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1 **Each-step activation of oxidative phosphorylation is**  
2 **necessary to explain muscle metabolic kinetic responses**  
3 **to exercise and recovery in humans**

4

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6

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15 Running title: Each-step activation of OXPHOS in human muscle during exercise transitions

16

17 Keywords: human skeletal muscle; exercise; oxygen uptake

18

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22 **Key points**

- 23
- The basic control mechanisms of oxidative phosphorylation (OXPHOS) and glycolysis  
24 during work transitions in human skeletal muscle are still a matter of debate
  - We used simulations of skeletal muscle bioenergetics to identify key system features  
25 that contribute to this debate, by comparing kinetic model outputs to experimental  
26 human data, including phosphocreatine (PCr), pH, pulmonary oxygen uptake and  
27 fluxes of ATP production by OXPHOS ( $v_{OX}$ ), anaerobic glycolysis and creatine  
28 kinase in moderate and severe intensity exercise transitions
  - We found that each-step activation (ESA) of particular OXPHOS complexes, NADH  
29 supply and glycolysis, and strong (third-order) glycolytic inhibition by protons, was  
30 required to reproduce observed PCr, pH, and  $v_{OX}$  kinetics during exercise
  - A slow decay of ESA during recovery, which was slowed further following [severe](#)  
31 exercise, was necessary to reproduce experimental findings
  - Well-tested computer models offer new insight in the control of the human skeletal  
32 muscle bioenergetic system during physical exercise
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38 **ABSTRACT**

39 To better understand muscle bioenergetic regulation, a previously-developed model  
40 of the skeletal muscle cell bioenergetic system was used to simulate the influence of: 1) each  
41 step activation (ESA) of NADH supply (including glycolysis) and oxidative phosphorylation  
42 (OXPHOS) complexes; and 2) glycolytic inhibition by protons, on the kinetics of ATP  
43 synthesis from OXPHOS, anaerobic glycolysis and creatine kinase (CK). Simulations were  
44 fitted to previously published experimental data of ATP production fluxes and metabolite  
45 concentrations during moderate and **severe** intensity exercise transitions in bilateral knee-  
46 extension in humans. Overall, computer simulations agreed well with experimental results.  
47 Specifically, a large (>5-fold) direct activation of all OXPHOS complexes was required to  
48 simulate measured phosphocreatine (PCr) and OXPHOS responses to both moderate and  
49 **severe** intensity exercise. In addition, slow decay of ESA was required to fit PCr recovery  
50 kinetics, and the time constant of ESA decay was slower following **severe** (180s) than  
51 moderate (90s) exercise. Additionally, a strong inhibition of (anaerobic) glycolysis by protons  
52 (glycolytic rate inversely proportional to the cube of proton concentration) provided the best  
53 fit to the experimental pH kinetics, and may contribute to the progressive increase in  
54 oxidative ATP supply during acidifying contractions. During **severe**-intensity exercise an  
55 'additional' ATP usage (a 27% increase at 8 min, above the initial ATP supply) was  
56 necessary to explain the observed  $\dot{V}O_2$  slow component. Thus parallel activation of ATP  
57 usage and ATP supply (ESA), and a strong inhibition of ATP supply by anaerobic glycolysis,  
58 were necessary to simulate the kinetics of muscle bioenergetics observed in humans.

## 59 **Abbreviations**

60  $A_{GL}$ , relative activation of glycolysis;  $A_{OX}$ , relative activation of oxidative phosphorylation;  $A_{UT}$ ,  
61 relative activation of ATP utilization; AK, adenylate kinase; CK, creatine kinase; ESA, each-  
62 step activation; KE, bilateral knee extension exercise; MAS, malate/aspartate shuttle;  
63 Moderate glycolysis inhibition by  $H^+$ , first-order dependence on  $H^+$ ; NADH, nicotinamide  
64 adenine dinucleotide; OXPHOS, oxidative phosphorylation; PCr, phosphocreatine;  $P_i$ ,  
65 inorganic phosphate; Strong glycolysis inhibition by  $H^+$ , third-order dependence on  $H^+$ ;  $S_{CK}$ ,  
66 stoichiometry of proton production/consumption by CK; TCA, tricarboxylic acid cycle;  $v_{CK}$ ,  
67 muscle ATP production by creatine kinase;  $v_{GL}$ , muscle ATP production by anaerobic  
68 glycolysis;  $v_{OX}$ , muscle ATP production by oxidative phosphorylation;  $v_{UT}$ , muscle ATP  
69 utilization (ATP hydrolysis);  $\dot{V}O_2$ , oxygen uptake (muscle or pulmonary).

## 70 INTRODUCTION

71 The basic mechanisms of the control of the skeletal muscle cell bioenergetic system,  
72 especially oxidative phosphorylation (OXPHOS), during work transitions is still a matter of  
73 debate. According to the original proposition by Chance and Williams (Chance & Williams,  
74 1955), based on studies on isolated mitochondria, only ATP usage (actomyosin-ATPase and  
75  $\text{Ca}^{2+}$ -ATPase) is directly activated by  $\text{Ca}^{2+}$  during rest-work transition in skeletal muscle,  
76 while the ATP-supply system, including  $\text{O}_2$ -consuming OXPHOS ( $\dot{V}\text{O}_2$ ), is activated indirectly  
77 through negative feedback via increased sarcoplasmic concentration of the products of ATP  
78 hydrolysis: ADP and  $\text{P}_i$ . Several theoretical models assume, explicitly or implicitly, this  
79 mechanism (see e.g., Wilson et al., 1979; [Wilson, 2015](#); Wu et al., 2007).

80 Jeneson and co-workers postulated that the mechanistic  $\dot{V}\text{O}_2$ -[ADP] dependence is at  
81 least second-order (Jeneson et al. 1996). The discovery that three TCA (tricarboxylic acid)  
82 cycle dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, 2-oxoglutarate  
83 dehydrogenase) are activated by  $\text{Ca}^{2+}$  ions (Denton & McCormack, 1990; Hansford, 1980)  
84 led to the postulate that the NADH-supply system is directly activated together with ATP  
85 usage. This possibility was supported by the discovery of a large stimulation of  $\dot{V}\text{O}_2$  in  
86 isolated brain mitochondria by  $\text{Ca}^{2+}$  ions acting through activation of the malate/aspartate  
87 shuttle (MAS) (Gellerich et al. 2012).

88 A general model of parallel activation of ATP usage and ATP supply during muscle  
89 contractions was postulated by Hochachka (1994), but this did not specify which particular  
90 enzymes/metabolic blocks in the ATP supply system were activated. It was subsequently  
91 proposed that not only ATP usage and NADH supply (including glycolysis), but also all  
92 OXPHOS complexes (complex I, complex III, complex IV, ATP synthase, ATP/ADP carrier,  $\text{P}_i$   
93 carrier) are directly activated by some cytosolic mechanism predominantly involving cytosolic  
94  $\text{Ca}^{2+}$  ions, calmodulin-like protein, and protein phosphorylation, during the rest-to-work  
95 transition in skeletal and heart muscle cells (Korzeniewski, 1998; Korzeniewski, 2003;  
96 Korzeniewski, 2007; Korzeniewski, 2014, Korzeniewski, 2015). This process is termed each-  
97 step-activation (ESA) (Korzeniewski, 2014). In skeletal muscle it is likely that a mixed

98 mechanism (MM) is manifest, in which all OXPHOS complexes are directly activated, but to a  
99 smaller extent than ATP usage, and therefore a moderate increase in [ADP] and [P<sub>i</sub>]  
100 cooperates with ESA to bring about OXPHOS activation (Korzeniewski, 2014). In intact heart  
101 muscle *in vivo* there is no (or extremely small) change in metabolite concentrations during  
102 work transitions (Katz et al. 1989). Therefore, while high expression of OXPHOS  
103 components may explain, in part, the a high sensitivity of ATP supply to very small changes  
104 in muscle metabolites, a 'perfect' ESA, directly activating both ATP usage and OXPHOS to  
105 the same extent, has been suggested to operate in intact heart *in vivo* (Korzeniewski, 2014).  
106 The possibility of the parallel activation of ATP demand and ATP supply during rest-work  
107 transition in skeletal muscle was supported by Wüst and co-workers (Wüst et al. 2011) on  
108 the basis of experimental measurement of changes of muscle  $\dot{V}O_2$  and [PCr] after the onset  
109 of electrically-stimulated contractions in the canine hind limb. Nevertheless, evidence  
110 supporting or refuting ESA has proven technically challenging in human muscle due to the  
111 complexity involved in determining, at the necessary high temporal resolution, instantaneous  
112 intramuscular metabolite concentrations, fluxes and relative activities of the various  
113 components of the bioenergetics systems during exercise.

114         Additionally, it has been demonstrated both *in vitro* (see Connet & Sahlin, 1996 for  
115 review) and in intact human skeletal muscle (Sutton et al. 1981) that cytosolic acidification  
116 inhibits (anaerobic) glycolysis. Thus, during high-intensity exercise characterised by a  
117 progressive metabolic acidosis, glycolytic inhibition may contribute to increasing the  
118 demands of ATP provision from OXPHOS.

119         The ESA mechanism was proposed mainly on the basis of theoretical studies carried  
120 out using a computer model of the skeletal muscle bioenergetic system developed previously  
121 (Korzeniewski, 1998; Korzeniewski & Zoladz, 2001, Korzeniewski & Liguzinski, 2004). This  
122 model includes a simple, semi-quantitative inhibition of glycolysis by protons, in which the  
123 rate of glycolysis is inversely proportional to the instantaneous H<sup>+</sup> concentration (the simplest  
124 possible description) (Korzeniewski & Liguziński, 2004). Recent investigation led to the  
125 proposal that the inhibition of ATP supply from anaerobic glycolysis by progressive H<sup>+</sup> ion

126 accumulation, together with a slow decrease of ATP supply by creatine kinase (CK) and an  
127 additional progressive increase in ATP demand, may contribute importantly to the  
128 progressive increase in  $\dot{V}O_2$  seen during high-intensity constant power exercise: the muscle  
129  $\dot{V}O_2$  slow component (Korzeniewski & Zoladz, 2015). The pulmonary  $\dot{V}O_2$  slow component is  
130 generated principally within the exercising skeletal muscles (Poole et al., 1991).

131 While this computational model was extensively verified by comparison with various  
132 experimentally measured parameter and variable values and system properties (see e.g.,  
133 Korzeniewski, 2007; Korzeniewski, 2014 for discussion), rarely has a direct comparison with  
134 biological data been made that would provide a satisfying, strictly quantitative, verification of  
135 the model and its postulates. This is mainly because the necessary *in vivo* measurements of  
136 several different variables during rest-work and work-rest transitions below [the lactate](#)  
137 [threshold](#) (moderate intensity) and above [critical power](#) ([severe](#) intensity), were not  
138 previously available. Recently, several different system variables, including time courses of  
139 pulmonary  $\dot{V}O_2$ , PCr and pH during transitions to and from moderate and [severe](#) intensity  
140 exercise as well as the rate of ATP supply by OXPHOS (vOX), CK (vCK) and anaerobic  
141 glycolysis (vGL) after 3 and 8 min of exercise during bilateral knee extension (KE) in humans  
142 were measured (Cannon et al. 2014). These data constitute an excellent reference point for  
143 computer model validation.

144 In this study, therefore, we tested the previously-developed computer model of the  
145 skeletal muscle bioenergetic system (Korzeniewski, 1998; Korzeniewski & Zoladz, 2001;  
146 Korzeniewski & Liguzinski, 2004) by direct comparison of computer simulations with  
147 published experimental data for transitions to and from moderate and [severe](#) intensity KE  
148 exercise in humans (Cannon et al. 2014). We hypothesized that a high intensity of ESA is  
149 necessary to account for the measured changes in fluxes and metabolite concentrations, as  
150 well as for the shape of time courses of these variables during transitions from rest to  
151 moderate or [severe](#) exercise and back to rest. We expected that ESA decays slowly during  
152 muscle recovery after exercise and that the characteristic decay time is longer after [severe](#)  
153 exercise than after moderate exercise. Finally, we tested the hypothesis that a progressive

154 increase in ATP usage during exercise and a strong glycolytic inhibition by protons in severe  
155 intensity exercise could account for the relative magnitude of the  $\dot{V}O_2$  and  $vOX$  slow  
156 component measured *in vivo*.

## 157 **METHODS**

158

### 159 ***Ethical approval***

160 The human data used in the present study for comparison with computational  
161 simulations were previously published (Cannon et al. 2014). All procedures were approved  
162 by The Biological Sciences Faculty Research Ethics Committee, University of Leeds, and the  
163 University of Liverpool Committee on Research Ethics, and complied with the latest revision  
164 of the Declaration of Helsinki. Written informed consent was obtained from all volunteers  
165 prior to their participation in the study. Further details on the experimental human data can  
166 be found in Cannon et al. (2014).

167

### 168 ***Computer model***

169 The theoretical model of the skeletal muscle cell bioenergetics including anaerobic  
170 glycolysis developed by Korzeniewski and Liguzinski (2004), based on earlier models by  
171 Korzeniewski and Zoladz (2001) and Korzeniewski (1998), was used in the present study.  
172 This model comprises particular OXPHOS complexes (complex I, complex III, complex IV,  
173 ATP synthase, ATP/ADP carrier, P<sub>i</sub> carrier), anaerobic glycolysis, CK, ATP usage, NADH  
174 supply, and proton efflux and influx.

175 The model has been broadly validated by comparison of its predictions with  
176 experimental data and used for numerous theoretical studies (see e.g., Korzeniewski, 2007;  
177 Korzeniewski, 2011, Korzeniewski 2014 for overview). The complete model description of the  
178 skeletal muscle bioenergetic system including anaerobic glycolysis is located on the web  
179 site: <http://awe.mol.uj.edu.pl/~benio/>.

180

### 181 ***Simulation procedures***

182 We aimed to model the on- (rest-to-work) and off- (work-to-rest) transition during  
183 exercise in human skeletal muscle during bilateral KE (Cannon et al. 2014). These published  
184 data used magnetic resonance spectroscopy of the quadriceps and pulmonary  $\dot{V}O_2$  to

185 measure the kinetics of muscle and whole-body bioenergetics during and following 3 and 8  
186 min of moderate and **severe** intensity KE exercise in healthy young humans (n=13, one  
187 female; age, 27±8 years (mean ± SD); height, 177±8 cm; mass, 75±12 kg).

188 Simulations were made for two exercise intensities: moderate-intensity exercise  
189 below the lactate threshold and **severe**-intensity exercise above **critical power**. It should be  
190 noted that **critical power** was not measured in the original study of Cannon et al. (2014).  
191 However, based on the non-steady-state behaviour of pulmonary  $\dot{V}O_2$  and intramuscular  
192 metabolism, the exercise intensity in Cannon et al. (2014) is here assumed to be above  
193 **critical power** (Poole et al. 1988; Jones et al. 2008): variably termed **very-heavy** or **severe**  
194 **intensity exercise** (see Rossiter, 2011 for discussion). Based on the experimental data we  
195 adjusted the activity (rate constant) of ATP usage (hydrolysis,  $A_{UT}$ ) to be elevated 22 fold  
196 during transition from rest to moderate-intensity exercise, and 47 fold during transition from  
197 rest to **severe**-intensity exercise. This gave the value of muscle  $\dot{V}O_2$  equal to about 2.6  
198 mM/min (58 ml/kg/min) after 8 min of moderate-intensity exercise and to about 6.7 mM/min  
199 (150 ml/kg/min) after 8 min of **severe**-intensity exercise. Following this, model parameter  
200 values were adjusted independently for moderate and **severe** exercise, in order to best fit the  
201 experimental data for the kinetics of muscle PCr, pH, vOX, vCK, and vGL.

202 The activation of oxidative phosphorylation ( $A_{OX}$ ; the relative increase of rate  
203 constants of complex I, complex III, complex IV, ATP synthase, ATP/ADP carrier,  $P_i$  carrier  
204 and NADH supply) (Korzeniewski, 1998; Korzeniewski, 2003; Korzeniewski, 2007;  
205 Korzeniewski, 2014) was adjusted following an exponential time course at exercise onset:

$$206 \quad m_{OX} = A_{OX} - (A_{OX} - 1) \cdot e^{-t/\tau(ON_{OX})} \quad (1)$$

207 where  $m_{OX}$  is the current activation (ratio of the current rate constant to the resting rate  
208 constant) of OXPHOS,  $A_{OX}$  is the relative activation of OXPHOS during moderate and  
209 **severe**-intensity exercise,  $\tau(ON_{OX}) = 3$  s is the characteristic time of the activation of oxidative  
210 phosphorylation (Korzeniewski, 2003) and  $t$  stands for the time after the onset of exercise.  
211  $\tau(ON_{OX})$  was small enough in order not to disturb the on-transient.

212 It was assumed that glycolysis was directly activated ( $A_{GL}$ ) during rest to moderate  
 213 and **severe** intensity exercise transitions, and the magnitude of  $A_{GL}$  was adjusted to best fit  
 214 the experimental data. The need for this strong direct parallel activation of glycolysis was  
 215 demonstrated previously (Korzeniewski & Liguzinski, 2004). The increase in the rate  
 216 constant of glycolysis was not instantaneous, but occurred exponentially:

$$217 \quad m_{GL} = A_{GL} - (A_{GL} - 1) \cdot e^{-t/\tau(ON_{GL})} \quad (2)$$

218 where  $m_{GL}$  is the current activation (ratio of the current rate constant to the resting rate  
 219 constant of glycolysis),  $A_{GL}$  is the relative activation of glycolysis during exercise,  $\tau(ON_{GL}) = 6$   
 220 s is the characteristic time for the activation of glycolysis and  $t$  stands for the time after the  
 221 onset of exercise.  $\tau(ON_{GL})$  was small enough in order not to disturb the on-transient.

222 After termination of exercise the rate constants of ATP usage and glycolysis were  
 223 decreased instantly to the initial (rest) values. The rate constants of oxidative  
 224 phosphorylation complexes decreased exponentially according to the following equation  
 225 (Korzeniewski, 2003):

$$226 \quad m_{OX} = 1 + (A_{OX} - 1) \cdot e^{-t/\tau(OFF_{OX})} \quad (3)$$

227 where  $m_{OX}$  is the current activation (ratio of the current rate constant to the resting rate  
 228 constant) of OXPHOS,  $A_{OX}$  is the relative direct activation of OXPHOS during exercise,  
 229  $\tau(OFF_{OX}) = 90$  s or  $180$  s is the characteristic decay time of the activation of oxidative  
 230 phosphorylation for moderate and severe exercise, respectively, and  $t$  stands for the time  
 231 after the onset of exercise.

232 There was no additional progressive component for ATP usage during moderate  
 233 exercise. During **severe** exercise, in order to fit experimental data, a linear increase in ATP  
 234 usage was included, beginning at 100 % of the 'fundamental' ATP usage rate (the rate at the  
 235 immediate onset of exercise) and continuing until the end of exercise at 8 min (Paterson &  
 236 Whipp, 1991; Barstow & Mole, 1991). This mechanism underlying the system behaviour of  
 237  $A_{UT}$  should not be confused with the behaviour of  $v_{OX}$  itself (which is commonly  
 238 approximated by a bi-exponential).

239 The influence of glycolytic inhibition by protons was interrogated using two different  
240 inhibitory states: moderate and strong. The rate of glycolysis for moderate glycolytic inhibition  
241 by protons was described by the following simple, semi-quantitative equation (as in  
242 Korzeniewski & Liguzinski, 2004):

$$243 \quad v_{GLYC} = k_{GLYC} \cdot ADP_{te} \cdot \left( \frac{H^+_{rest}}{H^+} \right) \quad (4)$$

244 where  $k_{GLYC}$  is the rate constant of glycolysis,  $ADP_{te}$  is the cytosolic total (magnesium-bound  
245 and magnesium free) free ADP concentration,  $H^+_{rest} = 10^{-7}$  M (pH = 7.0) is the resting proton  
246 concentration and  $H^+$  is the current proton concentration.

247 The rate of glycolysis for strong glycolytic inhibition by protons was described by the  
248 following simple equation:

$$249 \quad v_{GLYC} = k_{GLYC} \cdot ADP_{te} \cdot \left( \frac{H^+_{rest}}{H^+} \right)^3 \quad (5)$$

250 Therefore, it was assumed that the rate of glycolysis was inversely proportional to the cube  
251 of the current proton concentration.

252 Three computer simulations were carried out to identify the best-fit parameter values  
253 and conditions ( $A_{OX}$ ,  $A_{GL}$ ,  $\tau(OFF_{OX})$ , magnitude of 'additional' ATP usage, and moderate or  
254 strong inhibition of glycolysis by protons) during moderate and **severe** intensity exercise.

255 Simulation 1: **Severe** exercise with moderate glycolytic inhibition by protons.

256 Simulation 2: **Severe** exercise with strong glycolytic inhibition by protons.

257 Simulation 3: Moderate exercise with strong glycolytic inhibition by protons.

258 Simulations 1 and 2 first established the set of system characteristics that best fit the

259 experimental data under conditions where fluxes, metabolite concentrations and, in

260 particular, pH were most disturbed (**severe** exercise). Following this, simulation 3 was

261 conducted using moderate exercise with the parameters of glycolytic inhibition established by

262 the best fit from simulations 1 and 2.

## 263 RESULTS

264 The computer simulations performed in this study were compared with experimental  
265 data for transitions to and from moderate and severe intensity KE exercise in humans  
266 published previously (Cannon et al. 2014).

267 Figure 1 shows the results of Simulation 1. The best-fit model values with moderate  
268 glycolytic inhibition by protons (Equ. 4), were  $A_{UT} = 47$ ,  $A_{OX} = 47^{0.43}$  (5.24 fold),  $A_{GL} = 47^{0.65}$   
269 (12.21 fold),  $\tau(\text{OFF}_{OX}) = 180$  s, 'additional' ATP usage = 27 %. The simulated kinetics of PCr  
270 (% of resting value) and pH, as well as the values of ATP supply by OXPHOS (vOX), CK  
271 (vCK) and anaerobic glycolysis (vGL) at 3<sup>rd</sup> and 8<sup>th</sup> min of severe exercise with moderate  
272 glycolytic inhibition by protons (Equ. 4) agreed well with the experimental data. Muscle  $\dot{V}O_2$ ,  
273 PCr, pH, ADP, vOX, vCK, vGL and ATP usage (vUT) did not reach a steady state, but  
274 progressively changed during exercise ( $\dot{V}O_2$ , ADP, vUT and vOX increased, while PCr, pH,  
275 vCK and vGL decreased). The main small differences between the simulation and the  
276 experimental data were a slower decrease in pH and a smaller magnitude of the initial  
277 transient alkalosis in the simulation. The former is most probably the result of insufficient  
278 glycolytic inhibition by protons (see Simulation 2 below). The latter is likely due to the  
279 stoichiometry of proton consumption and production by the CK in the Lohmann reaction. The  
280 dependence of this stoichiometry on pH was extracted from the work of Kushmerick (1997)  
281 and is  $S_{CK} = 0.63 - (\text{pH} - 6.0) * 0.43$ ; which gives about 0.2 for pH ~7.0. For a higher value of  $S_{CK}$ ,  
282 a more pronounced initial transient alkalosis was obtained in computer simulations (not  
283 shown).

284 Additionally, a large slow component of the muscle  $\dot{V}O_2$  on kinetics appeared in this  
285 simulation (Simulation 1). It was caused mostly by the increase in ATP utilization during  
286 exercise (from 100 to 127 % of the 'fundamental' rate of ATP turnover), but also, to a small  
287 extent, by a moderate inhibition of ATP supply by anaerobic glycolysis (vGL) by  
288 accumulating protons as exercise progressed, necessitating a supplementary increase in the  
289 ATP supply from OXPHOS (vOX) (Korzeniewski & Zoladz, 2015).

290 The introduction of a strong glycolytic inhibition by protons (Equ. 5, glycolytic flux  
291 inversely proportional to the cube of the current proton concentration) for **severe** exercise,  
292 together with a stronger direct glycolytic activation at the onset of exercise (Simulation 2),  
293 significantly speeded the kinetics of the early intracellular pH response, but did not affect the  
294 pH value after 8 min of exercise. This can be seen in Fig. 2. In Simulation 2, with strong  
295 glycolytic inhibition by protons (Equ. 5), the model values were  $A_{UT} = 47$ ,  $A_{OX} = 47^{0.43}$  (5.24  
296 fold),  $A_{GL} = 47^{0.87}$  (28.49 fold),  $\tau(OFF_{OX}) = 180$  s, 'additional' ATP usage = 27 %. This  
297 simulation gave a much better fit to the experimentally-measured time course of pH without  
298 influencing significantly the time course of PCr relative to the good fit observed in Simulation  
299 1 (see Fig. 2). The high direct activation of (anaerobic) glycolysis led to a rapid decrease in  
300 pH after the onset of exercise, while the strong (anaerobic) glycolytic inhibition by protons  
301 that took place afterwards (slowing vGL) prevented excessive cytosolic acidification.  
302 Generally, an excellent agreement of theoretical predictions with experimental data was  
303 observed in this simulation. Not only time courses of PCr and pH, but also the values of ATP  
304 production by OXPHOS (vOX), CK (vCK) and anaerobic glycolysis (vGL) agreed well with  
305 the experimental data. The relative increase of the slow component of the muscle  $\dot{V}O_2$  on-  
306 kinetics measured between 3 and 8 minutes of exercise in this simulation (20%) was similar  
307 to that of the measured pulmonary  $\dot{V}O_2$  ( $22 \pm 8\%$ ; Cannon et al. 2014). Anaerobic glycolysis  
308 was strongly directly activated after the onset of exercise, which significantly elevated the  
309 initial ATP synthesis by this process. However, as exercise progressed, glycolysis was  
310 strongly (third-order dependence) inhibited by accumulating protons, meaning that a  
311 significant additional fraction of ATP supply was provided by oxidative phosphorylation. The  
312 additional requirement for vOX can be observed in the reduction of vGL in Fig. 2, between  
313 the peak at ~0.75 min and end-exercise (8 min). In Simulation 2 both the strong (anaerobic)  
314 glycolytic inhibition by protons and the 'additional' ATP usage increasing during exercise  
315 from 0 % of the 'fundamental' ATP usage at the onset of exercise to 27 % after 8 min of  
316 exercise contributed to the slow component of the muscle  $\dot{V}O_2$  on-kinetics.

317 Simulation 3 (for moderate exercise and strong glycolysis inhibition by protons) also  
318 generally agreed very well with experimental data obtained by Cannon et al. (2014). This is  
319 shown in Fig. 3. The model values for moderate exercise with strong glycolytic inhibition by  
320 protons (Equ. 5) were  $A_{UT} = 22$ ,  $A_{OX} = 22^{0.56}$  (5.64 fold),  $A_{GL} = 22^{0.85}$  (13.84 fold),  $\tau(OFF_{OX}) =$   
321 90 s, 'additional' ATP usage = 0 %. As expected, both experimental and simulated fluxes and  
322 metabolite concentrations during moderate exercise rest-to-work and work-to-rest transitions  
323 changed much less than during *severe* exercise. Additionally, unlike during *severe* exercise,  
324 a steady-state was achieved after ~3 minutes of exercise. The only exception seems to be  
325 the mean experimental measurement of vOX after 3 min of exercise is greater than the  
326 simulation. vOX is 35 % greater at 3 min than after 8 min of moderate exercise. However,  
327 while this difference is apparently big, as shown in the original presentation (Cannon et al.  
328 2014) it was not statistically significant (see error bars in Fig. 3). Of course, a difference in  
329 vOX between 3<sup>rd</sup> and 8<sup>th</sup> min of exercise could not be reconciled with the presence of the  
330 steady-state in the time course of the pulmonary  $\dot{V}O_2$ , PCr and pH observed in this  
331 experiment for moderate exercise (Cannon et al. 2014), and is likely the result of variability in  
332 the measurement of this variable within subjects. However, it is not possible to know whether  
333 the variability influences more the 3 min or 8 min values. Here, as in Cannon et al. (2014),  
334 we assumed that the 3 min pulmonary  $\dot{V}O_2$  value 'overshoots' the expected steady state,  
335 albeit, non-significantly. Nevertheless, adjusting the simulations to give vUT (and vOX) that  
336 was intermediate between the 3<sup>rd</sup> and 8<sup>th</sup> min of moderate exercise, namely 20 mM min<sup>-1</sup>, did  
337 not substantially change the relative agreement between the experimental and simulated  
338 fluxes and metabolite concentrations (simulation not shown).

## 339 DISCUSSION

340 The first objective of this theoretical study was to determine whether a computer  
341 model of the skeletal muscle bioenergetic system (Korzeniewski, 1998; Korzeniewski &  
342 Zoladz, 2001; Korzeniewski & Liguzinski, 2004), was able to reproduce, strictly quantitatively,  
343 a particular concrete set of experimental data. Using the data from the experiment conducted  
344 by Cannon et al. (2014) as the frame of reference (because it involved simultaneous  
345 measurements of the time courses of PCr and pH for rest-work-rest transitions during  
346 moderate and *severe* exercise as well as  $\dot{V}O_2$ , vCK and vGL after 3 and 8 min of exercise),  
347 we found that overall the computer simulations produced a very good agreement with the  
348 experimental data (Figs. 1-3). This proved that the model was able to reproduce correctly the  
349 complex set of the modelled system properties, and allowed us to test 4 specific hypotheses  
350 of the relative intensity of ESA and glycolytic inhibition by protons in contributing to the  
351 observed metabolite concentrations and fluxes during moderate and *severe* intensity  
352 exercise.

353 Specifically, the important new findings of this study were that: 1. high-intensity ESA  
354 accounted for the system behaviour (changes in fluxes and metabolite concentrations during  
355 rest-work-recovery transitions) in human skeletal muscle during *severe* and moderate  
356 exercise (Figs. 2 and 3, respectively); 2. the decay of ESA after exercise was slow, and  
357 slowed further by *severe*-intensity, compared to moderate-intensity, exercise (Figs. 2 and 3);  
358 3. strong (third-order dependence) glycolytic inhibition by protons better simulated the time  
359 course of pH during *severe* exercise (Fig. 2) than moderate glycolytic inhibition (Fig. 1); 4.  
360 the relative magnitude of the  $\dot{V}O_2$  slow component *in vivo* was better modelled using a large  
361 additional ATP usage increasing progressively during exercise together with a strong proton  
362 inhibition of (anaerobic) glycolytic flux.

363

364 *Each step activation (ESA) is obligatory for intramuscular bioenergetic flux control during*  
365 *exercise and recovery*

366 This study emphasizes that an intensive ESA during exercise and slow decay of ESA  
367 after termination of exercise are obligatory to reproduce quantitatively the muscle metabolite  
368 concentrations and fluxes of experimental data. The direct activation of all OXPHOS  
369 complexes and NADH supply ( $A_{OX}$  in computer simulations) is over 5-fold, namely 5.64-fold  
370 and 5.24-fold during transition from rest to moderate and **severe** work, respectively. The  $A_{OX}$   
371 is even slightly greater for moderate work, although the muscle  $\dot{V}O_2$  is much greater for  
372 severe work, because the stability of PCr and pH is much better in the former case.  $\dot{V}O_2$  is  
373 determined mostly by the ATP utilization rate ( $A_{UT}$ ; as long as the OXPHOS capacity for ATP  
374 synthesis is not saturated in the absence of ESA, see below), while ESA intensity affects  
375 predominantly the stability of metabolite concentrations and their kinetics (Korzeniewski &  
376 Zoladz, 2004). The high predicted ESA intensity is not surprising, because the muscle  $\dot{V}O_2$   
377 increases 9.04 fold during the moderate rest-exercise transition, and 23.45 fold during the  
378 severe rest-exercise transition, while [ADP] increases only 2.17 fold and 5.49 fold,  
379 respectively. Therefore the phenomenological  $\dot{V}O_2$ -[ADP] relationship is very steep, much  
380 steeper than possible from first- or even second-order rate reactions. Even a steeper  
381 phenomenological  $\dot{V}O_2$ -[ADP] relationship is observed in some experiments (Wüst et al.  
382 2011; Korzeniewski, 2014). For instance, it was observed that a 40-fold increase in  
383 electrically-stimulated dog muscle  $\dot{V}O_2$  is accompanied by only 2.5-fold increase in [ADP]  
384 (Zoladz et al. 2008).

385 The fact that  $A_{OX}$  was smaller than  $A_{UT}$  implies that OXPHOS is directly activated in  
386 parallel with ATP usage during rest-to-work transition, but that OXPHOS activation is less  
387 than that of ATP usage. This corresponds to the mixed mechanism (MM) of bioenergetics  
388 control, where direct activation co-operates with negative-feedback activation (through an  
389 increase in [ADP] and  $[P_i]$ ) in the control of OXPHOS (Korzeniewski, 2014) (see below).

390 Without ESA ( $A_{OX} = 1$ ) the system collapses during **severe** exercise ( $A_{UT} = 47$ ): [PCr]  
391 and [ATP] fall to zero (the latter is converted to ADP, and further, by adenylate kinase, AK, to  
392 AMP),  $[P_i]$  and [Cr] increase to maximal values, and a huge cytosolic acidification occurs  
393 while maximal muscle  $\dot{V}O_2$  becomes limited to about  $3.5 \text{ mM min}^{-1}$ . Of course, this is only a

394 virtual prediction: in reality, in the absence of ESA, exercise would be slowed or terminated  
395 shortly after onset. Without ESA ( $A_{OX} = 1$ ) during moderate exercise ( $A_{UT} = 22$ ) and for lower  
396 ESA (say, for  $A_{OX} = 3$ ) during moderate ( $A_{UT} = 22$ ) and **severe** ( $A_{UT} = 47$ ) exercise, the  
397 changes in metabolite concentrations predicted by the model are much greater than for high  
398 ESA, and therefore do not well fit the experimental data. Additionally, characteristic transition  
399 times ( $\tau$ ) for  $\dot{V}O_2$  and metabolite concentrations during rest-work transitions are lengthened  
400 under conditions where lower ESA activities are used (Korzeniewski & Zoladz, 2004).  
401 Therefore, a decrease in ESA intensity in computer simulations results in wide  
402 disagreements with experimental results.

403         It was demonstrated previously, in the bioenergetic system of the skeletal muscle cell  
404 that, without ESA, huge changes in metabolite (ADP, PCr,  $P_i$ ) concentrations take place  
405 when the relative ATP demand increases, and that the system collapses when the energy  
406 demand (rate constant of ATP usage) exceeds the relative value of about  $A_{UT} = 30$  fold  
407 above resting ATP demand. Under these conditions ATP and PCr concentrations fall to zero,  
408 Cr and  $P_i$  concentrations rise to the maximal values, [ADP] first rises to about 1500  $\mu\text{M}$  and  
409 then decreases near zero as it is converted to AMP (by AK) and there is no further increase  
410 in muscle  $\dot{V}O_2$  together with an increase of ATP demand (see Fig. 3A in Liguzinski &  
411 Korzeniewski, 2006). In the present study, the relative ATP demand ( $A_{UT}$ ) for **severe** exercise  
412 was 47 fold greater than resting, and is therefore far beyond this 'collapse threshold'. For this  
413 reason, in the absence of ESA, system collapse is observed in the simulations of the severe  
414 intensity exercise conditions. Strong ESA allows moderate changes in muscle metabolite  
415 concentrations and pH to take place, while  $\dot{V}O_2$  can reach values far above this threshold  
416 (see Fig. 3B in Liguzinski & Korzeniewski, 2006).

417         Our comparison of computer simulations with experimental data predicts that a slow  
418 decay of ESA during muscle recovery after exercise takes place. The simulated time course  
419 of [PCr] during recovery can be fitted to experimental data only when a slow decay of ESA is  
420 assumed – otherwise the PCr recovery would be much slower (see Korzeniewski & Zoladz,  
421 2014). The adjusted values of the characteristic ESA decay time  $\tau(\text{OFF}_{OX})$  were 90 s and 180

422 s for moderate and **severe** exercise, respectively. This is consistent with previous  
423 suggestions that a greater muscle metabolic strain during exercise lengthens  $\tau(\text{OFF}_{\text{OX}})$ ,  
424 sometimes leading to a transient overshoot in PCr recovery (Korzeniewski & Zoladz, 2005). It  
425 seems logical that more intensive exercise causes greater muscle metabolic stress and  
426 strain. Therefore, in our simulations ESA was necessary not only to account for the increase  
427 in muscle  $\dot{V}\text{O}_2$  and  $v\text{OX}$ , and changes in metabolites during the on-transient, but also to  
428 explain system behaviour during the off-transient.

429         The molecular mechanism of ESA remains in question. Glancy et al. (2013) showed  
430 that the activity of essentially all OXPHOS complexes was sensitive to  $\text{Ca}^{2+}$ : isolated skeletal  
431 muscle mitochondria incubated with glutamate/malate and exposed to increased  $\text{Ca}^{2+}$ ,  
432 increased overall OXPHOS activity by about 2 fold. In electrically-stimulated canine muscle  
433 direct measurement of the  $\dot{V}\text{O}_2$ -[ADP] relationship suggested an  $A_{\text{OX}}$  of ~3-4 fold with a  
434  $\tau(\text{ON}_{\text{OX}})$  of ~10 s (Wüst et al. 2011). The present study strongly suggests that OXPHOS  
435 complexes are activated directly over 5 fold during rest-to-work transitions in humans. In  
436 other muscles or experimental conditions this direct activation of OXPHOS can be even  
437 higher (Korzeniewski, 2014). It was proposed previously that cytosolic  $\text{Ca}^{2+}$  acts *in vivo*  
438 through some protein analogous to calmodulin that causes protein (e.g., OXPHOS  
439 complexes) phosphorylation and that is absent in the isolated mitochondrial system  
440 (Korzeniewski, 1998; Korzeniewski, 2007; Korzeniewski, 2014).

441         Generally, the consequence of ESA is that the regulation of OXPHOS in intact  
442 skeletal muscle is completely different than in isolated mitochondria (at least in the absence  
443 of  $\text{Ca}^{2+}$ ). In other words, the resting state in muscles is different from state 4 in isolated  
444 mitochondria: there is some ATP usage for 'basal' ATP usage by reactions that keep the cell  
445 alive (RNA/protein synthesis, ion circulation) that is responsible for about 40% of muscle  $\dot{V}\text{O}_2$   
446 in rat skeletal muscle, with the remaining  $\dot{V}\text{O}_2$  due to proton leak (Rolfe & Brand, 1996).  
447 Additionally, moderate and severe exercise states in intact muscle are different from state 3  
448 in isolated mitochondria:  $\dot{V}\text{O}_2$ ,  $\Delta p$  (protonmotive force) and NADH are much greater, while

449 [ADP] and [P<sub>i</sub>] are much less in intact muscle (Korzeniewski, 2015). Therefore, experimental  
450 data from isolated mitochondria (at least in the absence of Ca<sup>2+</sup>) concerning the regulation of  
451 OXPHOS during increase in energy (ATP) demand cannot be simply extrapolated to intact  
452 skeletal muscle.

453         During rest-to-work transitions in skeletal muscle, some moderate increase in [ADP]  
454 and [P<sub>i</sub>] takes place and therefore the negative-feedback activation by these metabolites co-  
455 operates with ESA in the regulation of OXPHOS (the mixed mechanism; Korzeniewski,  
456 2014). During work transitions in intact heart *in vivo* metabolite (PCr, P<sub>i</sub>, ADP, ATP, NADH)  
457 concentrations are essentially constant (see e.g. Katz et al., 1989); in the nomenclature used  
458 here, this reflects a 'pure' ESA-controlled system. This is related to the fact that ATP supply  
459 is directly activated during low-to-high work transition to the same extent as ATP usage  
460 (discussed in detail previously; Korzeniewski et al., 2005; Korzeniewski, 2006; Korzeniewski,  
461 2007).

462         While the each-step activation (ESA) mechanism was proposed previously, this study  
463 constitutes a very significant advance. Previous studies mostly used a semi-quantitative  
464 indirect validation of ESA in relation to steady-state changes in  $\dot{V}O_2$  and [ADP] or [PCr]  
465 during rest-to-work transitions. This study, however, offers a strictly quantitative direct  
466 validation, using: 1. Several different variables ( $\dot{V}O_2$ , PCr, pH, vOX, vCK, vGL); 2. Whole  
467 time courses during rest-work-recovery transitions; 3. Both moderate and **severe** intensity  
468 exercise. The excellent agreement of computer simulations with experimental data for such a  
469 broad range of system properties greatly supports the ESA mechanism and increases the  
470 computer model reliability.

471         It is in principle possible that a mechanism other than ESA could account for the  
472 discussed experimental data. On the other hand, we do not know any likely candidate for  
473 such a mechanism. Alternative proposals would have to also explain the great number of  
474 different system properties, time and intensity dependence, as explained by the ESA model  
475 with strong inhibition of glycolysis by protons, and it seems unlikely that two completely  
476 different mechanisms would be able to achieve this. **We mean not only the system properties**

477 simulated in the present study, but also numerous other properties, for instance the uniform  
478 distribution of metabolic control among OXPHOS complexes or PCr recovery overshoot,  
479 discussed previously (Korzeniewski, 2007; Korzeniewski ,2011; Korzeniewski 2014).

480         Recently Wilson (Wilson, 2015) presented a modified dynamic version of his previous  
481 static model (Wilson et al., 1979), involving e.g., the CK system. It was successful in  
482 reproducing semi-quantitatively some system properties, for instance the time course of PCr  
483 after the onset of exercise. Although the kinetic description of cytochrome oxidase in this  
484 model is very complex, the suggestion that cytochrome oxidase determines the rate of  
485 oxygen consumption is not well supported by Metabolic Control Analysis of isolated skeletal  
486 muscle mitochondria showing that flux control is more or less evenly distributed among  
487 OXPHOS complexes (Rossignol et al., 1999). While this model proposes to explain the 'lag  
488 phase' in pulmonary or muscle  $\dot{V}O_2$  observed in several studies, it remains uncertain the  
489 extent to which this lag reflects the mitochondria-lung or mitochondria-muscle vein delay in  
490 oxygen transport, rather than an actual lag in mitochondrial  $\dot{V}O_2$  on-kinetics. Interpretation of  
491 thermodynamic models is complicated because they do not distinguish differing effects of  
492 different metabolites e.g. the ADP and  $P_i$ . Therefore, in computer modelling it is crucial to  
493 validate a model for the broadest set of variable values and system properties possible, we  
494 have done here and previously (Korzeniewski, 2007; Korzeniewski ,2011; Korzeniewski  
495 2014; present study).

496

#### 497 *Mechanisms of the $\dot{V}O_2$ slow component in severe intensity exercise*

498         Our original model of the skeletal muscle bioenergetic system with ATP and  $H^+$   
499 production by anaerobic glycolysis (Korzeniewski & Liguzinski, 2004) included a simple  
500 kinetic description of glycolytic inhibition by protons. This description consists of an inverse  
501 linear dependence of the glycolytic flux on  $[H^+]$  (Equ. 4) and was used in Simulation 1 (Fig.  
502 1). However, Simulation 2 demonstrated that the time course of pH during on-transient  
503 measured by Cannon et al. (2014) was much better reproduced when a stronger glycolytic  
504 inhibition by  $[H^+]$  is assumed (Simulation 2, Fig. 2): specifically an inverse dependence of the

505 glycolytic flux on the cube of proton concentration (Equ. 5) (as well as a much stronger direct  
506 glycolysis activation after the onset of exercise). This assumption also worked well for  
507 moderate exercise (Simulation 3, Fig. 3). Therefore, this study provides an improved  
508 understanding of the kinetics of control and regulation of glycolytic flux by protons.

509 It should be stressed that the kinetic description of the glycolytic inhibition by  $H^+$  in the  
510 model is only phenomenological, and may involve many variables, including buffers whose  
511 relative contribution to glycolytic inhibition is intensity and/or pH dependent over the  
512 physiologic ranges investigated, such as ammonia or inorganic phosphate. The  
513 phenomenological proton buffering capacity is taken into account within the model. We found  
514 that the current phenomenological model of strong glycolytic inhibition explained well both  
515 moderate and **severe** intensity exercise system kinetics.

516 Korzeniewski & Zoladz (2015) proposed that the two main mechanisms underlying  
517 the  $\dot{V}O_2$  slow component in skeletal muscle (Poole et al. 1994), at least during cycling  
518 exercise, are the gradual inhibition of ATP supply by anaerobic glycolysis by protons  
519 accumulating during exercise (together with a slow decay of ATP supply by CK) and a  
520 progressive increase in ATP utilization during constant power exercise. While the latter has  
521 been frequently supported by experimental studies (for review see e.g., Rossiter et al. 2002;  
522 Rossiter, 2011; Poole & Jones, 2012; Cannon et al. 2014), the former proposition specifically  
523 linking progressive inhibition of glycolysis to the  $\dot{V}O_2$  slow component magnitude  
524 (Korzeniewski & Zoladz, 2015) is untested outside of computer simulations.

525 Here we found that the magnitude of the muscle  $\dot{V}O_2$  slow component in Simulations  
526 1 and 2 was similar to the magnitude of the pulmonary  $\dot{V}O_2$  slow component measured by  
527 Cannon et al. (2014) for bilateral KE exercise in humans. Specifically, the relative increase in  
528 muscle  $\dot{V}O_2$  (and  $vOX$ ) between 3<sup>rd</sup> and 8<sup>th</sup> min of exercise was 20 % in both simulations,  
529 and the relative increase in pulmonary  $\dot{V}O_2$  was 22 % in the measured data. This suggests  
530 that either moderate or strong glycolytic inhibition by protons likely contributes to the  
531 dynamics of *in vivo* muscle energetics in general, and to the dynamics of the  $\dot{V}O_2$  slow  
532 component in particular (with strong glycolytic inhibition better reproducing the dynamics of

533 pH), by necessitating a greater  $vOX$  as  $vGL$  becomes increasingly inhibited by proton  
534 accumulation in severe-intensity exercise in humans.

535 It has been proposed, on the basis of the lack of correlation between the magnitude  
536 of the pulmonary  $\dot{V}O_2$  slow component and the 'slow component' of the oxidative ATP supply  
537 ( $vOX$ ), that a decrease in the  $P/O$  ratio may contribute to the muscle  $\dot{V}O_2$  on-kinetics  
538 (Cannon et al. 2014). However, the interpretation of this finding is equivocal.

539 First, as discussed above, because the average relative increase in pulmonary  $\dot{V}O_2$   
540 and in  $vOX$  between 3<sup>rd</sup> and 8<sup>th</sup> min of exercise are very similar, varying  $P/O$  ratio among  
541 subjects would imply that in some subjects  $P/O$  decreases in the course of exercise, while in  
542 other subjects it increases. The latter seems unlikely. An alternative explanation could be a  
543 relatively large variability of  $vOX$  values (see error bars in Figs. 1-3) measured using the  
544 method based on the PCr recovery kinetics.

545 Second, as discussed in Cannon et al. (2014), there exist complexities associated  
546 with inferring muscle  $\dot{V}O_2$  kinetics from pulmonary  $\dot{V}O_2$  measurements. In this study the  
547 experimental pulmonary  $\dot{V}O_2$  (including whole-body  $O_2$  uptake) is, naturally, much greater  
548 than the simulated muscle  $\dot{V}O_2$  (isolated to the active muscle *in silico*) estimated from the  
549 oxidative ATP supply ( $vOX$ ). Using pulmonary gas exchange measurements to infer kinetic  
550 changes across the skeletal muscle requires a number of assumptions, including that the  
551 metabolic contribution of 'resting tissues' (basal metabolism in all bodily tissues) and  
552 'auxiliary tissues' (activity of respiratory muscles, stabilizing muscles, cardiac muscle etc.)  
553 are constant during constant power exercise. With cycling exercise or seated single leg KE  
554 exercise, this assumption appears reasonable (e.g. Grassi et al., 1996; Krstrup et al., 2009),  
555 and thus the  $\dot{V}O_2$  slow component can be inferred to be predominantly isolated to the active  
556 locomotor muscles (Poole et al. 1991). The study of Cannon et al. (2014) used bilateral KE  
557 exercise inside a superconducting magnet to measure phosphate metabolism by  $^{31}P$  MRS  
558 while maximizing the muscle mass engaged in the task and simulate conditions such as in  
559 walking or cycling. In this model, the contribution of 'axillary tissues' appears to be as much  
560 as approximately 35 to 45 % of the pulmonary  $\dot{V}O_2$  (assuming 6 or 5 kg of the mass of two

561 active quadriceps, respectively). Importantly it is not known whether oxygen consumption by  
562 these tissues changes substantially during exercise; any increase would contribute to the  
563 magnitude of the pulmonary  $\dot{V}O_2$  slow component, but not  $vOX$  or  $\dot{V}O_2$  in the muscles of  
564 interest.

565 During **severe** cycling exercise, working muscles are responsible for about 85 % of  
566 pulmonary  $\dot{V}O_2$  (Poole et al. 1992), while ATP supply by 'auxiliary tissues' is about 8 % (as  
567 estimated by Liguzinski & Korzeniewski, 2007), with the remainder being due to metabolism  
568 in 'resting tissues'. It seems that this relative contribution is much smaller in severe KE  
569 exercise, where the muscle mass activated for the external KE power production is smaller  
570 than in running or cycling. Additionally, the only known process that could decrease P/O,  
571 namely proton leak through the inner mitochondrial membrane, is estimated to be  
572 responsible for only about 1 % of muscle  $\dot{V}O_2$  during **severe** exercise (Korzeniewski &  
573 Zoladz, 2015), reducing the likelihood that mitochondrial uncoupling contributes substantially  
574 to the muscle  $\dot{V}O_2$  slow component magnitude. Finally, a significant (about 20 % at rest)  
575 fraction of  $O_2$  is consumed in the skeletal muscle cell by non-mitochondrial processes  
576 (residual oxygen consumption by e.g. NADPH oxidase, nitric oxide synthase, or xanthine  
577 oxidase), and not by OXPHOS in mitochondria (Rolfe et al. 1999). However, it cannot be  
578 excluded currently that a decrease in P/O contributes to some extent to the muscle  $\dot{V}O_2$  slow  
579 component on transition to KE exercise.

580 We did not model the experimentally-measured pulmonary  $\dot{V}O_2$  (Cannon et al. 2014),  
581 because, as discussed above, the ratio of 'active' to 'axillary' and 'resting' tissues  $\dot{V}O_2$  is  
582 unknown and may vary during exercise, and the outcome of the simulation rests entirely on  
583 the ratio selected. Additionally, it is likely that a slow decay of  $\dot{V}O_2$  by 'auxiliary tissues' and  
584 the contribution of circulatory dynamics to  $O_2$  transport may dissociate pulmonary  $\dot{V}O_2$   
585 kinetics from the muscle (Barstow et al., 1990; Benson et al. 2013; Korzeniewski & Zoladz,  
586 2013), especially during recovery (Krustrup et al. 2009). Instead, for **comparison** with  
587 simulated data, we relied on the on-transition  $vOX$  measured from  $^{31}P$  MRS in Cannon et al.

588 (2014), but we have no independent estimation of vOX in recovery other than that inferred  
589 from [PCr] recovery kinetics.

590

### 591 *Data variability*

592 We adjusted our model by parameter fitting to averaged data presented in Cannon et  
593 al. (2014). However, there is, naturally, some variability in the original data, which may derive  
594 either from real differences between individuals (“individual variability”) or from variability  
595 inherent in the measurement methods (“method variability”). The variability of most variables  
596 in Cannon et al. (2014) is moderate, reflecting mostly “individual variability”. The only  
597 exception is vOX, which was especially variable during moderate intensity exercise. vOX is  
598 determined from the initial rate of change of PCr recovery kinetics, and therefore is  
599 influenced by the magnitude of the exercise-induced PCr depletion (Rossiter et al., 2000). In  
600 this case, the “method variability” is likely the major contributor to the overall variability in  
601 moderate intensity vOX, and is within the variability expected for this method (Rossiter et al.,  
602 2000).

603 We investigated the potential role of “individual variability”, using slight modifications  
604 of relevant parameter values in the computational model, for instance  $A_{OX}$ ,  $A_{GL}$ ,  $A_{UT}$  and/or  
605 OXPHOS activity (there is no reason for these values to be identical among different  
606 individuals). The “individual variability” can be due to e.g. genetic differences or physical  
607 training. It has been proposed that training may lead not only to an increase in OXPHOS  
608 activity related to mitochondrial biogenesis, but also to elevation of ESA intensity (increase of  
609  $A_{OX}$ ) (Korzeniewski & Zoladz, 2003; Korzeniewski & Zoladz, 2004). It was demonstrated that  
610 both effects lead to acceleration of the  $\dot{V}O_2$  on-kinetics and to improvement of metabolite  
611 stability during rest-to-work transitions (Korzeniewski & Zoladz, 2003; Korzeniewski &  
612 Zoladz, 2004). Overall, we found that small modifications of relevant parameter values in the  
613 computational model could well account for the “individual variability” observed in several  
614 parameter values in moderate and severe intensity exercise. We also believe that the  
615 apparent (although not statistically significant) overshoot in vOX in moderate exercise is

616 predominately due to “method variability”; however a direct identification of the source of this  
617 variability remains to be determined.

618

### 619 *Conclusions*

620 The computer model of the skeletal muscle cell bioenergetic system developed  
621 previously (Korzeniewski, 1998; Korzeniewski & Zoladz, 2001; Korzeniewski & Liguzinski,  
622 2004) reproduces very well the experimental data of the time courses of [PCr] and pH during  
623 rest-work-rest transitions, as well as the ATP synthesis rate by OXPHOS (vOX), creatine  
624 kinase (vCK) and anaerobic glycolysis (vGL) after 3 and 8 min of moderate and severe  
625 bilateral knee extension (KE) exercise in humans (Cannon et al. 2014). It is demonstrated  
626 that an intensive each-step-activation (ESA) (over 5-fold direct activation of all OXPHOS  
627 complexes and NADH supply in parallel with the activation of ATP usage) was necessary to  
628 account for the changes in ATP synthesis fluxes, PCr and pH encountered in human  
629 muscles *in vivo*. Also a slow decay of ESA during recovery was necessary to fit experimental  
630 data. A strong inhibition of glycolysis by protons improves the agreement between the  
631 simulated and measured kinetics of pH after the onset of exercise in comparison to a  
632 moderate glycolytic inhibition. It is postulated that strong inhibition by accumulating protons  
633 of ATP supply by anaerobic glycolysis (together with a slow decay of ATP supply by CK) and  
634 an ‘additional’ ATP usage increasing gradually during **severe** exercise can explain the  
635 relative magnitude of the muscle  $\dot{V}O_2$  slow component, although some contribution of a  
636 decrease in P/O cannot be explicitly excluded. Overall, this well-tested computer model  
637 provides a useful tool for studying the dynamic behaviour of muscle metabolism during  
638 exercise and recovery.

639 **ADDITIONAL INFORMATION**

640 **Competing interests**

641 None.

642

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645 comparison with the simulations made in this study.

646

647 **Author contribution**

648 B.K. and H.B.R. designed computer simulations. H.B.R. prepared experimental data for  
649 presentation. B.K. performed computer simulations and prepared figures. B.K. and H.B.R.  
650 discussed the theoretical results and wrote the manuscript. Both authors critically reviewed  
651 and approved the final version of the manuscript.

652

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782 **FIGURE LEGENDS**

783 Fig. 1. Comparison of simulated (lines) and experimental (points) time course of PCr (% of  
784 resting values), pH and ATP supply flux during **severe** intensity rest-exercise-recovery with  
785 moderate glycolytic inhibition by protons (Simulation 1). Time course of simulated muscle  
786  $\dot{V}O_2$  and ADP are also presented. Experimental points for bilateral knee extension (KE)  
787 exercise in humans were taken from Cannon et al. (2014). Vertical lines in the upper panel  
788 indicate the onset and cessation of exercise. vOX, ATP supply rate by OXPHOS; vCK, ATP  
789 supply rate by CK; vGL, ATP supply rate by anaerobic glycolysis; vUT, muscle ATP  
790 utilization (ATP hydrolysis). 0.05 was subtracted from experimental pH values in order to  
791 scale experimental pH at rest to the value of 7.0, used in computer simulations.

792 Fig. 2. Comparison of simulated (lines) and experimental (points) time course of PCr (% of  
793 resting values), pH and ATP supply flux during **severe** intensity rest-exercise-recovery  
794 transitions with strong glycolytic inhibition by protons (Simulation 2). Time course of  
795 simulated muscle  $\dot{V}O_2$  and ADP are also presented. Experimental points for bilateral knee  
796 extension (KE) exercise in humans were taken from Cannon et al. (2014). Vertical lines in  
797 the upper panel indicate the onset and cessation of exercise. vOX, ATP supply rate by  
798 OXPHOS; vCK, ATP supply rate by CK; vGL, ATP supply rate by anaerobic glycolysis; vUT,  
799 muscle ATP utilization (ATP hydrolysis). 0.05 was subtracted from experimental pH values in  
800 order to scale experimental pH at rest to the value of 7.0, used in computer simulations.

801 Fig. 3. Comparison of simulated (lines) and experimental (points) time course of PCr (% of  
802 resting values), pH and ATP supply flux during moderate intensity rest-exercise-recovery  
803 transitions with strong glycolytic inhibition by protons (Simulation 3). Time course of  
804 simulated muscle  $\dot{V}O_2$  and ADP are also presented. Experimental points for bilateral knee  
805 extension (KE) exercise in humans were taken from Cannon et al. (2014). Vertical lines in  
806 the upper panel indicate the onset and cessation of exercise. vOX, ATP supply rate by  
807 OXPHOS; vCK, ATP supply rate by CK; vGL, ATP supply rate by anaerobic glycolysis; vUT,

808 muscle ATP utilization (ATP hydrolysis). 0.08 was subtracted from experimental pH values in  
809 order to scale experimental pH at rest to the value of 7.0, used in computer simulations.





