Chapter 2

Recruitment of single muscle fibres during submaximal cycling exercise
Abstract

In literature, an inconsistency exists in the submaximal exercise intensity at which type II fibres are activated. In the present study, the recruitment of type I and II fibres 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 fibres 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 fibres 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 fibre recruitment. Within 1 min of exercise both type I and, although to a lesser extent, type II fibres were recruited. Furthermore, the PCr/Cr ratio revealed that the same proportion of fibres was recruited during the whole 45 min of exercise, indicating a rather constant recruitment. The PAS staining, however, proved inadequate to fully demonstrate fibre recruitment even after 45 min of exercise. We conclude that during cycling exercise a greater proportion of type II fibres 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 fibre activation than the PAS stain intensity method.
Introduction
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 fibres, are recruited at low levels of force, and, as force increases, larger motor units (first fatigue resistant and later fast fatigable), containing type IIA and IIX fibres, are recruited. This is referred to as the size principle (Henneman et al, 1965). Beltman et al (2004a) investigated the initial activation of different muscle fibre types during repeated isometric contractions of the quadriceps muscle at different intensities and showed a predominant activation of type I fibres at 39 % of the maximal voluntary contractile (MVC) force, whereas a substantial activation of type II fibres was only detected at the exercise intensity of 72 % MVC and higher. Vøllestad et al (1984) investigated the activation pattern of type I and type II fibres during dynamic cycling exercise at an intensity of 75 % of the maximal oxygen uptake (Vo2max) at constant pedalling rate. According to Sargeant and Jones (1995) cycling at an exercise intensity of 75 % Vo2max requires only ~40 % of the maximal dynamic muscle force exerted on the pedals. In contrast to the results of Beltman et al (2004a), Vøllestad et al (1984) found activation of both type I and type II fibres during the dynamic cycling exercise at the exercise intensity using ~40 % of the maximal muscle force. Thus there seems to be an inconsistency in the submaximal exercise intensity at which type II fibres 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 fibre activation. In the studies of Vøllestad et al (1984), the glycogen depletion method is used to determine fibre activation, using the periodic acid-Schiff (PAS) reaction, whereas Beltman et al (2004a) assessed fibre activation using the phosphocreatine (PCr) – to – creatine (Cr) ratio (PCr/Cr) in muscle fibre fragments. The glycogen depletion method has the advantage that it is an accepted and commonly used method for the assessment of fibre recruitment. However, the glycogen depletion as indicator of fibre activation should be used with care (Kernell et al, 1995). Because of the relatively high concentration of glycogen in human muscle fibres, 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 (Infante et al, 1965), which allows one to assess single muscle fibre recruitment quantitatively after exercise of short duration. The
disadvantage is that this methodology may lead to an underestimation of the number of activated fibres as indicated in the study of Beltman et al (2004b).

Secondly, the mode of contraction may account for the differences between the studies of Vøllestad et al (1984) and Beltman et al (2004a). Where the subjects in the study of Beltman et al (2004a) performed isometric contractions, a dynamic cycling exercise, comprising of concentric contractions, was performed in the study of Vøllestad et al (1984). It has been shown that in rat muscle higher stimulation frequencies are needed for concentric compared with similar isometric contraction torques (de Haan, 1998). The larger degree of muscle activation needed for dynamic exercise could explain the additional recruitment of type II fibres in the study of Vøllestad et al (1984).

In the present study we wanted to make a comparison between the isometric exercise in the study of Beltman et al (2004a) and the dynamic exercise performed in the study of Vøllestad et al (1984). Moreover, we wanted to study the initial activation during dynamic exercise, which has never been studied. The first aim of this study was therefore to assess the changes in type I and II fibre recruitment from the very beginning and throughout longer term cycling exercise. We hypothesized that despite the relatively low exercise intensity (as percentage of the maximal dynamic muscle force) both type I and II fibres would be recruited from start of the exercise, because of the dynamic character of the exercise. The second aim of this study was to compare the glycogen depletion method with the PCr/Cr ratio method. With the glycogen depletion method a cumulative measurement of energy utilization is obtained, whereas with the PCr/Cr ratio method an acute measurement of the energy state is obtained, which has the potential to investigate rate coding or additional fibre activation.

**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 $V_{O2max}$ at a pedalling 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. $V_{O2max}$ had been determined previously during an incremental cycling test, during which the load required to elicit the 75 % $V_{O2max}$ had also been established. To overcome a slow rise in oxygen uptake throughout the 45-min cycle exercise (Krustrup et al, 2004), the load was decreased by 5 % after 10 min of exercise. The intensity of 75% $V_{O2max}$ was therefore maintained during the full 45-min exercise.

Experiment 1: Muscle biopsies
In the first experiment muscle fibre activation during a 45-min cycle exercise was measured. The exercise bout was performed on a mechanically braked bicycle ergometer. At 4 different time points two muscle samples were taken from the m. vastus lateralis. Exercise was interrupted for 1 min to take the biopsies; thereafter, subjects continued the cycling exercise. From each first muscle sample single fibres were analyzed for fibre type, PCr, Cr and glycogen content and whole muscle sections from each second muscle sample were analyzed for fibre type and glycogen content.

Experiment 2: Force and power
In the second experiment (changes in) maximal and submaximal dynamic leg forces and power were determined during a similar 45-min cycle exercise on an isokinetic ergometer by means of strain gauges in the pedals (Sargeant et al, 1981). Maximal dynamic leg forces and power were measured by exerting maximal force on the pedals at each revolution at the same four time points as in Experiment 1. Exercise was also interrupted for 1 min to perform the maximal dynamic force measurements. Submaximal dynamic forces during the exercise were measured prior to the maximal measurements by recording the submaximal force exerted on the pedals. The submaximal dynamic force values were expressed as percentage of the maximal dynamic force prior to the 45-min cycle exercise, to express exercise intensity as a percentage of maximal dynamic force.
Muscle sample collection and analysis

Muscle biopsy samples were obtained using a Bergström type biopsy needle (diameter of 5 mm, Popper Biomedical Instruments, Schuco International London Limited, London, UK) with suction. To facilitate immediate sampling, both legs were prepared for biopsy sampling before the experiment. Two small incisions were made in the skin and fascia in both legs (approximately one-third distal of the distance between the lateral femoral epicondyle and trochanter major, 5 cm in between two incisions) under local anesthesia (2 % lidocaine). At rest and after 1, 10 and 45 min of exercise, two biopsies were taken from one incision of the m. vastus lateralis, with tissue being collected from an adjacent location, with the needle directed either proximally or distally. Biopsies at the four different time points were alternately taken from both legs. 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 fibre 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 fibre fragment was then cut into 2 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 (Karatzafieri et al, 1999; Sant'ana Pereira et al, 1995).

Histochemistry

Twenty fibre fragments of each muscle sample were embedded in a double layer of a gelatin solution (Sant'ana Pereira et al, 1995). 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 (Brooke and Kaiser, 1970) 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, Germany). Optical density values (OD) of each fibre fragment were measured for each staining. Based on the OD values from mATPase stainings after preincubation at pH 4.4 and pH 4.7 fibres were classified into type I and II fibres (Sant'ana Pereira et al., 1995). The OD values of the PAS staining provided an indication of the glycogen content (Vøllestad et al., 1984) and served as a marker for fibre recruitment.

**Analysis of metabolites**

Fragments of characterized single fibres of at least 1 mm were analyzed for PCr and Cr using reverse-phase high-performance liquid chromatography with ultra-violet photometric detection, following overnight extraction in 60% methanol (Brooke and Kaiser, 1970; Karatzaferi et al., 1999). The ratio of PCr to Cr was used as a measure of the recruitment of individual muscle fibres (Beltman et al., 2004a; Beltman et al., 2004b).

**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 fibres 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 (Beelen et al., 1994). 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 (Beelen et al., 1994). 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 x 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 fibres during the exercise with the use of the PCr/Cr ratio method and the glycogen depletion method, cumulative distributions of the PCr/Cr ratios of individual fibres were calculated for both fibre 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 (Siegel, 1956). The level of significance of all statistical analysis was set at P < 0.05.

The Kolmogorov-Smirnov test was used to check for normality of the data. Because of the data of the OD PAS values of the whole muscle sections were not normally distributed, the non-parametric Kruskall Wallis test was used to test for significant differences in fibre activation per fibre group during the cycling exercise, followed by a Mann-Whitney U-test for post hoc comparisons. 100-150 fibres were analyzed from each whole muscle section for each subject at each time point. Fibres were grouped per subject (n = 6). From each first biopsy, 20-30 single fibre fragments of each type were analyzed for the single-fibre analysis for each subject at each time point. Single fibres were subsequently grouped per subject (n = 6). For the PCr/Cr values of each fibre group at rest, the 5th percentile value was determined. For the OD PAS values of the single fibres 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 fibres. Only 10.8 ± 9.2 % of all fibres 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 % $V_{O_2\text{max}}$ 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 pre-exercise ($P > 0.05$).

Single fibre analysis: PCr/Cr ratio & OD values PAS staining

Figure 2.1 shows the PCr/Cr ratios of the single fibre fragments of all subjects for both fibre types before and during the cycle exercise. The horizontal dashed lines represent the 5th percentile of the resting values of the type I and II fibres. Fibres with a PCr/Cr ratio below this 5th percentile value for the same fibre type show evidence of activation (Beltman et al, 2004b). PCr/Cr ratios of all fibre fragments for the type I and II fibres at rest and during exercise are shown in Table 2.1. It should be noted that, similar to the results of Beltman et al (2004a), there is a large variation in PCr/Cr ratio (Figure 2.1 and Table 2.1), which is a consequence of using a ratio with dependent variables. Any change in PCr will have an opposite change in Cr, leading to a larger variation compared to using e.g. only absolute PCr data. Methodological, it is however more accurate to use the ratio because of the large variation which will be introduced by inaccurate weighing of small single fibre fragments.

<table>
<thead>
<tr>
<th>Type</th>
<th>Rest</th>
<th>1 min</th>
<th>10 min</th>
<th>45 min</th>
</tr>
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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>
</tr>
</tbody>
</table>

Values are means ± SD. *Significantly different from rest ($p < 0.05$).

The cumulative distribution analysis showed for both fibre 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 fibres compared with the type I fibres (Figure 2.2). After 1, 10, and 45 min of exercise, 85 % of the type I fibres had a
50% lower PCr/Cr value compared with the mean resting value. For the type II fibres, only 70% of the fibres had a PCr/Cr value lower than 50% compared with the resting fibres. For the glycogen depletion method, the cumulative distribution analysis also showed a significant shift to lower OD PAS values after 1, 10 and 45 min of exercise compared with the resting distributions for both fibre types (Figure 2.3). Although the shifts in frequency distributions were smaller for the glycogen depletion method compared to the PCr/Cr ratio method, the shifts to lower OD PAS values were significant for both type I and II fibres, indicating that both fibre types were activated after 1 min of exercise.

![PCr/Cr ratios](image)

**Figure 2.1.** 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 fibres. Mean values for the group of fibres are shown by –. The horizontal dashed lines reflect the 5th percentile of resting value for the type I (1.09) and II (0.90) fibres.

**Whole muscle section analysis: OD values PAS staining**

Figure 2.4 shows the OD values of the PAS staining on the whole muscle sections. The OD PAS values for both type I and type II fibres decreased significantly after 1 min of exercise. These values decreased for both fibre 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 fibres (P = 0.072).
Figure 2.2. Cumulative frequency distribution of single fibre PCr/Cr ratios in type I (A) and II (B) fibres at rest (solid line), after 1 min (dotted line), 10 min (dashed line) and 45 min (dash-dot-dot line) of exercise. The closed circle represents the mean resting value ± SD. *Indicates significantly different distribution compared to 1, 10 and 45 min of exercise; †indicates significantly different in the shape of the distribution compared to 45 min of exercise (P < 0.05).
Figure 2.3. Cumulative frequency distribution of single fibre OD PAS values in type I (A) and II (B) fibres at rest (solid line), after 1 min (dotted line), 10 min (dashed line) and 45 min (dash-dot-dot line) of exercise. The closed circle represents the mean resting value ± SD. *Indicates significantly different distributions between rest, 1, 10 and 45 min of exercise (P < 0.05).
Figure 2.4. Mean values (± SD) for OD values of PAS staining of the type I (closed circles) and II (open circles) fibres of the whole muscle sections at rest and during exercise. *, †Significant differences from each time point for the type I and type II fibres, respectively (P < 0.05).

**Discussion**

The main finding of the present study was that both type I and type II fibres were recruited within 1 min of exercise at 75 % $V_{o_{2\text{max}}}$, 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 fibres remained activated during the whole 45 min of exercise.

**Force and Power**

In agreement with the study of Sargeant and Jones (1995), we observed that cycling at an exercise intensity of 75 % $V_{o_{2\text{max}}}$ 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 (2004b) developed the PCR/Cr ratio method to detect fibre activation and found that with this method muscle fibre 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 fibres was found
to be activated (Beltman et al, 2004a). To allow comparison of our results during exercise with the results of Beltman et al (2004a), the first biopsy in our experiment was taken after 1 min of cycle exercise, which was enough to properly detect fibre 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 fibres was lower compared to the 5th percentile of their respective 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 fibres, albeit that 15 % fewer type II fibres were activated compared with type I fibres after 45 min of exercise (Figure 2.2). After a rapid decrease within 1 min of exercise, in both type I and type II fibres, the PCr/Cr ratio remained unchanged during the rest of the exercise (Figures 2.1 and 2.2), demonstrating a constant proportion of activated type I and II fibres and hence a constant energy flow in the fibres during the cycle exercise.

Recruitment according to the glycogen depletion method
Vøllestad et al (1984) 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 fibre recruitment. OD PAS values for both type I and II fibres were significantly decreased after 1 min of exercise and decreased significantly further after 10 and 45 min for both fibre types (Figures 2.3 and 2.4), which was in line with the results of Vøllestad et al (1984).

Comparison of PCr/Cr ratio method and glycogen depletion method
With the use of the acute PCr/Cr ratio method larger shifts in the cumulative frequency distributions were shown compared to the slow cumulative glycogen depletion method (Figures 2.2 and 2.3). It can therefore be concluded that the PCr/Cr ratio method was more sensitive in determining fibre activation, especially during the initial phase of exercise.

Activation of type II fibres
We showed that not only the type I but also the II fibres were activated within 1 min of exercise at 75 % \( V_{O_{2\text{max}}} \), corresponding to 38 % of the maximal dynamic muscle force. According to de Haan (1998), higher stimulation frequencies are needed for concentric compared to 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 (Pasquet et al, 2006; Sogaard et al, 1998; Tax et al, 1989) and recruitment of additional motor units (Kato et al, 1985; Pasquet et al, 2006; Tax et al, 1989) were found during voluntary shortening compared to voluntary isometric contractions. Moreover, recruitment thresholds of motor units during concentric contractions were found to be lower compared to isometric contractions (Kossev and Christova, 1998; Sogaard et al, 1998; Tax et al, 1989). We conclude that, because of the dynamic character of the cycling exercise, a greater proportion of type II fibres was recruited than expected from isometric measurements. Furthermore, we conclude that the PCr/Cr method is more sensitive in determining fibre activation than the glycogen depletion method, especially during the initial phase of exercise.

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References


