Recruitment of single muscle fibers during submaximal cycling exercise

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1Institute for Biophysical and Clinical Research into Human Movement, Manchester Metropolitan University, Cheshire, United Kingdom; 2Research Institute MOVE, Faculty of Human Movement Sciences, VU University Amsterdam, The Netherlands; 3EMGO Institute and Department of Public and Occupational Health, VU University Medical Centre, The Netherlands

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Altenburg TM, Degens H, van Mechelen W, Sargeant AJ, de Haan A. Recruitment of single muscle fibers during submaximal cycling exercise. J Appl Physiol 103: 1752–1756, 2007. First published September 6, 2007; doi:10.1152/japplphysiol.00496.2007.—In literature, an inconsistency exists in the submaximal exercise intensity at which type II fibers are activated. In the present study, the recruitment of type I and II fibers was investigated from the very beginning and throughout a 45-min cycle exercise at 75% of the maximal oxygen uptake, which corresponded to 38% of the maximal dynamic muscle force. Biopsies of the vastus lateralis muscle were taken from six subjects at rest and during the exercise, two at each time point. From the first biopsy single fibers were isolated and characterized as type I and II, and phosphocreatine-to-creatine (PCr/Cr) ratios and periodic acid-Schiff (PAS) stain intensities were measured. Cross sections were cut from the second biopsy, individual fibers were characterized as type I and II, and PAS stain intensities were measured. A decline in PCr/Cr ratio and in PAS stain intensity was used as indication of fiber recruitment. Within 1 min of exercise both type I and, although to a lesser extent, type II fibers were recruited. Furthermore, the PCr/Cr ratio revealed that the same proportion of fibers was recruited during the whole 45 min of exercise, indicating a rather constant recruitment. The PAS staining, however, proved inadequate to fully demonstrate fiber recruitment even after 45 min of exercise. We conclude that during cycling exercise a greater proportion of type II fibers is recruited than previously reported for isometric contractions, probably because of the dynamic character of the exercise. Furthermore, the PCr/Cr ratio method is more sensitive in determining fiber activation than the PAS stain intensity method.

ONE OF THE MECHANISMS BY WHICH A MUSCLE CAN REGULATE FORCE IS BY CHANGING THE NUMBER OF ACTIVATED MOTOR UNITS. THE SMALLER MOTOR UNITS, CONTAINING TYPE I FIBERS, ARE RECRUITED AT LOW LEVELS OF FORCE, AND, AS FORCE INCREASES, LARGER MOTOR UNITS (FIRST FATIGUE RESISTANT AND LATER FAST FATIGABLE), CONTAINING TYPE II A AND IIX FIBERS, ARE RECRUITED. THIS IS REFERRED TO AS THE SIZE PRINCIPLE (6). BELTMAN ET AL. (2) INVESTIGATED THE INITIAL ACTIVATION OF DIFFERENT MUSCLE FIBER TYPES DURING REPEATED ISOmetrical CONTRACTIONS OF THE QUADRICEPS MUSCLE AT DIFFERENT INTENSITIES AND SHOWED A PREDOMINANT ACTIVATION OF TYPE I FIBERS AT 39% OF THE MAXIMAL VOLUNTARY CONTRACTILE (MVC) FORCE, WHEREAS A SUBSTANTIAL ACTIVATION OF TYPE II FIBERS WAS ONLY DETECTED AT THE EXERCISE INTENSITY OF 72% MVC AND HIGHER. VØLLESTAD ET AL. (20) INVESTIGATED THE ACTIVATION PATTERN OF TYPE I AND TYPE II FIBERS DURING DYNAMIC CYCLING EXERCISE AT AN INTENSITY OF 75% OF THE MAXIMAL OXYGEN UPTAKE (\(V_o_{2max}\)) AT CONSTANT PEDALING RATE. ACCORDING TO SARGEANT AND JONES (16), CYCLING AT AN EXERCISE INTENSITY OF 75% \(V_o_{2max}\) REQUIRES ONLY ~40% OF THE MAXIMAL DYNAMIC MUSCLE FORCE EXERTED ON THE PEDALS. IN CONTRAST TO THE RESULTS OF BELTMAN ET AL., VØLLESTAD ET AL. FOUND ACTIVATION OF BOTH TYPE I AND TYPE II FIBERS DURING THE DYNAMIC CYCLING EXERCISE AT THE EXERCISE INTENSITY USING ~40% OF THE MAXIMAL MUSCLE FORCE. THEREFORE, THERE IS AN INCONSISTENCY IN THE SUBMAXIMAL EXERCISE INTENSITY AT WHICH TYPE II FIBERS ARE ACTIVATED (EXPRESSED AS PERCENTAGE OF THE MAXIMAL MUSCLE FORCE).

THERE ARE TWO OBVIOUS EXPLANATIONS FOR THIS APPARENT DISCREPANCY. THE FIRST EXPLANATION IS RELATED TO THE METHODOLOGIES USED TO DETERMINE MUSCLE FIBER ACTIVATION. IN THE STUDIES OF VØLLESTAD ET AL. (20), THE GLYCOCEN DEPLETION METHOD IS USED TO DETERMINE FIBER ACTIVATION, USING THE PERIODIC ACID-SCHIFF (PAS) REACTION, WHEREAS BELTMAN ET AL. (2) ASSESSED FIBER ACTIVATION USING THE PHOSPHOCREATINE (PCR)-TO-CREATINE (CR) RATIO (PCR/CR) IN MUSCLE FIBER FRAGMENTS. THE GLYCOCEN DEPLETION METHOD HAS THE ADVANTAGE THAT IT IS AN ACCEPTED AND COMMONLY USED METHOD FOR THE ASSESSMENT OF FIBER RECRUITMENT. HOWEVER, THE GLYCOCEN DEPLETION AS INDICATOR OF FIBER ACTIVATION SHOULD BE USED WITH CARE (10). BECAUSE OF THE RELATIVELY HIGH CONCENTRATION OF GLYCOCEN IN HUMAN MUSCLE FIBERS, CHANGES ARE ONLY DETECTABLE AFTER SEVERAL MINUTES OF EXERCISE AND THEREFORE IT IS DIFFICULT TO SEPARATE THE INFLUENCE OF EXERCISE INTENSITY AND DURATION ON RECRUITMENT. ALTHOUGH THE PCr/CR RATIO METHOD IS LESS COMMONLY USED, IT HAS THE ADVANTAGE THAT A DECREASE IN PCr IS DIRECTLY LINKED TO ATP RESYNTHESIS DURING EXERCISE (7), WHICH ALLOWS ONE TO ASSESS SINGLE MUSCLE FIBER RECRUITMENT QUANTITATIVELY AFTER EXERCISE OF SHORT DURATION. THE DISADVANTAGE IS THAT THIS METHODOLOGY MAY LEAD TO AN UNDERESTIMATION OF THE NUMBER OF ACTIVATED FIBERS AS INDICATED IN THE STUDY OF BELTMAN ET AL. (3).


IN THE PRESENT STUDY WE WANTED TO MAKE A COMPARISON BETWEEN THE ISOMETRIC EXERCISE IN THE STUDY OF BELTMAN ET AL. (2) AND THE DYNAMIC EXERCISE PERFORMED IN THE STUDY OF VØLLESTAD...
The intensity of 75% V\text{O}_{2\text{max}} was therefore maintained during the full exercise (12), the load was decreased by 5% after 10 min of exercise. Overcome a slow rise in oxygen uptake throughout the 45-min cycle exercise.

**METHODS**

The present study was divided into two experiments consisting of 45 min of cycling exercise each. In the first experiment, muscle biopsies were taken before and during the cycling exercise, whereas in the second experiment maximal force and power were measured before and during the exercise. This study conformed to the Declaration of Helsinki and was approved by the ethics committee of the VU University Medical Centre in Amsterdam, The Netherlands.

**Subjects**

Six physically active, but not specifically trained, subjects (2 men, 4 women) participated in this study. Age, height, and body mass were, respectively, 24 ± 3 yr, 177 ± 9 cm, and 68 ± 8 kg (mean ± SD). None of the subjects had a history of muscle or metabolic diseases. After written and verbal explanations of the objectives and procedure of the experiment, the subjects signed an informed consent form. All subjects refrained from heavy exercise 24 h prior to the experiment.

**Experimental Protocol**

Subjects performed a 45-min cycle exercise at a constant intensity corresponding to 75% of the V\text{O}_{2\text{max}} at a pedaling rate of 90 rpm. Load was increased at a rate of 10 W/s from onset of the exercise, to reach the required load within 15–20 s. V\text{O}_{2\text{max}} had been determined previously during an incremental cycling test, during which the load required to elicit the 75% V\text{O}_{2\text{max}} had also been established. To overcome a slow rise in oxygen uptake throughout the 45-min cycle exercise (12), the load was decreased by 5% after 10 min of exercise. The intensity of 75% V\text{O}_{2\text{max}} was therefore maintained during the full 45-min exercise.

**Experiment 1: Muscle Biopsies**

In the first experiment muscle fiber activation during a 45-min cycle exercise was measured. The exercise bout was performed on a mechanically braked bicycle ergometer. At four different time points two muscle samples were taken from the m. vastus lateralis. Resting and exercise samples were taken with the subjects seated on the bicycle ergometer, with their leg almost straight. Altogether eight biopsy samples were obtained, four from each leg. The time needed for taking the two biopsies never exceeded 1 min.

Upon removal from the muscle, each first muscle sample was frozen in liquid nitrogen within 7.8 ± 3.0 s after interruption of the cycling exercise and freeze-dried overnight. The freeze-dried samples were stored desiccated in tubes. Each tube was placed in another small jar with some silica gel, sealed with laboratory film, and stored in liquid nitrogen vapor (−190°C) until analysis. Each second muscle sample was glued on cork before it was frozen in isopentane, cooled in liquid nitrogen. The frozen samples were stored at −80°C until further analysis.

**Analysis of Each First Muscle Sample**

After bringing the freeze-dried sample to room temperature in a vacuum chamber for 1 h, individual fiber fragments of 2–3 mm length (80 from each sample) were dissected under conditions of controlled ambient temperature (20–25°C) and relative humidity (<35%). Each fiber fragment was then cut into two parts. The first part, of at least 0.5 mm, was prepared for histochemistry and the second part, of at least 1.0 mm, was analyzed for metabolites (8, 14).

**Histochemistry.** Twenty fiber fragments of each muscle sample were embedded in a double layer of a gelatin solution (14). With a motor-driven cryostat (−20°C), serial sections of 10 μm were cut. Serial sections were stained for mATPase after preincubation at pH 4.4 and 4.7 (adapted from Ref. 4) and for glycogen by PAS staining. Image recordings and analysis of the stained sections were performed using a computer-enhanced image processing system (KS, Kontron Electronic). Optical density values (OD) of each fiber fragment were measured for each staining. Based on the OD values from mATPase stainings at pH 4.4 and pH 4.7 fibers were classified into type I and II fibers (20). The OD values of the PAS staining provided an indication of the glycogen content (20) and served as a marker for fiber recruitment.

**Analysis of metabolites.** Fragments of characterized single fibers of at least 1 mm were analyzed for PCr and Cr using reverse-phase high-performance liquid chromatography with ultraviolet photometric detection, following overnight extraction in 60% methanol (4, 8). The ratio of PCr to Cr was used as a measure of the recruitment of individual muscle fibers (2, 3).
Analysis of Each Second Muscle Sample

After bringing the frozen muscle samples to −20°C, serial sections of 10 μm were cut. Serial cross sections were stained for mATPase and glycogen content as described above. In each biopsy 100–150 fibers were analyzed.

Maximal and Submaximal Dynamic Leg Forces and Power

Maximal leg dynamic forces and power during the 45-min exercise protocol were measured on an isokinetic cycle ergometer that had two configurations: a conventionally electrically braked and an isokinetic configuration (1). Subjects were seated on the ergometer with their feet strapped to the pedals while the motor was switched on and the pedal frequency was set at 90 rpm. The electrically braked configuration was switched on during the 45-min protocol. For maximal dynamic force and power measurements, a coupling to the isokinetic system was made before exercise and after 1, 10, and 45 min of exercise. Subjects attempted to increase their pedal frequency by exerting maximal voluntary force to the pedals during every revolution for ~6 s. The isokinetic system maintained the pedal frequency (90 rpm) within 5%. Prior to and throughout the maximal dynamic force measurements, forces vertical and horizontal to the pedal surface were measured by means of strain gauges mounted inside the pedals (1). Force data were stored on disk for later analyses.

Peak tangential forces and peak power for each revolution were calculated. Peak tangential force was the greatest effective force (i.e., force exerted tangentially to the arc of crank rotation) in each revolution. Peak power was the power generated at the instant of the peak tangential force (peak power = peak tangential force × pedal frequency). For the maximal dynamic force measurements, maximal peak tangential force and power values were determined as the mean of three consecutive values in which the highest observed peak value occurred. An indication of fatigue was obtained by calculating maximal tangential forces prior to and during the 45-min protocol. To determine the exercise intensity throughout the 45-min protocol, peak tangential forces prior to the maximal dynamic force measurements at 1, 10, and 45 min of exercise were averaged and divided by the mean maximal peak tangential force prior to the exercise.

Statistics

Data are presented as mean values ± SD. To investigate the change in activation of the proportion of type I and II fibers during the exercise with the use of the PCr/Cr ratio method, cumulative distributions of the PCr/Cr ratios of individual fibers were calculated for both fiber types, using intervals of 0.1. Kolmogorov-Smirnov two-sample tests were performed on the cumulative distributions to test for differences in both the location and the shape of the distributions (17). The level of significance of all statistical analyses was set at P < 0.05.

The Kolmogorov-Smirnov test was used to check for normality of the data. Because the data of the OD PAS values of the whole muscle sections were not normally distributed, the nonparametric Kruskal-Wallis test was used to test for significant differences in fiber activation per fiber group during the cycling exercise, followed by a Mann-Whitney U-test for post hoc comparisons. At each time point 100–150 fibers were analyzed from each whole muscle section for each subject. Fibers were grouped per subject (n = 6). From each first biopsy, 20–30 single fiber fragments of each type were analyzed for the single-fiber analysis for each subject at each time point. Single fibers were subsequently grouped per subject (n = 6). For the PCr/Cr values of each fiber group at rest, the 5th percentile value was determined. For the OD PAS values of the single fibers univariate ANOVA was used, followed by a Bonferroni test for post hoc comparisons.

RESULTS

With the use of immunohistochemistry we investigated the presence of the IIX myosin heavy chains (MHC) in the single fibers. Only 10.8 ± 9.2% of all fibers contained the IIX MHC and some subjects did not express any IIX MHC at all. Therefore we did not distinguish between type II subgroups.

Force and Power

The exercise intensity of 75% \( \dot{V}_{O2\text{max}} \) corresponded to 38.3 ± 5.0% of the maximal dynamic muscle force. After 45 min of cycling maximal dynamic force and power on the pedals were 93 ± 16% of the maximum before the cycling exercise, which differed not significantly from preexercise (P > 0.05).

Single-Fiber Analysis: PCr/Cr Ratio and OD Values

PAS Staining

Figure 1 shows the PCr/Cr ratios of the single fiber fragments of all subjects for both fiber types before and during the cycling exercise. The horizontal dashed lines represent the 5th percentile of the resting values of the type I and II fibers. Fibers with a PCr/Cr ratio below this 5th percentile value for the same fiber type show evidence of activation (3). PCr/Cr ratios of all fiber fragments for the type I and II fibers at rest and during exercise are shown in Table 1. The cumulative distribution analysis showed for both fiber types a significant shift to lower PCr/Cr ratios after 1, 10, and 45 min of exercise compared with the resting distribution, albeit this shift was smaller for the type II fibers compared with the type I fibers. After 1, 10, and 45 min of exercise, 85% of the type I fibers had a 50% lower PCr/Cr value compared with the mean resting value. For the type II fibers, only 70% of the fibers had a PCr/Cr value lower than 50% compared with the resting fibers.

In Fig. 2, the relationship between the OD values of the PAS staining and the PCr/Cr ratios of the same single fibers is shown for the type I (Fig. 2A) and type II (Fig. 2B) fibers of all subjects after 45 min of exercise. The horizontal and vertical lines represent the 5th percentile of the resting values for, respectively, the OD PAS values and the PCr/Cr ratios. As explained above, fibers with PCr/Cr ratios below the 5th percentile of resting value for the type I (1.09) and II (0.90) fibers.

Fig. 1. Phosphocreatine-to-creatine (PCr/Cr) ratios of all subjects at rest and after 1, 10, and 45 min of exercise in type I and II fibers. Mean values for the group of fibers are shown by –. The horizontal dashed lines reflect the 5th percentile of resting value for the type I (1.09) and II (0.90) fibers.
percentile of this resting value show evidence of activation. To be able to compare both methods, we used the same 5th percentile criterion for the OD PAS values. For the type I fibers, 80% of the fibers were recruited according to the PCr/Cr ratio method, whereas according to the glycogen depletion method only 60% of the fibers were recruited. For the type II fibers, 55 and 25% of the fibers were recruited according to, respectively, the PCr/Cr ratio method and the glycogen depletion method. Note that the OD PAS data for the single fiber fibers were very similar to the ones for the whole muscle sections (see below).

Whole Muscle Section Analysis: OD Values PAS Staining

Figure 3 shows the OD values of the PAS staining on the whole muscle sections. The OD PAS values for both type I and type II fibers decreased significantly after 1 min of exercise. These values decreased for both fiber types significantly further after 10 and 45 min of exercise. Furthermore, there was a trend for higher OD PAS values for the type II compared with the type I fibers ($P < 0.072$).

DISCUSSION

The main finding of the present study was that both type I and type II fibers were recruited within 1 min of exercise at 75% $V_{O2\text{max}}$, which corresponded to 38.3% of the maximal dynamic muscle force. With the PCr/Cr ratio method it was further clear that the same proportion of fibers remained activated during the whole 45 min of exercise.

Force and Power

In agreement with the study of Sargeant and Jones (16), we observed that cycling at an exercise intensity of 75% $V_{O2\text{max}}$ required ~38% of the maximal available dynamic force. Although maximal dynamic force and power did not decline significantly during 45 min of cycling, this does not necessarily indicate that subjects were not fatigued at all during the cycle exercise. Some of the subjects reported that the exercise could not be sustained much longer, indicating that perhaps some fatigue occurred.

Recruitment According to the PCr/Cr Ratio Method

Beltman et al. (3) developed the PCr/Cr ratio method to detect fiber activation and found that with this method muscle fiber activation could be detected after only ~7 maximal voluntary isometric contractions of 1-s duration. In isometric contractions at an intensity of 39% MVC only a relative small proportion (11%) of type II fibers was found to be activated (2). To allow comparison of our results during exercise with the results of Beltman et al. (2), the first biopsy in our experiment was taken after 1 min of cycle exercise, which was enough to properly detect fiber recruitment on the basis of changes in the PCr/Cr ratio.

The PCr/Cr ratio of a number of both the type I and II fibers was lower compared with the 5th percentile of their respective

<table>
<thead>
<tr>
<th>Type</th>
<th>Rest</th>
<th>1 min</th>
<th>10 min</th>
<th>45 min</th>
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<tbody>
<tr>
<td>I</td>
<td>1.88±0.45</td>
<td>0.59±0.36*</td>
<td>0.57±0.36*</td>
<td>0.62±0.29*</td>
</tr>
<tr>
<td>II</td>
<td>1.61±0.55</td>
<td>0.66±0.43*</td>
<td>0.65±0.55*</td>
<td>0.60±0.44*</td>
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Values are means ± SD. *Significantly different from rest ($P < 0.05$).
resting values after already 1 min of exercise at only 38% of the maximal available dynamic force, which demonstrates recruitment of both type I and II fibers, albeit that 25% fewer type II fibers were activated compared with type I fibers after 45 min of exercise (Fig. 2). After a rapid decrease within 1 min of exercise, in both type I and type II fibers, the PCr/Cr ratio remained unchanged during the rest of the exercise (Fig. 1), demonstrating a constant proportion of activated type I and II fibers and hence a constant energy flow in the fibers during the cycle exercise.

Recruitment According to the Glycogen Depletion Method

Vøllestad et al. (20) demonstrated a close relationship between the optical density of the PAS stain and the glycogen concentration over a wide range of glycogen concentrations. A decline in OD PAS value is therefore a reliable indication of fiber recruitment. OD PAS values for both type I and II fibers were significantly decreased after 1 min of exercise and decreased significantly further after 10 and 45 min for both fiber types (Fig. 3), which was in line with the results of Vøllestad et al. (20).

Comparison of PCr/Cr Ratio Method and Glycogen Depletion Method

According to the acute PCr/Cr ratio method a substantial part of the type I and II fibers were activated after 45 min of exercise, whereas according to the glycogen depletion method fewer fibers were activated (Fig. 3). It can therefore be concluded that even after 45 min of exercise the glycogen depletion method could not fully demonstrate fiber activation, because of the slow cumulative character of this method.

Activation of Type II Fibers

We showed that not only the type I but also the type II fibers were activated within 1 min of exercise at 75% VO2max, corresponding to 38% of the maximal dynamic muscle force. According to De Haan (5), higher stimulation frequencies are needed for concentric compared with isometric contraction torques, which suggests that actual intracellular calcium concentration needs to be higher for dynamic than for isometric contractions at similar relative torques. More evidence comes from motor unit studies in which higher motor unit firing frequencies (13, 18, 19) and recruitment of additional motor units (9, 13, 19) were found during voluntary shortening compared with voluntary isometric contractions. Moreover, recruitment thresholds of motor units during concentric contractions were found to be lower compared with isometric contractions (11, 18, 19). We conclude that, because of the dynamic character of the cycling exercise, a greater proportion of type II fibers was recruited than expected from isometric measurements. Furthermore, we conclude that the PCr/Cr method is more accurate in determining fiber activation than the glycogen depletion method.

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