimed at elucidating the physiologic roles of AIG and ASG in human skeletal muscle, with AIG levels shown to be higher and more responsive than ASG to changes in glycogen levels, except when total muscle glycogen levels are elevated. Unfortunately, these studies have adopted a homogenization-free protocol that might have resulted in the incomplete extraction of ASG, thereby resulting in the contamination of AIG by ASG. Here, for the first time, the effects of homogenization-dependent and -independent acid extraction protocols on the patterns of change in ASG and AIG levels in human muscles were compared. Our findings show that the use of a homogenization-free glycogen extraction protocol markedly underestimates the proportion of ASG and that, with more thorough conditions of acid extraction, most of the glycogen in human muscles is extracted as ASG rather than AIG. More importantly, ASG levels in homogenized muscle extracts account for most of the changes in total glycogen levels in response to exercise and feeding postexercise; but AIG levels account for most when a homogenization-free extraction protocol is adopted. Altogether, these findings show that the pattern of change in ASG and AIG levels in response to changes in total muscle glycogen concentrations is dependent on whether muscles are homogenized to acid-extract glycogen and raise the issue of the physiologic significance of the many studies on ASG and AIG.

Although the patterns of change in ASG and AIG levels with exercise and refeeding found here differ greatly between extraction protocols, they are consistent with those reported in previous studies. When muscle samples are extracted using a homogenization-free protocol, our results show that there is a significant decrease in both ASG and AIG levels during exercise. During the first 2 hours of recovery, the rise in AIG levels accounts for the increase in total glycogen levels, whereas ASG remains at stable levels. However, during the 2- to 24-hour period postexercise, both ASG and AIG contribute to the increase in total glycogen levels. These patterns of AIG and ASG responses to exercise and recovery are similar to those reported in a number of studies based on homogenization-free acid extraction protocols [12,14,20,23,26,28,30]. In contrast, we show here that when muscles are homogenized to extract glycogen, ASG rather than AIG accounts for all the changes in total glycogen, with AIG remaining at stable and low levels throughout both exercise and refeeding. These findings corroborate our earlier work in starved-to-fed rats and those of other studies using homogenization-dependent protocols to acid-extract glycogen, where ASG levels have been reported to be the most responsive fraction to a wide range of conditions affecting muscle glycogen levels, such as adrenaline administration, electrostimulation, starvation, and refeeding after a prolonged fast [2,3,5,6,7,21,37]. It is noteworthy that, although muscle glycogen levels in humans are much higher (3- to 6-fold) than those reported in rats [21], the concentrations of AIG extracted here with a homogenization-dependent protocol in humans are similar to those measured previously in rats using a similar protocol [21]. However, the proportion of AIG is much lower in humans because of the large excess of ASG that accounts for the large difference in total muscle glycogen levels between humans and rats.

Except when muscle glycogen levels are elevated, the low proportion of ASG obtained from glycogen extracted using a homogenization-free protocol raises the question of the factors explaining such a low but variable relative extraction yield. If the only factor determining the extraction of ASG was the size of glycogen particle relative to glycogenin, as originally proposed by Lomako et al [8], the levels and proportions of ASG and AIG should not be affected by the protocol of glycogen extraction. However, against this interpretation are the compelling evidence that AIG is not a discrete species of low molecular weight [44,45] and the recent evidence that ASG and AIG have a similar molecular weight [21]. As mentioned in an earlier study in rats [21], it is possible that the poor yield of ASG using a homogenization-free acid extraction protocol might be due to some of the glycogen precipitating not because of its poor acid solubility per se, but simply because it is trapped within the dense mesh of undisrupted myofibrils that precipitate during

centrifugation in the presence of acid. When total muscle glycogen levels are low or moderate, extracting glycogen without a homogenization step would liberate only a small proportion of the pool of ASG, with the resulting AIG contaminated by ASG accounting for most of the change in total muscle glycogen levels. However, when total glycogen increases to levels that exceed the capacity of this mesh of muscle myofibrils to trap glycogen as effectively, a disproportionate and marked rise in the release of ASG would be expected to occur with an increase in glycogen content as reported here and other studies [13,14,19,23,36]. Because our results show that there are no marked changes in ASG levels when glycogen concentrations are less than 200 mmol/kg dry weight, this suggests that the limit of the proposed capacity of myofibrils to trap glycogen is somewhere between 200 and 400 mmol/kg dry weight under our experimental conditions.

Although the above interpretation implies that the patterns of change in ASG and AIG levels obtained using a homogenization-free extraction protocol could be the result of an artifact of tissue extraction, the results obtained using such a protocol might still be highly physiologically significant. Indeed, this would be the case if the ASG and AIG fractions thus obtained and their patterns of response to changes in glycogen levels were to reflect the behaviors of distinct and labile subpopulations of glycogen that are vulnerable to homogenization-dependent extraction. For instance, because each glycogen particle binds a number of proteins including those involved in its synthesis and degradation to form a complex known as *glycosome* [46], with some of these glycosomes being associated with the sarcoplasmic reticulum and with some proteins whose binding (eg, glycogen synthase) is affected by factors such as glycogen levels [47], it is possible that AIG and ASG correspond to distinct protein/sarcoplasmic reticulum–associated glycosomes. The disruption of these structures and associated fall in protein to glycogen ratio when muscles are homogenized could result in an increase in the proportion of glycogen extracted as ASG. Another possibility is that AIG and ASG extracted without homogenization may reflect, at least in part, glycogen from different locations as suggested by the uneven distribution of glycogen between the subsarcolemmal compartment, the intra- and intermyofibrillar spaces, and the newly discovered intracellular cytoskeleton-associated compartment [47] where glycogen differs in concentration and is metabolized at different rates [23]. Clearly, more work is required to explain the factors determining the pattern of AIG and ASG extraction obtained under conditions where muscles are not subjected to a homogenization step to assess the physiologic significance of these glycogen fractions and explain their responses to homogenization.

Under conditions where muscle glycogen is extracted using a homogenization-dependent protocol, the low levels and absence of change in AIG levels raise the question of the factors underlying the behavior of this glycogen pool. One possibility is that AIG is a less metabolically active and responsive pool of glycogen. In this respect, glycogen levels in the subsarcolemmal space was reported to be unresponsive to exercise of submaximal intensity [48], thus suggesting that AIG corresponds to this glycogen fraction. Against this interpretation, however, is the recent work of Marchand and colleagues [23] that showed that glycogen levels in this and other cellular compartments in skeletal muscles are markedly depleted in response to exercise of submaximal intensity. Another possibility is that AIG corresponds, at least in part, to the fraction of muscle glycogen entrapped inside lysosomes [49]. Indeed, the fact that lysosomal glycogen is not metabolized by glycogen phosphorylase or synthase [50] might explain, in part, the absence of rapid changes in the levels of AIG in response to exercise and refeeding. The problem with this interpretation, however, is that the work of Huang and colleagues [51] shows using isotopically labeled glucose in rats that AIG obtained from homogenized extracts is a highly metabolically active pool of glycogen [51] and is thus unlikely to represent lysosomal glycogen. Their findings also highlight the very important point that the absence of net changes in AIG levels does not exclude the possibility that the turnover rate of this glycogen fraction might be elevated, with AIG synthesis and breakdown occurring at similar and high rates. Finally, as discussed above, AIG obtained after homogenization may also represent a distinct metabolically active subfraction of glycosomes with a unique complement of proteins and cellular location compared not only to ASG but also to AIG obtained without a homogenization step. Clearly, more work is required not only to elucidate the structural differences between ASG and AIG obtained from homogenized muscle extracts, but also to determine the physiologic significance of all findings based on such an extraction protocol.

In conclusion, this study corroborates earlier findings in rats that the protocol used to acid-extract muscle glycogen affects the proportion of AIG and ASG. Surprisingly, however, our findings show that despite large differences in muscle glycogen levels between rats and humans, they carry comparable levels of AIG, with ASG levels accounting for their differences in muscle glycogen content. In addition, this study compares for the first time the effect of these extraction protocols on the patterns of response of AIG and ASG to changes in glycogen concentrations and shows that these patterns are highly dependent on the protocol used to acid-extract glycogen. Furthermore, it highlights the fact that, although the findings of the many studies on ASG and AIG could be physiologically meaningful, none of these studies including this one has excluded the possibility that their reported patterns of change in AIG and ASG levels could be the result of an artifact of tissue extraction. Clearly, more work is required to elucidate the mechanisms underlying the acid solubility of muscle glycogen across extraction conditions (with or without homogenization) to establish once and for all the physiologic significance of the findings of the large number of studies performed since the start of last century on AIG and ASG.

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