No limiting role for glycogenin in determining maximal attainable glycogen levels in rat skeletal muscle

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Hansen, Bo Falck, Wim Derave, Pia Jensen, and Erik A. Richter. No limiting role for glycogenin in determining maximal attainable glycogen levels in rat skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 278: E398–E404, 2000.—We examined whether the protein level and/or activity of glycogenin, the protein core upon which glycogen is synthesized, is limiting for maximal attainable glycogen levels in rat skeletal muscle. Glycogenin activity was 27.5 ± 1.4, 34.7 ± 1.7, and 39.7 ± 1.3 mU/mg protein in white gastrocnemius, red gastrocnemius, and soleus muscles, respectively. A similar fiber type dependency of glycogenin protein levels was seen. Neither glycogenin protein level nor the activity of glycogenin correlated with previously determined maximal attainable glycogen levels, which were 69.3 ± 5.8, 137.4 ± 10.1, and 80.0 ± 5.4 µmol/g wet wt in white gastrocnemius, red gastrocnemius, and soleus muscles, respectively. In additional experiments, rats were exercise trained by swimming, which resulted in a significant increase in the maximal attainable glycogen levels in soleus muscles (−25%).

The fiber type study. Fed male Wistar rats (≈210 g) from Møllegård (Lille Skensved, Denmark) were anesthetized by a brief exposure to CO2 and killed by cervical dislocation. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

β-particle has a molecular weight of ~1·107, which means that the molecular weight of glycogenin is 0.35% of the β-particle. Interestingly, when the protein content of glycogen was measured, it came to exactly that number: 0.35%, suggesting that glycogenin and β-particles exist in a 1:1 stoichiometric relationship (14, 28). Thus it follows that there is an average of one glycogen synthase catalytic subunit bound to each glycogen molecule (32). In skeletal muscle it was observed that almost all of the glycogenin was bound covalently to the carbohydrate moiety and that no reservoir of free glycogenin molecules exist (17, 33). Therefore, the amount of glycogenin present in muscle could be the factor ultimately limiting glycogen deposition, because glycogen synthesis would stop if all glycogenin molecules were saturated (32).

Skurat et al. failed to observe an increase in total cell glycogen in COS (31) or rat 1 fibroblasts (30) overexpressing glycogenin. However, in these studies cells were only stimulated with glucose, not with insulin, and cells were only studied at a single time point. Therefore, in these studies it is unknown whether the maximal glycogen level was reached. In fact, a small but significant increase in maximal attainable glycogen level was observed in L6 cells overexpressing glycogenin (Hansen, unpublished observations). Nevertheless, neither of these studies allows any firm conclusions with respect to skeletal muscle, because major differences exist between cultured cells and skeletal muscle.

Thus the purpose of this study was to investigate whether maximal attainable glycogen levels in skeletal muscle are determined by the protein level and/or activity of glycogenin. For this purpose we studied glycogenin protein levels and activity in different fiber types and in soleus muscles after exercise training. Furthermore, we examined the proportion of pro- and macroglycogen in skeletal muscles from rats with very different levels of glycogen.

MATERIALS AND METHODS

All experiments were approved by the Danish Animal Experiments Inspectorate and complied with the “European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes” (Council of Europe no. 123, Strasbourg, France, 1985).

The fiber type study. Fed male Wistar rats (~210 g) from Møllegård (Lille Skensved, Denmark) were anesthetized by a brief exposure to CO2 and killed by cervical dislocation. The superficial part of the white gastrocnemius muscle (WG),
which consists mainly of fast-twitch white (FTW) fibers (4), was cut off and freeze-damped. Then the soleus muscle (SOL), which consists mainly of slow-twitch red (STR) fibers (4), was reflected and clamped. Finally, a portion of the deep part of the medial head of the red gastrocnemius (RG), consisting mainly of fast-twitch red (FTR) fibers (4), was cut out and clamped. Muscles were stored at −80°C until analyzed.

Glycogen loading in soleus muscles. In preparation for the training study, rat soleus muscles were incubated in high glucose and insulin concentrations for <6 h to determine the time course for saturation of glycogen stores. Male Wistar rats (~50 g) from Møllegård were anesthetized by a brief exposure to CO₂ and killed by cervical dislocation. Soleus muscles were quickly removed (~1 min) and placed in 2 ml (31°C) of a pregassed (95% O₂-5% CO₂) Krebs Henseleit (KRH) buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 0.1% BSA, and 5 mM HEPES) supplemented with 0.15 mM pyruvate, amino acids at concentrations corresponding to DMEM (13), and 25 mM mannitol. The muscles were preincubated for 30 min to equilibrate. At time 0 muscles were changed to a new identical KRH buffer now containing high insulin (60 nM) and glucose (20 mM) concentrations, as well as 5 mM mannitol, and incubated for 0–6 h. Throughout the incubation, vials were gassed with 95% O₂-5% CO₂. The medium was replaced with a fresh one every hour. At time 0 and 1, 2, 3, 4, 5, and 6 h, muscles were quickly freeze-damped between tongs cooled in liquid nitrogen. All muscles were kept at −80°C until analyzed.

Glycogen synthesis rate was measured in 1-h intervals as follows. At 0–1 h, 1–2 h, 2–3 h, 3–4 h, 4–5 h, and 5–6 h, −0.5 μCi ([1-⁴⁰C]glucose was added to the incubation medium at the beginning of each interval. At the end of the 1-h exposure to labeled glucose, muscles were quickly freeze-damped and weighed. Biopsies were boiled in 1 ml NaOH (1 N) for 30 min, and glycogen precipitated overnight in ethanol (−20°C) after addition of unlabeled glycogen (0.35 μg/μl). After centrifugation for 15 min at 2,500 g, the glycogen pellet was washed once in ice-cold ethanol, and finally the glycogen pellet was dissolved in water and counted in a β-counter (Packard, TRI-CARB 1500).

The training study. Male Wistar rats (50 g) were purchased from Panum Institute Breeding Centre (Copenhagen University, Copenhagen, Denmark) and randomly assigned to either a sedentary control group or a training group. The rats were kept in cages (4 rats/cage) measuring 30 × 45 × 25 cm in a temperature (21°C)-controlled room with a 12:12-h light-dark cycle. The rats had free access to water and rat chow. The rats assigned to the exercise training were acclimated to swimming by swimming in water maintained at 35 ± 1°C for 10 min the 1st day, 1 h the 2nd day, and 3 h the 3rd day. From days 4–8 (5 days in total), the rats swam 2 times for 3 h separated by a 1-h rest period per day. During the rest period, rats were towel dried, kept warm, and given food and water. The rats in both groups were weighed before and after the training period. A similar training regimen has previously been demonstrated to induce pronounced effects on glucose metabolism in skeletal muscle (23, 24). After the last training session, rats were allowed to rest for ≥40 h before experiments started.

At 40–45 h after the last training session, rats in the fed state were anesthetized by a brief exposure to CO₂ and were killed by cervical dislocation. Soleus muscles were incubated exactly as described above. However, because the rats weighed between 76 and 123 g (mean 96.0 ± 2.0 g) after the exercise training, it was necessary to split the soleus muscle longitudi-
glycogenin protein level. Equal amounts of protein (150 µg) were subjected to amylolysis for 1 h at 37°C with α-amylase (Sigma, final concentration 10 µg/ml). Subsequently, the activity of glycogenin was measured. The incubation mixture contained the following components in a final volume of 60 µl: 8 µM UDP-[14C]glucose (287 mCi/mmol, NEN), 17 mM MES (pH 7.0), 5 mM MnSO₄, 0.2 mM n-dodecyl β-D-maltoside (DBM; Sigma), and 50 µl homogenate (150 µg protein). The glucosylation was allowed to proceed for 10 min at 30°C, and the reaction was terminated by addition of 16 µl of 0.1 M EDTA. The samples were made 1 mM in glucose and 2 mM in cold UDP-glucose in a final volume of 200 µl. Total radioactivity was measured in 10 of these 200-µl samples, and the remainder were passed through a prewashed C₁₈ cartridge (Waters). The cartridges were washed with 10 ml of water, and the 14C-labeled glucosylated DBM was eluted with 3 times 1 ml methanol. The three fractions per sample were counted (Packard, TRI-CARB 1500) individually after addition of 10 ml scintillation liquid (Ultima Gold). The majority (>95%) of the radioactivity was recovered in the first two fractions, indicating that all glucosylated DBM was eluted from the cartridges. One unit of activity is defined as 1 nmol of [14C]glucose incorporated into DBM per minute (8).

Statistics. Statistical evaluation of the data was done by unpaired t-tests or one-way ANOVA by use of the Student-Newman-Keuls method for post hoc multiple comparisons, where appropriate. Data shown in Fig. 6 were fitted using the SigmaPlot for Windows version 4.0 (SSPS). Data are presented as means ± SE, and the level of significance was chosen at 0.05.

RESULTS

Glycogenin activity was found to be significantly different in the three fiber types (P < 0.001). The glycogenin activity was highest in STR fibers, lowest in FTW fibers, and intermediate in FTR fibers (Fig. 1). A quite similar fiber type dependency of glycogenin protein levels was seen (ANOVA, P < 0.05, Fig. 1).

Initial experiments showed that glycogen levels in SOL muscle could be saturated within 5 h if incubated in a medium containing 20 mM glucose and 60 nM insulin (data not shown). At the same time as glycogen stores were saturated, glycogen synthesis rate decreased to 20% of the initial value (Fig. 2). Therefore, SOL muscles from exercise-trained and untrained rats were incubated for 5 h under these conditions (Fig. 3). In both trained and untrained muscles, glycogen levels were found to be saturated within 5 h, because no significant increase was seen from 4 to 5 h. Analysis of variance (ANOVA) revealed a significant overall difference in glycogen between the two groups (P < 0.001). Despite this increase in glycogen deposition in trained muscle, no increase in glycogenin protein level or glycogenin activity could be observed (Fig. 4).

The concentrations of proglycogen and macroglycogen in the fed state in the three different fiber types are shown in Fig. 5A. In all three fiber types, most glycogen was found to be present as proglycogen. A fiber type dependency of the proglycogen level was observed. Interestingly, the proglycogen-to-macroglycogen ratio was found to be ~8 in all three fiber types (Fig. 5B). The relative proportion of proglycogen and macroglycogen was dependent on the total glycogen concentration (Fig. 6). Thus, in muscles with a low total glycogen concentration, almost all glycogen was present as proglycogen. The relative proportion of proglycogen decreased from values close to 100% down to ~30% as the total glycogen concentration increased. Interestingly, this relationship between the relative proportion of proglycogen (or macroglycogen) and total glycogen seems to be independent of fiber type (Fig. 6). Furthermore, it should be noted that, despite a decrease in the relative proportion of proglycogen, an increase in the
absolute proglycogen concentration was in fact observed as total glycogen increased.

DISCUSSION

This study shows that the protein level and the activity of glycogenin are significantly different in the three major muscle fiber types (Fig. 1). Previously we showed that maximal glycogen levels were reached within 5 h in rat skeletal muscle when perfused with 11–13 mM glucose and insulin concentrations of maximal stimulation (25). The maximal attainable glycogen levels were found to be 137.4 ± 10.1, 80.0 ± 5.4, and 69.3 ± 5.8 µmol/g wet wt in FTR, STR, and FTW fibers, respectively (25). Thus there seems to be a poor correlation between maximal attainable glycogen levels during insulin stimulation and the protein level/activity of glycogenin in the three different fiber types.

Exercise training is normally associated with increased glycogen levels in muscle (12, 26). However, from these studies it is not possible to conclude that exercise training increases maximal attainable glycogen levels, because it is unknown whether maximal glycogen levels were in fact reached. Therefore, we incubated trained and untrained soleus muscles in high glucose and insulin concentrations for 5 h to saturate glycogen stores. Here we show that even short-term exercise training (5 days) does in fact increase maximal attainable glycogen levels (Fig. 3). Despite this increase in maximal glycogen levels, we

Fig. 3. Glycogen accumulation in trained and untrained rat soleus muscles. Male Wistar rats (50 g) were either trained for 6 h/day for 5 days by swimming (trained) or allowed to rest (sedentary). After the last training session, rats were allowed to rest for 40–45 h before experiments began. Soleus muscles were incubated for 5 h in a medium containing high insulin (60 nM) and glucose (20 mM). At t = 1, 2, 4, and 5 h, muscles were quickly freeze-clamped between tongs cooled in liquid nitrogen. Samples of fresh muscles were also collected (time 0). Values are means ± SE, n = 6.

Fig. 4. Glycogenin activity and expression in trained and untrained rat soleus muscles. See legend to Fig. 1. Values are means ± SE; n = 7 (untrained) and n = 9 (trained).

Fig. 5. A: proglycogen and macroglycogen in muscles representing the 3 different rat muscle fiber types. B: proglycogen-to-macroglycogen ratio for the 3 fiber types. Values are means ± SE, n = 8.
failed to observe any increase in glycogenin protein level and activity. In fact, a small decrease in glycogenin activity was seen in trained muscle (Fig. 4). Thus, from the study of glycogenin protein level and activity in different rat muscle fiber types as well as in trained vs. untrained rat soleus muscle, it is suggested that glycogenin does not play a direct role for maximal attainable glycogen levels.

This conclusion is further supported by the study of relative proportions of proglycogen and macroglycogen in skeletal muscle. In the present study, an ~8:1 glycogen by weight pro/macro ratio was found in all three fiber types in the normal fed state (Fig. 5), which corresponds nicely to the values obtained by Adamo and Graham (1) in mixed human and rat muscle. The comparable pro/macro glycogen ratio found in the three fiber types, despite quite different levels of maximal attainable glycogen levels, indicates that glycogen synthesis stops before all proglycogen is converted to macroglycogen. This is further supported by the relationship between total glycogen and the relative proportions of proglycogen and macroglycogen (Fig. 6). Macroglycogen accounted for a minor portion of total glycogen at normal glycogen levels (~30 µmol/g wet wt), but the proportion of macroglycogen increased gradually. However, the proportion of macroglycogen never exceeded 70% of the total glycogen, even if total glycogen levels further increased from 100 to 170 µmol/g wet wt. So, even at very high glycogen levels, there is still up to 30% of the total glycogen that is in the proglycogen form, and the absolute proglycogen concentrations showed no sign of a decrease. Similar results were obtained by Adamo and colleagues (1, 2). In other words, even at very high glycogen concentrations, a considerable number of the glycogen particles are still unsaturated.

In the normal fed resting state, we found an ~8:1 glycogen by weight pro/macro ratio in all three fiber types. With the assumption of a molecular mass of 400,000 Da for proglycogen and 107 Da for macroglycogen (3), it can be calculated that, if all the remaining proglycogen were to be converted to macroglycogen, the glycogen levels would theoretically be increased ~20-fold to ~600 µmol/g wet wt. Interestingly, when the maximal glycogen concentration is estimated (when macroglycogen equals 100%) from the fit of the data on Fig. 6, a value of ~700 µmol/g wet wt is obtained. Therefore, we believe that the theoretical maximal glycogen concentration in rat skeletal muscle is ~600–700 µmol/g wet wt, which is three- to fourfold higher than the highest observed values (25). Thus, from the data in this study, it seems safe to conclude that glycogenin does not play a limiting role in determining maximal glycogen levels in rat skeletal muscle, because glycogen synthesis stops before all glycogen particles are saturated.

According to the model proposed by Alonso et al. (3), glycogen exists in various stages between proglycogen (400 kDa) and macroglycogen (107 Da). Furthermore, proglycogen is proposed to be a stable intermediate in the synthesis of macroglycogen, and it is never broken down to glycogenin (3). If it is assumed that the number of glycogenin molecules is constant, another interesting finding can be gained from this study, that the average size of proglycogen molecules increases with increasing total glycogen levels, because the absolute proglycogen concentration in fact increases. Thus the term proglycogen does not represent a distinct fraction (400 kDa) of the glycogen pool. If the theoretical maximal glycogen concentration is 700 µmol/g wet wt (corresponding to 12.6 mmol/g glycogenin), it can be calculated from the fit that, at normal fed glycogen levels (20–30 µmol/g wet wt), the average size of proglycogen molecules should be ~270 kDa, whereas the average size increases to ~575 kDa at a total glycogen of 90 µmol/g wet wt. At 650 µmol/g wet wt (just before the very last proglycogen molecules are converted into macroglycogen), the average size of proglycogen is 940 kDa, indicating that the critical size of proglycogen is 1,000 kDa, corresponding to a protein content of ~4%.

If glycogenin is not the factor ultimately limiting further synthesis of glycogen, despite ongoing stimulation with insulin and glucose, it must be assumed that some process during the storage of glycogen induces a state of insulin resistance responsible for the downregulation of glycogen synthesis. These processes could be partly responsible for the insulin resistance seen in type II diabetes. Alternatively, it could be speculated that glycogen breakdown was accelerated, which limited further increase in the glycogen levels despite the ongoing glycogen synthesis.
glycogen stores saturate, suggests that synthesis was in fact decreased (Fig. 2). Furthermore, glycogen synthase (GS) activity was found to be severely decreased in all three fiber types after 5 h of rat hindquarter perfusion with moderately high glucose (11–13 mM) and maximal stimulations of insulin (25). Therefore, we believe that prolonged exposure to high glucose and insulin induces a state of insulin resistance to glycogen synthesis in skeletal muscle.

Numerous studies have shown that, as glycogen stores increase after glycogen-lowering exercise, GS activity and glycogen synthesis rate decrease (5, 6, 9–11, 15, 19, 22, 29, 34). Furthermore, a significant negative correlation between GS activity and the glycogen concentration has been demonstrated (5, 6, 9, 10, 19, 34). This negative relationship between glycogen levels and glycogen synthesis was also found when glycogen levels were increased above normal values by stimulation with insulin and glucose (25; and Hansen, unpublished observations). Although these findings do not prove a direct role of glycogen in determining glycogen synthesis, they do suggest it. How such a mechanism might operate can only be speculated on at the present time, but it may be related to the binding of glycogen-metabolizing enzymes to the glycogen molecules. One hypothesis could be that the activity of GS is under the influence of the interaction with glycogenin. If GS activity is high only so long as glycogenin and GS are bound to each other, that circumstance could explain why GS activity decreases as the glycogen particle grows. Although there is no evidence for such a mechanism at the present time, it is intriguing that, in vitro, it was shown that GS only efficiently elongated the primer if complexed to glycogenin (33) and that the GS activity with respect to proglycogen and macroglycogen appears to be different (2, 18).

In summary, it was found in rat skeletal muscle that the protein level and activity of glycogenin correlated poorly with the maximal attainable glycogen level. In addition, a training-induced increase in maximal glycogen levels was not accompanied by an increase in either glycogenin protein levels or activity. Finally, even at very high glycogen levels, a considerable number of the glycogen particles are still unsaturated. Thus, from the data in this study, it seems safe to conclude that glycogenin does not play a limiting role in determining maximal glycogen levels in rat skeletal muscle.

The authors thank Betina Bolmgreen and Irene Bech Nielsen for expert technical assistance. The present study was supported partly by Grant 504–14 from the Danish National Research Foundation.

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Received 8 March 1999; accepted in final form 13 October 1999.

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