

The Experimental Type 2 Diabetes Therapy Glycogen Phosphorylase Inhibition Can Impair Aerobic Muscle Function During Prolonged Contraction

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Glycogen phosphorylase inhibition represents a promising strategy to suppress inappropriate hepatic glucose output, while muscle glycogen is a major source of fuel during contraction. Glycogen phosphorylase inhibitors (GPI) currently being investigated for the treatment of type 2 diabetes do not demonstrate hepatic versus muscle glycogen phosphorylase isoform selectivity and may therefore impair patient aerobic exercise capabilities. Skeletal muscle energy metabolism and function are not impaired by GPI during high-intensity contraction in rat skeletal muscle; however, it is unknown whether glycogen phosphorylase inhibitors would impair function during prolonged lower-intensity contraction. Utilizing a novel red cell-perfused rodent gastrocnemius-plantaris-soleus system, muscle was pretreated for 60 min with either 3 $\mu\text{mol/l}$ free drug GPI ($n = 8$) or vehicle control ($n = 7$). During 60 min of aerobic contraction, GPI treatment resulted in $\sim 35\%$ greater fatigue. Muscle glycogen phosphorylase a form ($P < 0.01$) and maximal activity ($P < 0.01$) were reduced in the GPI group, and postcontraction glycogen (121.8 ± 16.1 vs. 168.3 ± 8.5 mmol/kg dry muscle, $P < 0.05$) was greater. Furthermore, lower muscle lactate efflux and glucose uptake ($P < 0.01$), yet higher muscle V_{O_2} , support the conclusion that carbohydrate utilization was impaired during contraction. Our data provide new confirmation that muscle glycogen plays an essential role during submaximal contraction. Given the critical role of exercise prescription in the treatment of type 2 diabetes, it will be important to monitor endurance capacity during the clinical evaluation of nonselective GPI. Alternatively, greater effort should be devoted toward the discovery of hepatic-selective GPI, hepatic-specific drug delivery strategies, and/or alternative strategies for controlling excess hepatic glucose production in type 2 diabetes. *Diabetes* 55:1855–1861, 2006

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Received for publication 28 December 2005 and accepted in revised form 13 March 2006.

GPI, glycogen phosphorylase inhibitors; GPS, gastrocnemius-plantaris-soleus; HMP, hexosemonophosphate; QOL, quality of life.

DOI: 10.2337/db05-1687

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Development of novel treatments for type 2 diabetes remains an extremely challenging process. Research strategies aim to enhance peripheral glucose uptake from the bloodstream, suppress inappropriately high glucose output from the liver, or prevent the inevitable decline in pancreatic β -cell mass/function. Pharmacological inhibition of excessive glycogen phosphorylase activity is one strategy that has been pursued for the development of new antidiabetic agents (1). Inhibition of glycogen phosphorylase activity reduces glucose production in cell culture (2) and in murine models of type 2 diabetes (1,3,4). Compensating for a failure of insulin's action on hepatic glucose output may be a more logical strategy than stimulating muscle glucose transport (5,6) because a drug promoting muscle glycogen storage is likely to produce a rather short-lived effect in characteristically inactive patients (7), since unused glycogen would give negative feedback on glucose transport or potentially lead to muscle pathology (8). Given the variety of potential approaches aimed at improving regulation of hepatic glucose metabolism (9,10), there is a need to carefully consider the various drug discovery strategies targeting this aspect of insulin resistance.

One of the merits of the glycogen phosphorylase inhibition approach is that certain glycogen phosphorylase inhibitors (GPI) have been shown to be more potent at reducing hepatic glucose output in the presence of high glucose concentrations. Thus, as blood glucose concentration diminishes, compound potency is attenuated, and the patient is better protected from periods of hypoglycemia (1,11). In addition to this valuable property, certain GPI also demonstrate cardioprotective effects (12) and thus demonstrate an attractive clinical profile for a patient population that is at risk for cardiovascular disease. In recent years, patient quality of life (QOL) has been emphasized as being an important consideration for any new therapy (13). It is widely accepted that any new chronic therapy should, at worst, be neutral for QOL and that adequate skeletal muscle function is an essential component of factors that determine QOL (14).

In humans, muscle glycogen stores represent an essential energy source during submaximal exercise, and depletion of muscle glycogen stores promotes muscle fatigue (15–18). Due to the close homology between the liver and skeletal muscle glycogen phosphorylase isoenzymes, the available GPI do not demonstrate tissue specificity (1,3,19) and thus may impair glycogen mobilization in skeletal muscle. Despite this, we recently demonstrated that dur-

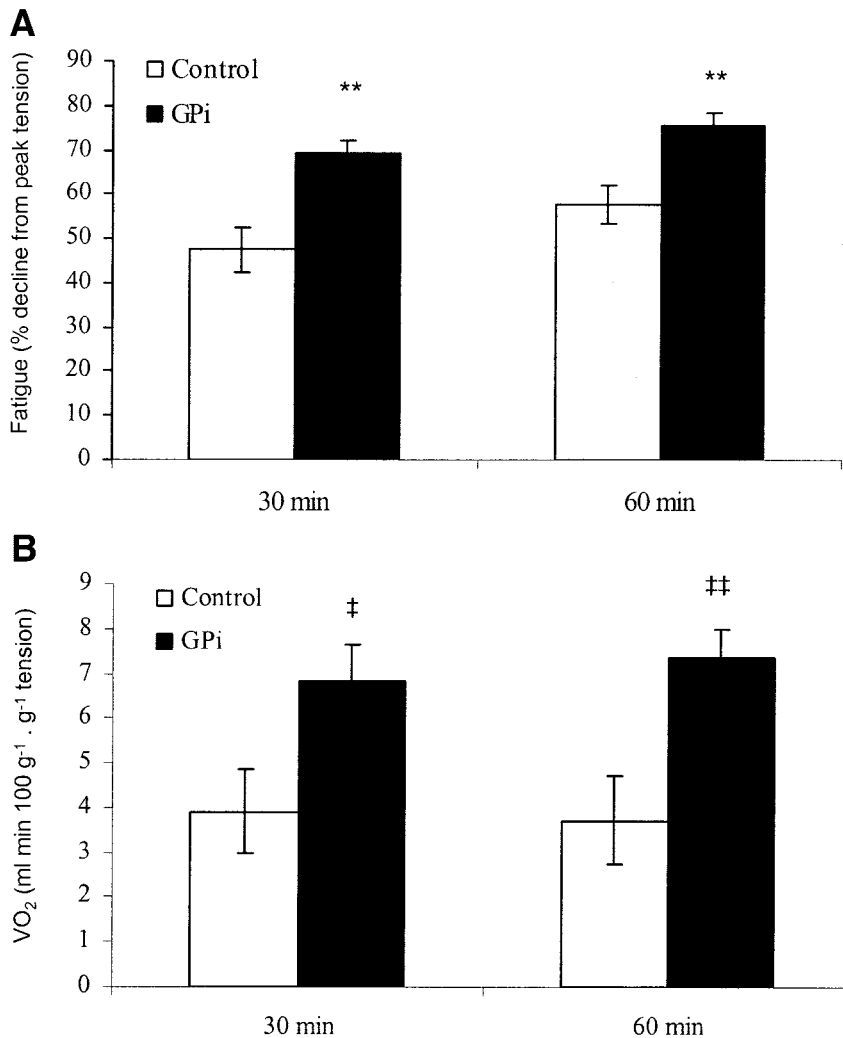


FIG. 1. Muscle function during 60 min of aerobic endurance contraction. Data are means \pm SE. **A:** Percent decline in muscle fatigue from peak tension. *Different from control ($P < 0.05$). **B:** Muscle oxygen consumption (V_{O_2}) normalized to muscle tension after 30 and 60 min of contraction in control and GPI-treated groups. Values are means \pm SE ($\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g tissue}^{-1} \cdot \text{g of tension}^{-1}$). ‡Significantly different from corresponding control value; $P < 0.05$. ‡‡Significantly different from corresponding control value; $P < 0.01$.

ing short maximal contractions and high-intensity submaximal contraction, muscle function and energy metabolism were not impaired by the GPI, CP-316819, in an in situ rat hind-limb perfusion model (20). This indicates that acute, high-intensity exercise capacity is likely to be unimpeded in type 2 diabetic patients treated with a GPI, an (acute) observation that would be easy to verify during a clinical trial. However, we did note that muscle lactate production was reduced following GPI administration, indicating some inhibition of muscle glycogenolysis. While we hypothesized that endogenous activation of glycogen phosphorylase by calcium and adenine nucleotides during intense muscle contraction overcomes the action of CP-316819 (20), this may not occur during lower-intensity exercise, where glycogen phosphorylase inhibition could be sufficient to impair skeletal muscle function. This is an important issue, as patients with type 2 diabetes are encouraged to undertake aerobic exercise training (21). Our aim, therefore, was to characterize the effect of a representative GPI, CP-316819, on muscle function, energy metabolism, and glycogen phosphorylase activation status during prolonged submaximal contraction. The information generated could be used to facilitate clinical trial design for this category of type 2 diabetes therapy and to influence selection of the most promising strategy aimed at suppressing excess hepatic glucose output.

RESULTS

Muscle function. Peak tension development occurred during the first minute of contraction and was similar between control- and GPI-treated groups (3.4 ± 0.4 vs. 3.5 ± 0.6 kg/100 g wet muscle mass, respectively). Figure 1A demonstrates that after 30 min of contraction, muscle fatigue (defined as percent decline from peak tension) was $47.5 \pm 4.9\%$ in the control group, and this was significantly less than that observed in the GPI-treated group ($69.5 \pm 2.8\%$, $P < 0.05$). By 60 min, muscle fatigue was $57.9 \pm 4.2\%$ in the control group, while the GPI-treated group demonstrated substantially more fatigue ($75.6 \pm 2.8\%$, $P < 0.01$). Muscle force production was impaired by CP-316819, yet the muscle consumed significantly more oxygen (Fig. 1B) **Glycogen phosphorylase activity and muscle metabolism.** Figure 2 presents the muscle glycogen phosphorylase a form and the in vitro AMP-mediated activation status for each treatment group at the end of contraction. After 60 min of submaximal intensity contraction, muscle a form activity was $\sim 70\%$ lower in the GPI-treated group compared with control ($P < 0.01$). Furthermore, in vitro maximal glycogen phosphorylase activity, measured in the presence of AMP, was reduced by $\sim 40\%$ following GPI treatment compared with control ($P < 0.01$). This implies that greater compound delivery was achieved with the longer experimental protocol. Figure 3A demonstrates

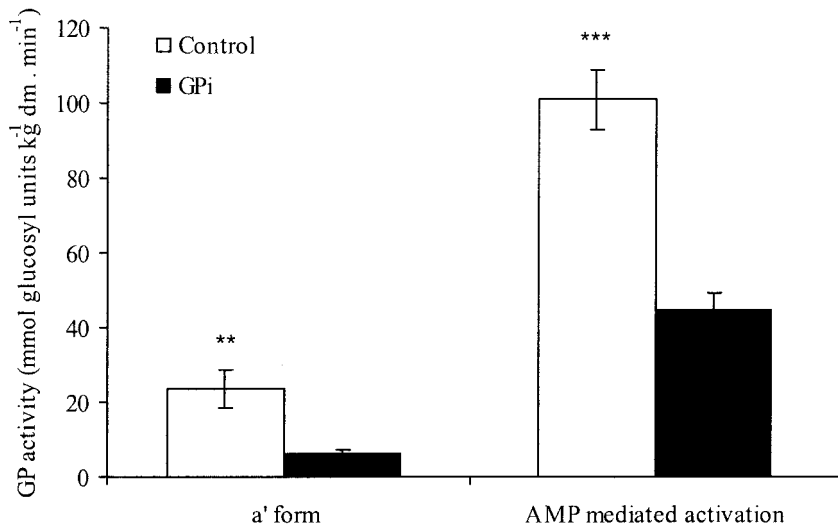


FIG. 2. Ex vivo glycogen phosphorylase activity and transformation. Data are means \pm SE of glycogen phosphorylase (GP) activation status (mmol glucosyl units \cdot kg dry muscle⁻¹ \cdot min⁻¹). ** $P < 0.01$ and *** $P < 0.001$ different from control.

that muscle lactate efflux in the GPi-treated group was approximately threefold lower than that recorded in the control group after 15 min of submaximal intensity contraction and remained lower thereafter ($P < 0.01$). Similarly, muscle glucose uptake was also reduced throughout contraction in the GPi-treated group (Fig. 3B). These

responses may be secondary to the impairment of muscle function or be a direct consequence of GPi.

We have previously demonstrated that 60 min of exposure of muscle to CP-316819 in this model does not alter resting skeletal muscle metabolite and substrate concentrations (20). Table 1 presents muscle metabolite and

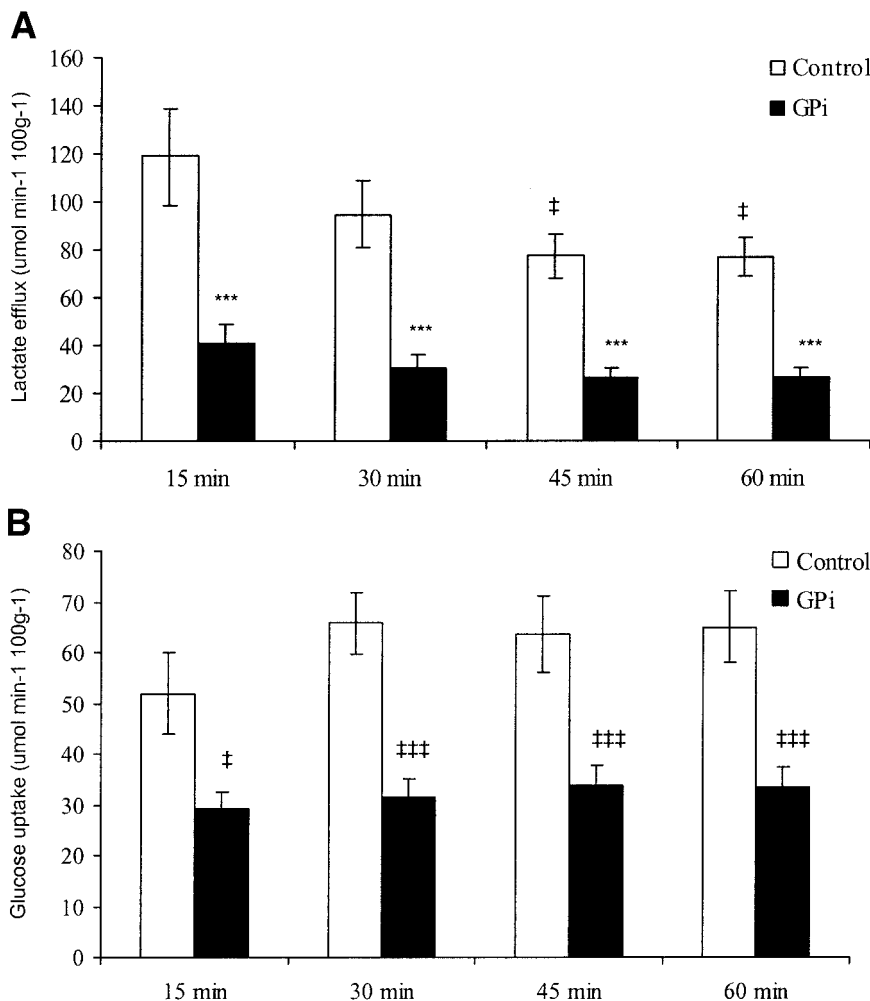


FIG. 3. Muscle glucose and lactate exchange during contraction. Data are means \pm SE. A: Muscle lactate efflux after 15, 30, 45, and 60 min of contraction for control and GPi-treated muscles ($\mu\text{mol} \cdot \text{min}^{-1} \cdot 100 \text{ g wet muscle}^{-1}$). B: Muscle glucose uptake after 15, 30, 45, and 60 min of contraction for control and GPi-treated muscles. *** $P < 0.001$ different from control. † $P < 0.05$ different from 15-min value within treatment group. ‡Significantly different from corresponding control value, $P < 0.05$. §§Significantly different from corresponding control value, $P < 0.001$.

TABLE 1
Metabolite concentrations in skeletal muscle after 60 min of contraction

	Control	GPI
<i>n</i>	7	8
ATP	28.4 ± 1.1	28.8 ± 0.8
ADP	3.8 ± 0.4	3.8 ± 0.2
AMP	0.2 ± 0.1	0.2 ± 0.1
IMP	1.2 ± 0.4	1.2 ± 0.5
INO	3.2 ± 0.1	2.99 ± 0.3
HYP	0.06 ± 0.01	0.06 ± 0.01
XAN	0.3 ± 0.03	0.3 ± 0.1
Uric acid	1.2 ± 0.2	1.1 ± 0.1
Phosphocreatine	54.9 ± 6.1	56.2 ± 5.0
Total creatine	102.3 ± 2.7	95.9 ± 2.0
HMP	3.3 ± 0.5	2.1 ± 0.2*
Glycogen	121.8 ± 16.1	168.3 ± 8.5*
Lactate	45.6 ± 10.2	30.1 ± 4.0

Data are means ± SE (mmol/kg dry muscle). *Significantly different from control value using an unpaired *t* test; *P* < 0.05. HYP, hypoxanthine; IMP, inosine monophosphate; INO, inosine; XAN, xanthine.

substrate concentrations determined at the end of 60 min of contraction. Muscle adenine nucleotides and the breakdown products (ATP, ADP, AMP, inosine monophosphate, inosine, hypoxanthine, xanthine, and uric acid) were not different between treatments following 60 min of submaximal contraction. Similarly, muscle phosphocreatine and total creatine did not differ between treatment groups. Muscle glycogen content was ~35% greater in the GPI-treated group compared with control (*P* < 0.05), and, consistent with this reduction in glycogenolysis during contraction, muscle hexosemonophosphate (HMP) content was ~35% lower in the GPI-treated group compared with control (*P* < 0.05). Accordingly, muscle lactate content in the GPI-treated group was ~30% lower than in the control group; however, this did not reach statistical significance (probably reflecting the time course of peak muscle lactate production and efflux).

DISCUSSION

While the molecular mechanisms underpinning the benefits of exercise therapy are still poorly understood (22,23), there is little doubt that medical treatment for type 2 diabetes should include prescription of endurance exercise activities. Thus for optimal benefit, any new pharmacological agent should be compatible with exercise therapy. We recently demonstrated that muscle function was unaffected by GPI treatment during short-term maximal and submaximal intensity contraction (20), despite a modest impairment of muscle glycogen phosphorylase a form activation and reduced lactate accumulation. We concluded that as long as the endogenous activation of muscle glycogen phosphorylase during contraction was sufficient to overcome any drug-mediated inhibition of glycogen phosphorylase, a non-tissue-selective GPI would not impair muscle function. However, based on these data (20), we also considered that GPI may compromise glycogen metabolism and hence muscle function during low contraction intensities, as contraction-induced activation of muscle glycogen phosphorylase should be lower under such circumstances. Accordingly, in the present study we observed that muscle tension development and aerobic efficiency were impaired during prolonged low-intensity contraction, raising the possibility that non-tissue-selective

GPI may preclude patients from adopting a more active lifestyle.

Precipitation of fatigue and impaired aerobic efficiency. In the present study, we demonstrate that during prolonged submaximal intensity contraction, muscle glycogen utilization was reduced by GPI, and this was associated with greater muscle fatigue development. This was linked to lower muscle HMP accumulation and lactate efflux, indicating that glycolytic flux was impaired by GPI treatment during submaximal contraction. When muscle oxygen consumption was related to muscle tension development (absolute rate of $\dot{V}O_2$ did not differ, statistically), the GPI-treated group demonstrated a poorer aerobic efficiency. The magnitude of this aerobic inefficiency appears greater than could be expected from merely increased lipid oxidation, which presumably increased in an effort to compensate for impaired carbohydrate availability (~12% more oxygen is required per mole of ATP regenerated when oxidizing lipid as opposed to carbohydrate). Since there was no impact of GPI administration on muscle AMP or phosphocreatine concentrations, a role for altered AMPK activation controlling a substrate switch appears less likely. Rather, since the decline in muscle function occurred rather early during contraction (between 1 and 30 min), we can speculate that impaired steady-state aerobic efficiency (force declined modestly from 30 to 60 min) reflects an altered relative contribution to tension development from the various fiber types within the muscle group (24,25). This may have been precipitated by an early failure of glycolysis to support aerobic fiber function during the transition from rest, since it has been postulated that glycolytic flux per se is important for the modulation of mitochondrial redox state (26,27).

Thus, we can propose the following sequence of events. As carbohydrate substrate availability (glycolytic flux) is limited early during contraction, force development declined. This decline in force then attenuates the endogenous contraction-mediated activation of muscle glycogen phosphorylase, further promoting GPI-mediated impairment of muscle glycogen utilization during steady-state contraction. What remains unexplained is why, given the above scenario, phosphocreatine degradation was not more substantial in the GPI group. The unaltered phosphocreatine utilization may transpire to reflect an interaction between glycolytic H⁺ generation (which would be impaired) and creatine kinase activity, an observation that deserves further investigation.

Carbohydrate metabolism, fatigue, and interspecies considerations. It is important that we reflect on the clinical relevance of our observations. The present findings are consistent with the observations that muscle glycogen availability is a determinant of human endurance performance (17,18,28), and thus our model appears to be consistent with human data. Furthermore, loss of muscle adenine nucleotides is modest during fatiguing glycogen-depleting exercise in humans (29,30), consistent with the metabolic profile we observed. This suggests that loss of muscle nucleotides requires activation of AMP deaminase activity, and despite the impaired energy metabolism, the parallel impairment of glycolysis may explain a lack of flux through this catabolic route. Indeed, data are available to indicate that only when substantial glycolysis and lactate accumulation occurs (hence lowering of muscle pH) is flux through AMP deaminase high, resulting in severe changes in the muscle adenylate pool (31,32). Indeed, within a mixed fiber muscle group, muscle nucleotide loss

does not occur to any appreciable extent until the muscle lactate concentration is in excess of 40 mmol/kg dry muscle (32), consistent with present experiment. It has also been suggested that glycogen depletion could limit Krebs's cycle intermediate availability and hence oxidative phosphorylation (29); however, based on recent studies this explanation now seems an unlikely fatigue mechanism (30,33,34).

In using a rodent model to study muscle function, we are of course extrapolating muscle fatigue mechanisms across species and contrasting voluntary with involuntary fatigue. Furthermore, it is plausible that we have underestimated the potential for fatty acids to support muscle energy metabolism (35,36), as we did not specifically enrich the perfusate with free fatty acids in the present study (although intramuscular stores would be plentiful). It would seem, from the higher oxygen consumption and lower glucose and glycogen utilization, that muscle lipid utilization must have been increased to some extent. The concurrent reduction in muscle glucose uptake (measured from 15 to 60 min of contraction) could be explained by reduced muscle force; however, this is not entirely satisfactory as, relatively speaking, muscle metabolic rate (oxygen consumption) was greater. It is possible that the elevated muscle glycogen content in the GPI group may have suppressed glucose transporter function, thereby impairing muscle glucose uptake. Finally, it has been postulated that glucose fluxes through the glycogen pool, before being utilized during contraction, and thus direct inhibition of glycogen degradation would predictably also impair muscle glucose consumption, if the "glycogen shunt" theory is correct (27).

It can also be argued that by utilizing a rodent model, we are underestimating the potential side effects of a nonselective GPI on endurance capacity, as data indicate that rodent or murine skeletal muscle relies less on endogenous muscle glycogen stores during prolonged exercise (37–39). Interestingly, in mice engineered to have greater muscle glycogen stores, endurance capacity is not enhanced and fatigue did not correspond with glycogen depletion (38). Furthermore, when glycogen synthase activity is genetically ablated (37), endurance exercise remains unchanged. In contrast, disruption of protein phosphatase PP1G/R_{GL} (which activates glycogen synthase) reduces muscle glycogen content by 50% and function by >50% in an alternative background strain of mouse (40). As with all transgenic studies, evidence based on a single mutation with a single background strain can be challenging to interpret. In addition, when *GYS1* (muscle glycogen synthase gene) is ablated (37), 90% of the animals die with cardiac abnormalities, while the survivors demonstrate an enhanced capacity for lipid oxidation (41), suggesting that genetic subgroup selection contributes to a net neutral endurance performance phenotype (37,41). Based on our data and the above discussion, it can be proposed that GPI compounds that show no tissue selectivity (1,3) may be detrimental to skeletal muscle energy metabolism and function during prolonged submaximal contraction.

In conclusion, this is the first study to evaluate the impact of direct pharmacological inhibition of glycogen phosphorylase on skeletal muscle metabolism and function during prolonged submaximal contraction. Given the negative effect of GPI under these conditions, and the importance of exercise prescription in the treatment of type 2 diabetes, we caution that quantification of patient

endurance capacity and perception of effort be evaluated during the clinical testing of nonselective GPI to determine whether our observations translate to humans. Furthermore, alternative strategies devoted toward hepatic specific drug delivery systems (42) for nonselective GPI compounds and/or the identification of liver-selective GPI for the treatment of type 2 diabetes should be pursued, while greater focus could be placed on alternative strategies for controlling excess hepatic glucose production (9,10,43).

RESEARCH DESIGN AND METHODS

Fifteen female Wistar rats (250–350 g body mass) were terminally anesthetized with a long-acting barbiturate (120 mg/kg body mass, Inactin; Sigma-Aldrich) and surgically prepared for perfusion and subsequent contraction of the gastrocnemius-plantaris-soleus (GPS) muscle group, as described previously (20). (3S,2R)-3-(5-chloroindole-2-carbonyl)amino-2-hydroxy-4-phenylbutyric acid *N*-methyl-*N*-methoxyamide (CP-316819) was synthesized at Pfizer Global Research and Development as described previously (44). The GPS was perfused for 60 min with a red cell suspension (Hct 47%) containing isolated canine erythrocytes, suspended in a modified Krebs buffer containing 5% BSA, insulin (100 μ U/ml), 0.15 mmol/l pyruvate, and 6 mmol/l glucose (adjusted to pH 7.4). In one group of animals ($n = 8$), the potent GPI, CP-316819 (half-maximal inhibitory concentration against glycogen phosphorylase = \sim 100 nmol/l), was added to the perfusion medium to give an unbound free drug concentration of 3 μ mol/l (plasma protein binding was estimated to be 97%) and DMSO concentration of 0.01%. The remaining animals (control group, $n = 7$) received only vehicle (0.01% DMSO).

Following 60 min of pretreatment with GPI or vehicle, electrically evoked submaximal isometric muscle contractions were induced (1 Hz, 0.3 ms, 2 V) for 60 min according to methods previously described (20,45), and tension development was recorded. At the start of contraction, muscle blood flow was increased twofold from the resting rate (15 ml \cdot min⁻¹ \cdot 100 g muscle tissue⁻¹) to improve oxygen and substrate delivery. Arterial and venous blood samples were drawn into 1-ml syringes at rest and every 15 min during contraction for the determination of oxygen content, blood glucose, and blood lactate concentrations using a blood gas and metabolite analyzer (ABL600; Radiometer Copenhagen). Previously described measurement methods were used to calculate muscle lactate efflux and glucose uptake (20,46). Immediately at the end of 60 min of contraction, the GPS was snap frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analysis. All experiments were conducted in accordance with the U.K. Home Office Animals (Scientific Procedures) Act of 1986 and approved by the local ethics committee.

Muscle metabolite analysis. The GPS muscle group was crushed under liquid nitrogen and thoroughly mixed to create a homogenous mix of fiber types. An aliquot of this pool of muscle was then freeze dried overnight and stored at -80°C until powdered and extracted (47) for determination of muscle ATP, ADP, AMP, inosine monophosphate, inosine, hypoxanthine, xanthine, and uric acid content by high-performance liquid chromatography, as previously described (48). Muscle phosphocreatine, free creatine, HMP, lactate, and glycogen content were determined by spectrophotometry, as previously described (47).

Glycogen phosphorylase activation status. Aliquots of freeze-dried and powdered muscle tissue were extracted at -35°C (60% glycerol, 30 mmol/l Na- β -glycerolphosphate, 50 mmol/l NaF, 5 mmol/l EDTA, 0.05% BSA, and pH 7.0 containing 30 mmol/l dithiothreitol) and used for determination of both fractional and maximal (incubation with AMP) glycogen phosphorylase activity. This was calculated by measuring the generation of HMP from glycogen breakdown in aliquots of muscle homogenate during incubation at 35°C (49). In brief, 40 μ l of muscle homogenate was added to a prewarmed incubation buffer (800 μ l of assay buffer [30 mmol/l Na- β -glycerolphosphate, 4 μ l H₂O, 15 mmol/l NaF, 2 mmol/l EDTA, and 11 mmol/l KH₂PO₄], 10 mmol/l dithiothreitol, and 60 μ l distilled H₂O) for measurement of glycogen phosphorylase a form (fractional) status and to another incubation buffer (800 μ l assay buffer [as above], 10 mmol/l dithiothreitol, 2 mmol/l AMP, and 10 μ l distilled H₂O) for measurement of total (a + b) (maximal) status, before the addition of glycogen and subsequent aliquot removal for HMP analysis by spectrophotometry (49).

Statistics. A comparison of differences between means was performed using Student's unpaired *t* test (end point metabolites) and two-way ANOVA when appropriate (treatment versus time). When the ANOVA resulted in a significant *F* statistic, a least significant difference post hoc test was used to locate differences. Significance was set at $P < 0.05$.

ACKNOWLEDGMENTS

We would like to thank Pfizer Global Research and Development, Groton Laboratories, Groton, Connecticut, for the gift of CP-316819. J.A.T. is supported by the Swedish Diabetes Association.

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