Enhanced catalytic activity of hexokinase by work-induced mitochondrial binding in fast-twitch muscle of rat

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Abstract **Using a teased muscle fiber preparation, we determined the activity of mitochondrially bound hexokinase in rat fast-twitch muscle under control conditions and after low-frequency stimulation periods for up to 2 h. As compared to soluble hexokinase, mitochondrial binding led to stimulation of glucose 6-phosphate production. Low-frequency stimulation greatly enhanced glucose 6-phosphate formation which was 100% and 250% elevated after 1 and 2 h, respectively. These observations point to a mechanism which rapidly increases the catalytic activity of hexokinase through binding to the mitochondrial surface.**

Key words: Fast-twitch muscle; Hexokinase; Low-frequency stimulation; Mitochondrial binding; Rat

1. Introduction

Chronic low-frequency stimulation of muscle induces changes in gene expression that affect all functional elements of the muscle fiber. As a result, a fast-twitch, fast-fatigable muscle is gradually transformed into a slower contracting, less fatigable muscle [1]. These changes occur in a time-dependent manner, some of them soon after the onset of stimulation and others later. Enhanced synthesis of hexokinase (HK) II, leading to significant elevations in HKII protein and total cellular HK activity within several days, represent early responses of the muscle to forced contractile activity by low-frequency stimulation. These early alterations in gene expression are preceded by changes in the intracellular distribution of HK and the glucose transporter GLUT4 [2]. Low-frequency stimulation significantly augmented the fraction of structure-bound HK [3,4]. We interpret these changes as contributing to an elevated glucose utilization during contractile activity. The enhanced mitochondrial binding of HK is thought to channel mitochondrially generated ATP into glucose phosphorylation, as well as to augment the capacity to phosphorylate glucose before de novo synthesis of HKII leads to increases in total cellular HK activity.

The present study was undertaken in order to investigate in short-term stimulated tibialis anterior muscle of rat the effect which mitochondrial binding exerts on the catalytic activity of HK. Because intact mitochondria are difficult to isolate from these muscles and bound HK activity might be lost during the isolation procedure, we assessed mitochondrially bound HK activity according to Kunz et al. [5] in fiber bundles prepared from control muscles and muscles stimulated for up to 2 h at 10 Hz. To assess mitochondrially bound HK activity, respiratory rates were measured polarographically and compared with enzymatically determined amounts of glucose 6-phosphate (Glc6P) formed in the presence or absence of glucose, ADP or ATP. We presumed that mitochondrially bound HK preferentially uses mitochondrially generated (endogenous) ATP for glucose phosphorylation. Therefore, exogenous ADP should stimulate respiration in the presence of glucose, whereas no such stimulation should occur in the presence of exogenous ATP. In addition, we measured the total HK activity of the fiber bundles after desorption of the structure-bound enzyme by Triton X-100 and compared this value to the catalytic activity derived from the rate of Glc6P formation under conditions when HK is bound to the mitochondria.

2. Materials and methods

2.1. Animals, low-frequency stimulation, preparation of muscle fiber bundles

Adult male rats (Wistar) weighing 250–300 g were used. One week before the acute stimulation experiment, electrodes were implanted laterally to the peroneal nerve of the left hindlimb [6]. Acute stimulation at 10 Hz (impulse width 0.15 ms) was performed during 1 or 2 h. After disconnection from the stimulator, the rats were killed, and stimulated and contralateral tibialis anterior (TA) muscles were quickly excised and kept in ice-cooled isolation buffer (10 mM EGTA, 9.5 mM $MgCl_2$, 3 mM KH_2PO_4, 20 mM taurine, 49 mM K-Mes, 20 mM imidazole, pH 7.10). For preparing skinned fiber bundles according to [5], a piece from the midbelly region was cut and teased apart with a pair of tweezers to produce fiber bundles of similar size. To permeabilize the sarcolemmal membrane, fiber bundles were transferred to fresh isolation buffer containing 50 μg/ml digitonin and agitated gently for 20 min on a rocking table at 4°C. Thereafter, the isolation buffer was decanted and replaced with respiration buffer (75 mM mannitol, 25 mM sucrose, 10 mM KH_2PO_4, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 20 mM Tris-HCl, pH 7.40) in order to wash out the digitonin. This wash procedure was repeated three times.

2.2. Respiration analyses, fluorometric determination of glucose 6-phosphate

The respiration analyses were carried out in respiration buffer using a thermostatted cuvette (25°C) of a Cyclobios oxygraph. All biochemicals used were from Boehringer Mannheim. Muscle fibers were used at a concentration of approximately 15 mg wet weight in 2 ml assay volume. After completing the measurements, their protein concentration was measured by using the Micro BCA Protein Assay Kit (Pierce). Stimulation of oxidative phosphorylation was performed in the presence of (final concentrations) 5 mM succinate, 0.1 mM ADP or 2.0 mM ADP. For measuring glucose-stimulated respiration, glucose was added at a final concentration of 5 mM. Subsequently, respiratory activity was recorded in the presence of low (0.1 mM) and high (2.0 mM) ADP. Finally, atractyloside was added at a final concentration of 0.25 mM in order to determine state 4 respiration. Bound and solubilized HK activities were also measured in the presence of 2 mM external ATP. To assess Glc6P formation, the assay

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mixture also contained 0.5 mM NADP and 5 U glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. Small volumes (20 μl) of the assay mixture were sampled at defined time points (see Fig. 1) and used for fluorometric determination of NADPH.

2.3. Statistical analyses

Four animals were analyzed in both the 1 h and 2 h stimulation groups. The contralateral muscles were used as controls. The results are given as means ± S.D. Statistical significance was calculated by applying ANOVA and Dunnett's multiple comparison post-tests.

3. Results and discussion

A typical experiment in which respiratory rates were measured polarographically after specific additions is shown in Fig. 1. The time points are indicated at which 20 μl samples were withdrawn from the assay for fluorometric determination of NADPH (Glc6P) formation. Addition of glucose to fiber bundles of a control muscle did not increase respiration above the rate determined in the presence of succinate. However, subsequent addition of 0.1 mM ADP led to a pronounced, although not maximal activation of state 3 respiration. After addition of 2 mM ADP, respiration was maximal and could be inhibited by atractyloside (Fig. 1).

Quantitative analyses of respiratory rates recorded in control muscles and in muscles stimulated at 10 Hz for 1 or 2 h are depicted in Fig. 2. Obviously, succinate-dependent respiration was elevated in the stimulated muscles although no differences existed between 1 h and 2 h stimulation periods. As already shown in Fig. 1, glucose did not further stimulate respiration. The elevated rates in the stimulated muscles corresponded to those recorded in the presence of succinate. Addition of ADP in the presence of succinate and glucose moderately stimulated respiration of the control muscles. This effect was more pronounced in the stimulated muscles at both low and high ADP concentrations. At high ADP, the 2 h stimulated muscle displayed a slightly stronger activation (not significant) than the 1 h stimulated muscle. The addition of atractyloside reduced oxygen consumption to about 20% of the maximum state 3 respiration (Fig. 2), proving that approximately 80% of the recorded oxygen uptake resulted from coupled respiration. The values obtained after addition of atractyloside were surprisingly low compared to succinate-dependent respiration. This difference suggested coupled respiration due to endogenous adenine nucleotides in the presence of succinate. Most likely, the presence of actomyosin ATPase under the conditions of our assay contributed to the observed basal ATP turnover.

Thus, it appeared impossible to calculate from the polarographic measurements the full extent of the HK-dependent stimulation of oxidative phosphorylation. We therefore decided to use Glc6P production as a direct measure of mitochondrially bound HK activity. In order to assure that the Glc6P formed under the various incubation conditions corresponded to the activity of bound HK, we made use of an additional control. Beitner and Lilling have shown that only soluble but not mitochondrially bound HK is inhibited by glucose 1,6P_2 [7]. Indeed, addition of glucose 1,6P_2 did not significantly inhibit Glc6P formation, except when the bound enzyme was liberated by the addition of Triton X-100 (Fig. 3, right panel). Therefore, we were sure that Glc6P production in the skinned fibers reflected the activity of bound HK. Low-frequency stimulation for 1 and 2 h led to significant increases in Glc6P production (Fig. 3, left panel). Compared to the control, Glc6P formation was elevated almost 100% after 1 h and 250% after 2 h.

Interestingly, exogenous ATP resulted in much lower Glc6P formation both in control and stimulated muscles (Fig. 3, middle panel) than ATP generated by oxidative phosphorylation from added ADP (endogenous ATP) (Fig. 3, left panel). Thus, the bound enzyme appeared to be less accessible to exogenous ATP, but it was more active with internal ATP. The higher activity of the bound enzyme could be due to an interaction with the membrane, i.e. with porine and the adenine nucleotide translocator. This interaction might facilitate substrate channeling between oxidative phosphorylation and the bound enzyme. This suggestion is further emphasized by the results obtained when the bound enzyme was solubilized by Triton X-100. The Glc6P production by solubilized HK in

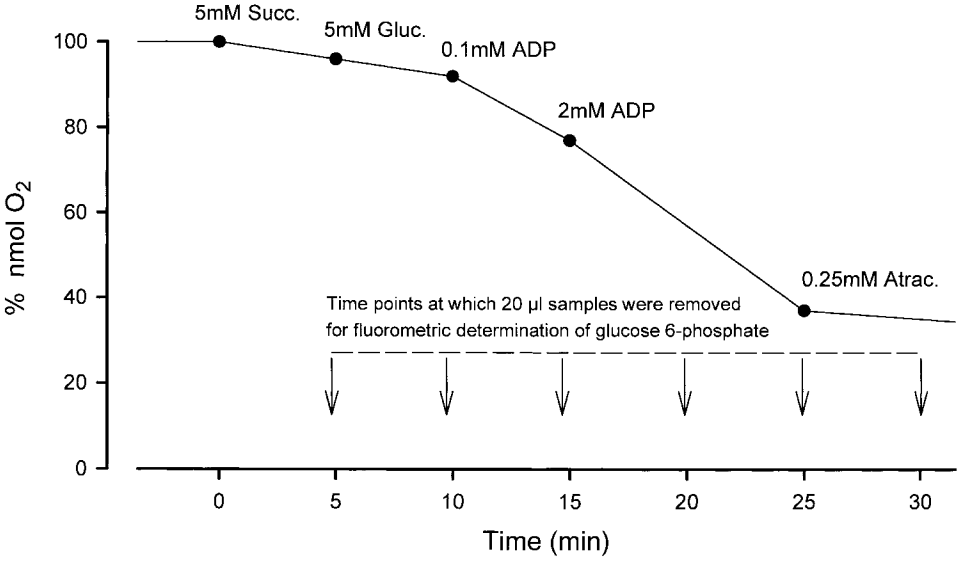

Fig. 1. Recording of respiratory rates of skinned fibers from control tibialis anterior muscle of rat. Oxygen consumption was recorded polarographically after additions as indicated. Arrows mark the time points at which 20 μl samples were withdrawn from the assay mixture for fluorometric determination of glucose 6-phosphate production.

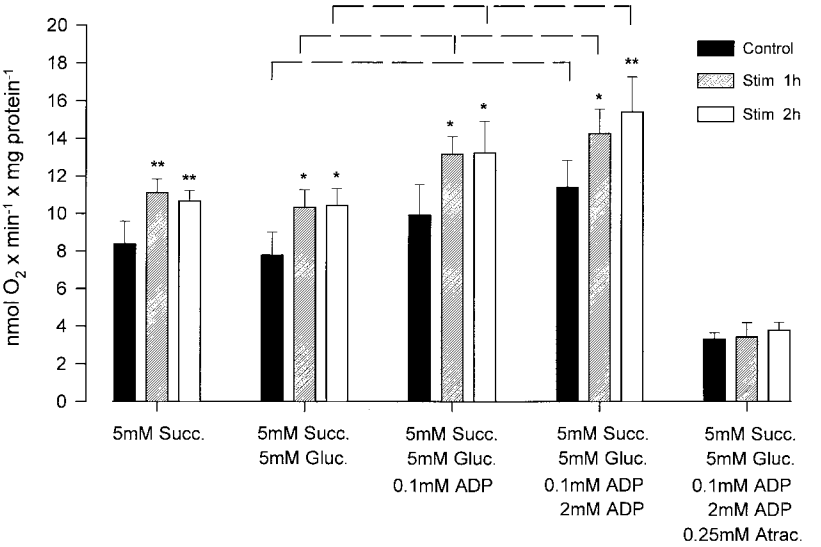

Fig. 2. Respiration analyses of control, 1 h, and 2 h low-frequency stimulated tibialis anterior muscle of rat. Oxygen consumption of the different skinned fiber samples was determined polarographically in the presence of different substrates listed below the bars. Values are means ± S.D., $n = 4$. Asterisks denote significant ($^{*}P < 0.05$, $^{**}P < 0.01$) changes between stimulated muscles and their corresponding controls for each condition. The significance ($P < 0.05$) of the stimulatory effect of high ADP is indicated by the broken lines above the bars.

the presence of added ATP (exogenous ATP) was in the same range as, or slightly higher than, determined in the bound state with exogenous ATP. That the enzyme was truly solubilized by Triton X-100 was proved by its almost complete inhibition by glucose 1,6P_2 (Fig. 3).

As can be seen from the measurement of Glc6P formation by the solubilized enzyme (Fig. 3, right panel), total HK activity was only slightly elevated after 1 (25%) and 2 (50%) h of low-frequency stimulation. These increases most probably resulted from the previously observed upregulation of HKII expression by enhanced transcription and translation in muscles exposed to chronic low-frequency stimulation [2]. However, these early elevations in total cellular HK activity were small when compared to the pronounced increases in HK activity resulting from mitochondrial binding as determined by Glc6P formation with endogenous ATP (Fig. 3, left panel). The reason for the elevated activity of HK, therefore, resides in its mitochondrial binding and this has previously been shown to be greatly enhanced during the first hours of contractile activity [4].

It is known from in vitro studies that HK isozyme I binds to a specific protein at the mitochondrial surface [8] which corresponds to the pore protein of the outer mitochondrial membrane [9]. Moreover, the enzyme has been shown to be specifically activated by binding to contact sites between the two boundary membranes of liver mitochondria [10]. The interaction between HK and mitochondrial membrane leads to a compartmentation of the ADP from the HK reaction. This

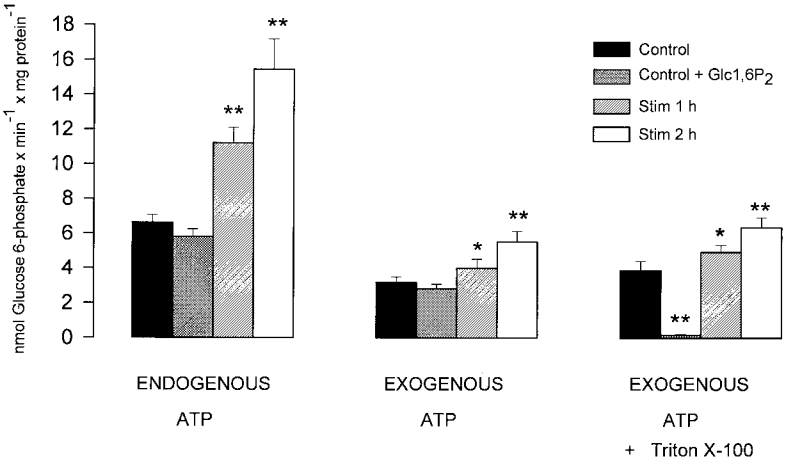

Fig. 3. Glucose 6-phosphate formation of skinned fibers from control and low-frequency stimulated tibialis anterior muscles. Glucose 6-phosphate production was measured fluorometrically by NADPH formation under conditions at which HK was mitochondrially bound and ATP was generated either intramitochondrially in the presence of 5 mM succinate, 5 mM glucose, and 2 mM ADP (endogenous ATP, left panel) or provided externally from added 2 mM ATP (exogenous ATP, middle panel). The solubilized activity after treatment of the preparation with Triton X-100 was measured in the presence of exogenous (2 mM) ATP and 5 mM glucose (right panel). Measurements in the presence of glucose 1,6P_2 were used to determine the degree of HK binding. Note that only the soluble enzyme was inhibited by glucose 1,6P_2. Values are means ± S.D., $n = 4$ for each condition.

was recently demonstrated under conditions favoring the formation of contacts between the two mitochondrial membranes [11].

In summary, using a skinned muscle fiber preparation we have established an assay for direct measurement of mitochondrially bound hexokinase activity in skeletal muscle. We show that contractile activity for 2 h leads to a 250% increase in Glc6P formation. This stimulation most likely represented the activity of bound HK supplied with ATP from oxidative phosphorylation. It could be ruled out that the 250% increase in the flux rate of HK resulted from de novo synthesis of the enzyme. The stimulation of HK is solely explained by its binding to the mitochondrial surface. It remains to be seen whether the increase in catalytic activity is a direct result of the enzyme/membrane interaction or whether it is due to the channeling of mitochondrially generated ATP to the membrane-bound enzyme. In any case, our results show that the effective activity of HK can be substantially elevated as a first response to the enhanced energy demand of contracting muscle.

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