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Altered glucose 1,6-bisphosphate and fructose 2,6-biphosphate levels in low-frequency stimulated rabbit fast-twitch muscle

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Glucose 1,6-bisphosphate (Glc-1,6-P_2) and fructose 2,6-bisphospate (Fru-2,6-P_2) concentrations display pronounced increases in rabbit fast-twitch muscle during chronic low-frequency stimulation. These increases are first seen after stimulation periods exceeding 3 h and reach maxima after 12–24 h of stimulation (approximately 3-fold for Glc-1,6-P_2 and 5-fold for Fru-2,6-P_2). Both metabolites regress to normal values after stimulation periods longer than 4 days. The fact that their increases coincide with the replenishment of glycogen after its initial depletion, could point to a role of Glc-1,6-P_2 and Fru-2,6-P_2 in glycogen metabolism.

Chronic low-frequency stimulation; Rabbit fast-twitch muscle; Glucose 1,6-bisphosphate; Fructose 2,6-bisphosphate

1. INTRODUCTION

In a recent study [1] we investigated changes in levels of metabolites of energy supply in low-frequency stimulated rabbit fast-twitch muscle. An important finding was that ATP and creatine phosphate decayed steeply during the first 15 min after the onset of stimulation, but recovered almost completely during the next 2–4 days with uninterrupted stimulation. Similarly, glycogen was nearly depleted after 60 min, recovered thereafter, and displayed an overshoot after 2 days with ongoing stimulation. Lactate increased dramatically during the first 15 min, but regressed to control levels thereafter. In view of both the initial glycogen depletion and lactate formation and the successive replenishing of the glycogen store in the absence of increases in lactate, the question arose as to the fuel source and control of energy metabolism during this time period. The several-fold increase in the concentration of glucose [1] indicated that exogenous glucose became a major fuel of the contracting muscle by this time. Since glucose 1,6-bisphosphate (Glc-1,6-P_2) has been postulated to be a powerful regulator of carbohydrate metabolism in muscle (for review see [2]) and more recently fructose 2,6-bisphosphate (Fru-2,6-P_2) as well [3], we have investigated the changes in the concentrations of these two effectors in chronically stimulated rabbit tibialis anterior muscle.

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2. MATERIALS AND METHODS

2.1. *Animals and chronic stimulation*

Adult male and female White New Zealand rabbits were used. Nine different periods of continuous (24 h/day) low-frequency (10 Hz) stimulation (15 min, 60 min, 3 h, 12 h, 24 h, 2 days, 4 days, 10 days and 50–60 days) were selected for investigation. Three to four animals were examined at each time point. Following each stimulation period, animals were anesthetized and the right, non-stimulated and left, stimulated tibialis anterior (TA) muscles were rapidly sampled. In addition, both left and right TA muscles were sampled from control animals that had been implanted with electrodes, but were not stimulated.

Implantation of electrodes laterally to the peroneal nerve and chronic low-frequency stimulation with the use of a telestimulation set-up have been described previously [4]. Stimulation (10 Hz, 0.15 ms single pulse duration, 24 h/day) was started one week after implantation of the electrodes. Prior to muscle sampling, the actual voltage used in the telestimulation was measured. The animal was then removed from the cage, quickly transferred to a surgical table and the same voltage previously delivered by telestimulation was administered directly from a Grass stimulator. The animal was then anesthetized and both contracting (left) and unstimulated (right) TA muscles isolated; extreme caution was taken to avoid disruption of the nerve and blood supply. When the muscles were prepared for sampling, the skin flaps were carefully folded over the muscle and the left muscle allowed to contract for an additional 5 min. Thereafter, the muscles were exposed and two investigators simultaneously froze the lower sections of both the unstimulated and contracting TA muscles using modified Wollenberger clamps pre-cooled in liquid nitrogen. The muscles were placed in liquid nitrogen, transferred to vials and stored at −70 to −80°C until analysed.

2.2. *Analytical procedures*

Muscle samples were pulverized under liquid nitrogen and extracted using previously published procedures [5]. For Fru-2,6-P_2, an alkaline extraction was used. Approximately 20 mg of muscle powder were

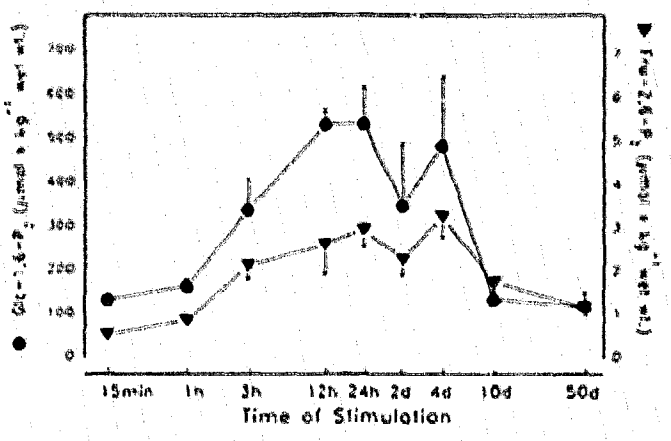

Fig. 1. Time-dependent changes in Glc-1,6-P_2 and Fru-2,6-P_2 concentrations in rabbit fast-twitch tibialis anterior muscle during chronic low-frequency stimulation (10 Hz, 24 h/day). Values are given as means ± S.E. from 3-4 animals per time point.

homogenized in 10 vols of 50 mM NaOH and maintained at 90°C for 10 min. Neutralization of the extract was performed on ice by the addition of 250 mM sodium acetate, pH 4.0. The supernatant fraction used for the measurement of Fru-2,6-P_2 was obtained after 5 min centrifugation at 4°C in an Eppendorf centrifuge. The procedure of van Schaftingen et al. [6] was used to measure Fru-2,6-P_2 under mild acidic conditions. In the case of Glc-1,6-P_2, glucose 6-phosphate (Glc-6P) and fructose 6-phosphate (Fru-6P), approximately 50 mg of frozen muscle powder was homogenized at 0°C in 5 vols of 10% (v/v) $HClO_4$. The homogenate was centrifuged at 3000 rpm for 20 min and the supernatant fraction was neutralized with 3 M KOH/$KHCO_3$ [5]. For Glc-1,-6-P_2, the samples were heated for 1 h at 80°C at alkaline pH (0.1 M KOH) and then neutralized with $HClO_4$. Glc-1,6-P_2 was determined as described by Passonneau et al. [7]. Glucose 6-phosphate and fructose 6-phosphate were determined by standard photometric methods.

A one-way analysis of variance was used to determine whether significant differences existed between the metabolite concentrations in control and stimulated muscles at different time periods. A Newman-Keuls post hoc procedure was used to determine the significance of differences between specific means. All comparisons were performed at the 95% level of confidence.

3. RESULTS

Pronounced elevations in both Glc-1,6-P_2 and Fru-2,6-P_2 occurred with increased contractile activity in a time-dependent fashion (Fig. 1). In the case of Glc-1,6-P_2, the elevations ($P<0.05$) did not occur until 12 h of stimulation. By 12 h, there was an approximate 3-fold increase in Glc-1,6-P_2. With the exception of the 2 day time point, this elevation persisted through the 4th day. By the 10th day, Glc-1,6-P_2 had declined to control levels and then remained unchanged. The pattern of change in Fru-2,6-P_2 concentration was qualitatively similar (Fig. 1) to the changes in Glc-1,6-P_2. The peak value observed at 4 days was approximately 5-6-fold higher than the control value. Like Glc-1,6-P_2, Fru-2,6-P_2 at 50 days did not remain elevated over control and the early stimulation periods (15 and 60 min).

Analyses were also performed to follow stimulation-induced changes in Glc-6P, Fru-6P and glycogen concentrations (Table I). The first 15 min of stimulation resulted in an approximately 5-fold increase ($P<0.05$) in the concentration of Glc-6P (Table I). As stimulation continued, its concentration declined and stabilized at rest values through the 10th day. At 50 days, Glc-6P was higher ($P<0.05$) than at all other time points, except at 15 min. No difference was found between the 15-min and 50-day values. In the case of Fru-6P, the only significant change was an increase in the 50-day stimulated muscle (Table I).

Conversely, low-frequency stimulation had pronounced effects on muscle glycogen. By 15 min and 60 min, glycogen was reduced approximately 76% and 90%, respectively. However, with persistent stimulation glycogen stores were not only replenished, but found to be higher than in normal TA. At 2 days, the glycogen concentration of the stimulated muscle ex-

Table 1

Time-dependent changes in glucose 6-phosphate, fructose 6-phosphate and glycogen concentrations in unstimulated, chronically low-frequency stimulated tibialis anterior muscle (TA), and normal soleus muscle of rabbit. The values for glycogen are from [1]. Values are means ± S.E. (mmol · kg^{-1} wet wt., except for glycogen which is given as mmol glucosyl unists · kg^{-1} dry wt.)

	Glc-6P	Fru-6P	Glycogen
Unstimulated TA			
	0.110 ± 0.02	0.039 ± 0.004	224 ± 6.9
Stimulated TA			
15 min	0.573 ± 0.22[a]	0.089 ± 0.03	63.7 ± 10[a]
60 min	0.089 ± 0.03[b]	0.027 ± 0.01	26.1 ± 5.7[a]
3 h	0.143 ± 0.02[b]	0.042 ± 0.004	70.3 ± 22[ac]
12 h	0.127 ± 0.03[b]	0.037 ± 0.004	85.5 ± 14[ac]
24 h	0.078 ± 0.01[b]	0.048 ± 0.02	129 ± 23[ac]
2 d	0.070 ± 0.01[b]	0.039 ± 0.02	326 ± 17[abc]
4 d	0.092 ± 0.03[b]	0.027 ± 0.002	286 ± 17[bc]
10 d	0.085 ± 0.04[b]	0.084 ± 0.06	115 ± 19[ac]
50 d	0.387 ± 0.11[ac]	0.130 ± 0.02[ac]	122 ± 15[ac]

[a]significantly different from unstimulated TA ($P<0.05$)
[b]significantly different from 15 min stimulated TA
[c]significantly different from 60 min stimulated TA

ceeded that of the normal TA by approximately 25% ($P < 0.05$). Thereafter, glycogen declined, ultimately reaching a value at 10 days that was less than 50% of the initial concentration. No further alterations were found with stimulation up to 50 days.

4. DISCUSSION

This study reveals several novel findings with regard to the pattern of changes in both Fru-2,6-P_2 and Glc-1,6-P_2 in rabbit fast-twitch muscle subjected to persistently sustained contractile activity. The concentrations of these two effector molecules display similar time-dependent changes, consisting of a delayed increase which does not occur until 3 h of activity and which, with the exception of one time point, persists over several days before declining to control values.

Elevations in Glc-1,6-P_2 concentration with increased contractile activity have been observed previously in rat fast-twitch muscle [5] and in human muscle of mixed fiber type [8,9]. However, it appears that these increases were transient, being observed only during the first several seconds after the onset of activity. This transient response may explain why initial increases in Glc-1,6-P_2 were not detected in the present study, since the first time point investigated was at 15 min. Our inability to detect increases in Fru-2,6-P_2 early after the onset of contractile activity may be due to the same reason, since rapid but extremely transient increases in Fru-2,6-P_2 were previously reported to occur immediately after the onset of exercise [5,10].

The novel finding in this study is the delayed and sustained increase in the concentrations of these two regulators of carbohydrate metabolism for several days. These increases occur during a time period when anaerobic glycolysis appears to be considerably blunted as shown by the low lactate concentration of the contracting muscle [1]. Thus, it appears that the increases in Glc-1,6-P_2 and Fru-2,6-P_2 are not related to stimulation of anaerobic glycolysis. Nevertheless, the changes in the two hexose bisphosphates may be associated with a stimulation of phosphofructokinase in conjunction with an elevated aerobic glycolytic flux rate. Analyses performed on the same samples used in the present study have shown that muscle glucose is elevated several-fold and a net glycogen synthesis occurs during the same time period [1]. Glycogen which is virtually depleted after one hour, recovers to values above control during the subsequent period of 4 days of activity [1]. However, glucose appears not only to be the substrate for glycogen synthesis, but also the major fuel for mitochondrial substrate oxidation after the initial dependency of energy supply on glycogen breakdown and anearobic glycolysis.

The suggested use of glucose for both aerobic glycolysis and glycogen synthesis is difficult to reconcile with the large elevation in Glc-1,6-P_2, an inhibitor of the hexokinase isozyme II [11,12], especially of its mitochondrially bound form [13]. Increases in the fraction of structure-bound hexokinase in combination with several-fold elevations in the total amount of hexokinase II have been noticed during the first days after the onset of stimulation [14]. It may be speculated that the increase in total hexokinase II compensates for the inhibition by Glc-1,6-P_2. Another possibility to be considered in compartmentation, which would make glucose phosphorylation inaccessible to the inhibitory effect of Glc-1,6-P_2. The low levels of both Glc-6P and Fru-6P may indicate a high flux rate at the phosphofructokinase reaction, caused by the increased levels of the two activators. The increases in both Glc-1,6-P_2 and Fru-2,6-P_2 during chronic low-frequency stimulation with ungoing metabolic recovery [1] may have additional, but as yet unidentified, metabolic functions, e.g. in glycogen metabolism. Such functions are suggested by the relationship which exists between the time course of the observed changes in concentrations of the two hexose bisphosphates and glycogen.

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