



Title	Effect of repeated forearm muscle cooling on the adaptation of skeletal muscle metabolism in humans
Author(s)	Wakabayashi, Hitoshi; Nishimura, Takayuki; Wijayanto, Titis; Watanuki, Shigeki; Tochihara, Yutaka
Citation	International journal of biometeorology, 61(7), 1261-1267 <a href="https://doi.org/10.1007/s00484-016-1303-z">https://doi.org/10.1007/s00484-016-1303-z</a>
Issue Date	2017-07
Doc URL	<a href="http://hdl.handle.net/2115/70879">http://hdl.handle.net/2115/70879</a>
Rights	The original publication is available at <a href="http://www.springerlink.com">www.springerlink.com</a>
Type	article (author version)
File Information	Wakabayashi_manuscript_HUSCAP.pdf



[Instructions for use](#)

1 **Title:**

2 Effect of repeated forearm muscle cooling on the adaptation of skeletal muscle metabolism in humans

3

4 **Authors:**

5 Hitoshi Wakabayashi<sup>1\*</sup>, Takayuki Nishimura<sup>2</sup>, Titis Wijayanto<sup>3</sup>, Shigeki Watanuki<sup>4</sup>, Yutaka Tochihara<sup>4</sup>

6

7 1) Faculty of Engineering, Hokkaido University, Sapporo, Japan

8 2) Department of Public Health, Nagasaki University Graduate School of Biomedical Sciences,

9 Nagasaki, Japan

10 3) Department of Mechanical and Industrial Engineering, Gadjah Mada University, Indonesia

11 4) Faculty of Design, Kyushu University, Fukuoka, Japan

12

13 **Running head:** cold adaptation in muscle metabolism

14

15 **Corresponding author:**

16 Hitoshi Wakabayashi, Ph.D.

17 Laboratory of Environmental Ergonomics, Faculty of Engineering, Hokkaido University

18 N13 W8, Kita-ku, Sapporo, Hokkaido, 060-8628, Japan

19 Tel&Fax: +81-11-706-6280

20 E-mail: wakabayashi@eng.hokudai.ac.jp

21

22 **Abstract**

23 This study aimed to investigate the effect of repeated cooling of forearm muscle on adaptation in  
24 skeletal muscle metabolism. It is hypothesized that repeated decreases of muscle temperature would  
25 increase the oxygen consumption in hypothermic skeletal muscle.

26 Sixteen healthy males participated in this study. Their right forearm muscles were locally cooled to  
27 25°C by cooling pads attached to the skin. This local cooling was repeated eight times on separate  
28 days for eight participants (experimental group), whereas eight controls received no cold exposure.  
29 To evaluate adaptation in skeletal muscle metabolism, a local cooling test was conducted before and  
30 after the repeated cooling period. Change in oxy-hemoglobin content in the flexor digitorum at rest  
31 and during a 25-second isometric handgrip (10% maximal voluntary construction) was measured using  
32 near-infrared spectroscopy at every 2°C reduction in forearm muscle temperature. The arterial blood  
33 flow was occluded for 15 seconds by upper-arm cuff inflation at rest and during the isometric handgrip.  
34 The oxygen consumption in the flexor digitorum muscle was evaluated by a slope of the  
35 oxy-hemoglobin change during the arterial occlusion.

36 In the experimental group, resting oxygen consumption in skeletal muscle did not show any difference  
37 between pre- and post-intervention, whereas muscle oxygen consumption during the isometric  
38 handgrip was significantly higher post-intervention than pre-test from thermoneutral baseline to 31°C  
39 muscle temperature ( $P < 0.05$ ). This result indicated that repeated local muscle cooling might facilitate  
40 oxidative metabolism in the skeletal muscle.

41 In summary, skeletal muscle metabolism during submaximal isometric handgrip was facilitated after  
42 repeated local muscle cooling.

43

44 **Key Words:** hypothermic skeletal muscle; tissue oxygenation; non-shivering thermogenesis; local  
45 cold exposure; cold adaptation

## 46 **Introduction**

47 A number of researchers have studied the adaptation of non-shivering thermogenesis (NST) after  
48 repeated cold exposures in humans (Vybiral et al. 2000; van Marken Lichtenbelt and Daanen 2003;  
49 van Marken Lichtenbelt and Schrauwen 2011; van der Lans et al. 2013; Yoneshiro et al. 2013;  
50 Nishimura et al. 2015). Recently, brown adipose tissue (BAT) have been focused on as one of the  
51 major components of energy expenditure, since cold-induced activity of BAT in adult humans had  
52 been identified in studies using PET/CT scanning (Saito et al. 2009; van Marken Lichtenbelt et al.  
53 2009). On the other hand, it is necessary to study the skeletal muscle's contribution to the adaptation  
54 of cold induced thermogenesis, concerning their large volume and wide distribution to human body.

55 A recent study reported increases of human BAT activity and NST after 10 consecutive days of  
56 mild cold exposure to 15-16°C in air for six hours a day, whereas no significant change was observed  
57 in skeletal muscle mitochondrial uncoupling in vitro (van der Lans et al. 2013). However, in the case  
58 of acute response to cold, a significant positive relationship has been reported previously between the  
59 increase of total daily energy expenditure during mild cold exposure (16°C in air for 48 hours) and the  
60 increase of mitochondrial uncoupling (state 4 respiration) of isolated human skeletal muscle biopsies  
61 taken after the cold exposure (Wijers et al. 2008; Wijers et al. 2011). Furthermore, recent studies  
62 reported an increase of skeletal muscle oxidative capacity in UCP1 knockout mice or BAT partial  
63 ablation mice after repeated cold exposure (Meyer et al. 2010; Mineo et al. 2012), that indicated the  
64 contribution of skeletal muscle to the increase of NST after chronic cold exposure. However, as was  
65 mentioned above, it was reported that there was no significant change in human skeletal muscle  
66 mitochondrial uncoupling after repeated mild cold exposure (van der Lans et al. 2013). Concerning  
67 these studies, it is suggested that mild cold exposure (defined here as cold stimulus which only  
68 decreases skin temperature and induces NST) would activate mitochondrial uncoupling in the skeletal  
69 muscle, but it is not cold enough to induce a significant increase in UCP activation after repeated

70 exposure to mild cold. The necessary condition for inducing metabolic adaptation in the skeletal  
71 muscle might be a repetition of severe enough cold stimulus which would decrease deep body  
72 temperature and/or initiate shivering. However, Cannon and Nedergaard (2004) insisted that the  
73 increase of muscle oxidative capacity observed after repeated cold exposure was a by-product of  
74 muscle training induced by repeated shivering. Thus, in this study, repeated local severe cold exposure  
75 was used as a thermal adaptation impulse for potentially inducing metabolic adaptation in the skeletal  
76 muscle. This method enables repetition of a strong cold stimulus locally in skeletal muscle without  
77 shivering. Increased muscle capillary density after repeated cold exposure was reported in rats which  
78 were exposed to 5°C in air for four weeks (Suzuki et al. 1997) and in human free-divers who routinely  
79 have been immersed into cold water for more than 20 years (Bae et al. 2003). This might be one  
80 potential mechanism to increase muscle oxidative capacity following repeated cold exposure. It is  
81 probably because of a decline of muscle temperature, which reduces enzyme activity as a result of the  
82 Q10 effect. To compensate for the reduction of the enzyme activity in each muscle fiber, more muscle  
83 fibers would be recruited and/or capillary density might be increased to satisfy an energy (oxygen)  
84 demand. Thus, reduction of muscle temperature might be an essential qualification to increase  
85 oxidative capacity after repeated cold exposure.

86 This study aimed to investigate the effect of a repeated severe local cold exposure (reduction of  
87 forearm muscle temperature) on metabolic adaptation of hypothermic skeletal muscle. Because the  
88 energy demand of resting muscle seems too small to be affected by the suppression of metabolism due  
89 to local cooling, submaximal isometric handgrip exercise conditioning was tested pre- and  
90 post-intervention. It is hypothesized that a repeated decrease of local skeletal muscle temperature will  
91 increase muscle-based thermogenesis in humans.

92

## 93 **Methods**

94 **Participants**

95 Sixteen healthy males participated in this study. They were all right handed and divided into two  
96 groups (experimental cold adaptation and control group) that had no statistical group difference in the  
97 following parameters; height, weight, body mass index, right forearm maximal girth, skin fold  
98 thickness and handgrip strength during maximal voluntary contraction (MVC). Skinfold thickness was  
99 measured at seven sites (forearm, upper arm, subscapular, abdomen, iliac spine, thigh and calf) by one  
100 experienced examiner using a skinfold caliper (Eiyoken-type; Meikosha Co. Ltd., Japan). Physical  
101 characteristics measured pre- and post-intervention are shown in Table 1. All experimental protocols in  
102 this study were designed according to the principle of the Helsinki Declaration and approved by the  
103 Institutional Review Board of Kyushu University. All participants were informed of the experimental  
104 procedures and gave their written informed consent before participation.

105

106 **Procedures**

107 The experimental cold adaptation group repeated forearm muscle cooling eight times on separate  
108 days in three weeks, while no cold exposure was conducted in the control group. Their right forearm  
109 was locally cooled by using cooling pads attached to the skin until their forearm muscle temperature  
110 fell to 25°C. Whole surface of the right forearm between the wrist and elbow was wrapped by several  
111 water perfusion cooling pads, in which 5°C water was circulated using a thermostatic bath system  
112 (LTB-400, ASONE, Japan). On average, about 70 minute was required to cool forearm muscle to 25°C.  
113 The forearm muscle temperature was measured by a deep body temperature monitor (CM-210,  
114 TERUMO, Japan) which detects the tissue temperature 5-10 mm below the skin surface using the zero  
115 heat flow method (Yamakage and Namiki 2003). This monitor measures skin surface temperature  
116 beneath a thermal insulating pad containing a heater, which equilibrates the skin temperature with the  
117 deep tissue temperature when heat flow from the skin is maintained to zero.

118 To evaluate adaptation in metabolism in the skeletal muscle, both groups undertook two  
119 experimental forearm cooling tests, three weeks apart (Pre and Post), until their right forearm muscle  
120 temperature fell to 25°C. Before the first local cooling test, all participants practiced the protocol  
121 including submaximal (10% of predetermined MVC) isometric handgrip exercise using a handgrip  
122 dynamometer (T.K.K.5710b, Takei Scientific Instruments, Japan). On the day of the forearm cooling  
123 test, participants came to the laboratory at least one hour prior to starting the protocol. After changing  
124 into shorts and T-shirts, they rested on a chair in a climate chamber controlled at 24°C and 50%  
125 relative humidity, and sensors were attached to them. During the forearm cooling test they sat on a  
126 chair and placed their right arm on a table holding the hand grip dynamometer fixed on the table. At  
127 the beginning of the test, normothermic baselines of measurement items described below at rest and  
128 during submaximal isometric contraction (10%MVC) were measured before starting forearm cooling.  
129 After the measurement of normothermic baseline, their right forearms were locally cooled by the  
130 water perfusion cooling pads attached to the skin, as the same manner of the repeated forearm cooling  
131 described above. During the cooling protocol, participants repeated 25-second isometric handgrip  
132 (10% MVC) for every 2°C reduction in the forearm muscle temperature (around 35, 33, 31, 29, 27 and  
133 25°C).

134

### 135 **Measurements**

136 Changes in muscle hemodynamics (changes in oxy- and deoxy-hemoglobin content) were  
137 continuously measured by spatially resolved near-infrared spectroscopy (NIRS) (NIRO-200,  
138 Hamamatsu Photonics, Japan) every 0.5 seconds. The optodes of the NIRS, with a 3 cm distance  
139 between an illuminant and a detector, were housed in an optically dense black vinyl holder to ensure  
140 the position and were attached on the skin above the flexor digitorum (around 5-10 cm distal from the  
141 cubital fossa) using double-sided adhesive tape. A fixed path length (10.6 cm) was used in this study

142 as suggested by the manufacturer. The resting oxygen consumption in normothermic forearm muscle  
143 was evaluated by the slope of the concentration change of oxy-hemoglobin (O<sub>2</sub>Hb) during 15-second  
144 arterial occlusion using an upper-arm cuff inflated to 220 mmHg with a rapid cuff inflator system (E20  
145 and AG101, Hokanson, USA). The calculation of tissue oxygen consumption (tissueVO<sub>2</sub>) was based  
146 on previous studies (van Beekvelt et al. 2001; van Beekvelt et al. 2002). The rate of decrease in  
147 concentration of O<sub>2</sub>Hb (-d(O<sub>2</sub>Hb)/dt) was manually detected using a 5-second stable linear slope of  
148 O<sub>2</sub>Hb around 5-10 seconds after occlusion. Then, the unit of -d(O<sub>2</sub>Hb)/dt [μmol Hb/L/sec] was  
149 converted into milliliters O<sub>2</sub> per 100 gram tissue per minute [mLO<sub>2</sub>/100g/min] taking into account that  
150 each Hb molecule binds four O<sub>2</sub> molecules, the molar gas volume in STPD is 22.4 L and muscle  
151 density is 1.04 kg/L, using the following equation:

$$152 \quad \text{TissueVO}_2 = -d(\text{O}_2\text{Hb})/dt \cdot 4 \cdot 22.4/1000 / 1.04/10 \cdot 60 [\text{mLO}_2/100\text{g}/\text{min}]$$

153 TissueVO<sub>2</sub> of the normothermic forearm during isometric handgrip (10% MVC) was also  
154 evaluated by a similar technique. Participants were asked to keep constant handgrip strength for 25  
155 seconds with arterial occlusion for the latter 15 seconds. After starting forearm cooling, tissueVO<sub>2</sub> of  
156 the right forearm at rest and during isometric handgrip (10% MVC) was evaluated for every 2°C  
157 reduction in the forearm muscle temperature using the same technique as for the normothermic  
158 baseline.

159 Skin temperature on the right forearm ( $T_{\text{forearm}}$ ) was measured by thermistor sensors and a data  
160 logger (LT-8A, Gram Corporation, Japan) every two seconds. Skin blood flow (SkBF) in the forearm  
161 skin above the flexor digitorum was measured by laser Doppler flowmetry (FLO-C1, OMEGAWAVE,  
162 Japan). The right forearm skin thermistor and a laser Doppler probe were attached on the skin around  
163 10 mm distal site from the NIRS probe. The SkBF data were sampled using an A/D converter  
164 (Powerlab/16SP, AD Instruments, Australia) and recorded at 0.5 second intervals using a personal  
165 computer. Since the laser Doppler flow signal does not provide an absolute measurement of blood flow,



166 the voltage output of the laser Doppler measurement was normalized (%SkBF) relative to maximal  
167 levels (100%), which were measured during reactive hyperemia after right arm arterial occlusion, and  
168 the minimum value (0%) during the occlusion conducted before starting cooling. To minimize the  
169 artifact due to the movement of the laser Doppler probe, participants were asked to keep their right  
170 arm as stable as possible on a fixed table. The 15-second averaged %SkBF and skin temperature just  
171 before the arterial occlusion for evaluating resting muscle oxygenation for every 2°C reduction in  
172 forearm muscle temperature was analyzed later.

173

## 174 **Statistics**

175 Data representative for baseline prior to cooling and each 2°C reduction in the forearm muscle  
176 temperature (tissue  $\dot{V}O_2$ ,  $T_{\text{forearm}}$  and %SkBF) were analyzed by a repeated measures two-way (muscle  
177 temperature  $\times$  pre-post intervention) analysis of variance (ANOVA). This analysis was conducted  
178 separately for each control and experimental cold adaptation group. After determining the main effects,  
179 pair-wise post-hoc tests were conducted between pre- and post-intervention tests at indicated muscle  
180 temperatures, and among muscle temperatures within pre- and post-test groups. Additionally,  
181 pre-intervention group differences were tested by two-way (muscle temperature  $\times$  group) ANOVA  
182 followed by Student's *t*-tests between groups at each muscle temperature, using the measured pre-test  
183 data. Significant differences were established at  $P < 0.05$ . All data are presented as mean values and  
184 standard error (SE).

185

## 186 **Results**

### 187 **Physical characteristics**

188 No difference was observed in any physical characteristics between groups or in pre- and  
189 post-intervention forearm cooling within a group (Table 1).

190

## 191 **Control group**

192 Based on the data measured in the pre-intervention forearm cooling test, there was no statistical  
193 difference between the control and experimental groups in any variables (tissue  $\dot{V}O_2$ ,  $T_{\text{forearm}}$   
194 and %SkBF) at each muscle temperature. The control group did not show significant differences in  
195 any variables at each muscle temperature between pre and post forearm cooling tests (Fig. 1a-4a).

196

## 197 **Tissue oxygen consumption in the experimental group**

198 The resting forearm tissue  $\dot{V}O_2$  of the experimental group at each 2°C muscle temperature reduction  
199 pre- and post-cooling is shown in Figure 1b. There is a significant main effect of muscle temperature  
200 ( $P=0.014$ ) in the two-way ANOVA (Fig 1b). However, post-hoc analysis indicated no statistical  
201 difference among tissue  $\dot{V}O_2$  at any muscle temperatures between pre- and post-intervention tests.

202 Forearm tissue  $\dot{V}O_2$  during submaximal isometric handgrip in the experimental group showed a  
203 significant main effect of muscle temperature ( $P<0.001$ ), pre- and post-cooling ( $P=0.001$ ), and a  
204 significant interaction between those factors ( $P=0.022$ ) in the two-way ANOVA (Fig 2b). Tissue  $\dot{V}O_2$   
205 during submaximal handgrip was significantly higher after local cooling than pre-test for baseline, 35,  
206 33 and 31°C muscle temperature ( $P<0.05$ ). In the pre-test, tissue  $\dot{V}O_2$  during submaximal handgrip at  
207 27 and 25°C muscle temperature was significantly lower than that at baseline ( $P<0.05$ ). In the post-test,  
208 tissue  $\dot{V}O_2$  during handgrip at 31 to 25°C muscle temperature was significantly lower than that at  
209 baseline ( $P<0.05$ ).

210

## 211 **Forearm skin blood flow and skin temperature**

212 The resting %SkBF in the right forearm just before occlusion at each 2°C muscle temperature  
213 reduction pre- and post-cooling is shown in Figure 3. In the experimental group, a significant main

214 effect of muscle temperature ( $P<0.001$ ) was observed in the %SkBF in the two-way ANOVA (Fig  
215 3b). %SkBF at 35°C (when cooling just was started) to 25°C muscle temperature was significantly  
216 lower than at baseline both pre- and post-intervention ( $P<0.05$ , Fig 3b). No statistical difference was  
217 observed between pre- and post-intervention at each muscle temperature level.

218 Right forearm skin temperature was gradually decreased with the reduction of muscle temperature;  
219 a significant main effect of muscle temperature ( $P<0.001$ ), pre- and post-intervention ( $P=0.039$ ), and a  
220 significant interaction between those factors ( $P=0.008$ ), was shown in the two-way ANOVA in the  
221 experimental group (Fig 4b). Forearm skin temperature tended to be higher post-test at each muscle  
222 temperature level and a significant difference was observed at 33°C ( $P<0.05$ , Fig 4b).

223

## 224 **Discussion**

225 This study investigated the effect of repeated local forearm muscle cooling on the adaptation in the  
226 skeletal muscle metabolism. The major finding is that the forearm tissue oxygen consumption during  
227 submaximal isometric handgrip was significantly greater post-intervention local cooling test than  
228 pre-test in the range of thermoneutral baseline to 31°C muscle temperature level ( $P<0.05$ ). This result  
229 indicated that repeated forearm muscle cooling may facilitate metabolism in skeletal muscle. On the  
230 other hand, resting oxygen consumption during forearm muscle cooling was relatively constant among  
231 muscle temperature levels and no change was found between pre and post intervention. This might be  
232 because the energy demand of resting muscle was too small to be affected by the suppression of  
233 metabolism in hypothermic muscle.

234 In this study, the change in skeletal muscle metabolism was examined after repeated local severe  
235 cold exposure which did not induce shivering. Thus, the present results were not affected by the  
236 muscle training effect due to repeated shivering during the adaptation process, as suggested by Cannon  
237 and Nedergaard (2004). Additionally, as there were no morphological change in the girth and skinfold

238 thickness on the forearm between pre- and post-intervention (Table 1), there would be no muscular  
239 hypertrophy. Thus, the increased tissue $\dot{V}O_2$  after the repeated forearm cooling intervention would  
240 mainly be due to some qualitative changes in the skeletal muscle tissue.

241 It is well known that a decline of muscle temperature slows enzymatic processes as a result of the  
242 Q10 effect (Barany 1967; Bennett 1985). Additionally, suppression of O<sub>2</sub>-uploading from hemoglobin  
243 in lower temperature tissue (the Bohr Effect) would restrict the supply of O<sub>2</sub> to the muscle fibers.  
244 Moreover, O<sub>2</sub> supply to the muscle tissue is restricted by the lower muscle blood flow in the  
245 hypothermic muscle (Abramson et al. 1958; Thorsson et al. 1985). The suppressed muscle metabolism  
246 would be a factor in the impairment of physical performance in the cold (Bennett 1985; Wakabayashi  
247 et al. 2015). In this study, a significantly greater tissue $\dot{V}O_2$  during submaximal isometric handgrip was  
248 observed after the repeated forearm muscle cooling in the experimental group, who repeatedly  
249 experienced these metabolic suppressions in their skeletal muscle.

250 We propose a potential mechanism for this adaptation. Distribution of muscle fiber type was  
251 assessed in Korean diving women who routinely exposed their body to cold water (Bae et al. 2003).  
252 Divers had a greater percentage of type IIx and a lower proportion of type IIa fibers in the vastus  
253 lateralis than control active women, whereas no group difference was observed in the percentage of  
254 type I fibers. This suggested that repeated cold water immersion might induce the shift of type II  
255 muscle fibers to the faster subgroup. In an animal study, a similar shift in fiber type from type I to type  
256 IIa fibers was observed in rat soleus muscle (predominantly Type I) after intermittent cold exposure  
257 (Walters and Constable 1993). Although, there is a discrepancy between the previous and the present  
258 studies in the adaptation process with or without exercise, it is speculated that a greater number of  
259 fast-twitch fibers might be recruited for the handgrip exercise following the intervention. This might  
260 cause the increase of tissue $\dot{V}O_2$  after the cold acclimation period, as fast-twitch fibers are less  
261 economical than slow-twitch fibers (Crow and Kushmerick 1982; Krstrup et al. 2008).

262 Another potential mechanism for the greater tissue $VO_2$  after cold adaptation is an increment of  $O_2$   
263 delivery caused by the growth of capillary density. Bae et al. (2003) reported that Korean diving  
264 women had a significantly greater capillary number per muscle fiber of the vastus lateralis compared  
265 to that in the active control group. In animal studies, chronic cold exposure induced an increase in  
266 capillary density and/or the capillary to fiber ratio in rats (Suzuki et al. 1997; Deveci and Egginton  
267 2002) and in guinea pigs (Sillau et al. 1980). The greater tissue $VO_2$  after cold adaptation could be  
268 explained by the improvement of the oxygen supply to the muscle tissues by microvascular  
269 remodeling (angiogenesis). In this study, since the tissue $VO_2$  was evaluated by a slope of the  
270 oxy-hemoglobin change during arterial occlusion, the blood circulation was limited in the local body  
271 part. However, better blood perfusion before occlusion might enable greater oxygen uptake in the  
272 occluded muscle tissue.

273 Additionally, a significant positive relationship between the increase of total daily energy  
274 expenditure during mild cold exposure and the increase of mitochondrial uncoupling of isolated  
275 human skeletal muscle biopsies taken after the cold exposure has been reported (Wijers et al. 2008;  
276 Wijers et al. 2011). On the other hand, no significant change in human skeletal muscle mitochondrial  
277 uncoupling was observed after repeated mild cold exposure (van der Lans et al. 2013). The timing of  
278 the muscle biopsy, when the sample was taken with (Wijers et al. 2008) or without (van der Lans et al.  
279 2013) mild cold exposure, could influence the result of muscle respiration. The increased muscle  
280 metabolism observed in the present study might be due to enhanced muscle oxidative capacity via  
281 mitochondrial uncoupling and/or a greater number of mitochondria (Bruton et al. 2010). In this study,  
282 skin temperature of the cooled forearm tended to be higher after intervention, whereas there was no  
283 change in the skin blood flow. Thus, the higher skin temperature would be due to a greater  
284 thermogenesis in the subcutaneous skeletal muscle tissue, rather than to skin vasodilation.

285 In conclusion, after repeated local forearm muscle cooling, the forearm muscle oxygen

286 consumption during a submaximal isometric handgrip was facilitated at muscle temperatures higher  
287 than 31°C.

288

289 **Acknowledgements**

290 The authors wish to thank all those who participated in this study. We would also like to express  
291 our gratitude to Mr Mutsuhiro Fujiwara for his technical support. This study was supported by a  
292 Grant-in-Aid for Scientific Research (No. 09J03584, No. 26291099) from the Japan Society for the  
293 Promotion of Science, and the DESCENTE and ISHIMOTO Memorial Foundation for the Promotion  
294 of Sports Science. There is no conflict of interest in relation to this work.

295 **Table 1** Physical characteristic of the experimental and control groups pre- and post-intervention

		Experimental group		Control group	
		Pre	Post	Pre	Post
Age	yrs	21.9 (0.4)	-	22.1 (1.1)	-
Height	cm	171.3 (2.6)	171.3 (2.6)	168.5 (1.2)	168.5 (1.2)
Body weight	kg	59.8 (2.4)	60.2 (2.4)	55.3 (1.3)	55.4 (1.3)
Body mass index	kg·m <sup>-2</sup>	20.4 (0.5)	20.5 (0.5)	19.5 (0.4)	19.5 (0.5)
Mean skin fold thickness	mm	7.6 (0.8)	7.4 (0.8)	6.6 (1.0)	6.5 (1.0)
Forearm maximal girth	cm	24.3 (0.4)	24.3 (0.3)	24.0 (0.4)	24.1 (0.3)
Forearm skin fold thickness	mm	3.0 (0.2)	3.2 (0.3)	3.3 (0.2)	3.1 (0.2)
Handgrip MVC	kg	47.4 (2.5)	-	45.1 (3.3)	-

296

297 Values are means (standard error). Mean skin fold thickness was calculated from the measured values  
 298 at seven sites (forearm, upper arm, subscapular, abdomen, iliac spine, thigh and calf). Maximal  
 299 voluntary contraction (MVC) during isometric handgrip was measured. No statistical difference was  
 300 observed in any physical characteristics between groups or between pre- and post-intervention.



301 **Figure captions**

302 **Fig. 1** Resting forearm tissue oxygen consumption at each 2°C muscle temperature reduction pre- and  
303 post-intervention. Values are mean  $\pm$  SE of each control (a) and experimental group (b).

304

305 **Fig. 2** Forearm tissue oxygen consumption during isometric handgrip at each 2°C muscle temperature  
306 reduction pre- and post-intervention. Values are mean  $\pm$  SE of each control (a) and experimental group  
307 (b). \*Significant difference between pre- and post-intervention tests.

308

309 **Fig. 3** Normalized skin blood flow in the forearm just before occlusion at each 2°C muscle  
310 temperature reduction pre- and post-intervention. Values are mean  $\pm$  SE of each control (a) and  
311 experimental group (b). The skin blood flow data are normalized relative to peak value.

312

313 **Fig. 4** Forearm skin temperature just before occlusion at each 2°C muscle temperature reduction pre-  
314 and post-intervention. Values are mean  $\pm$  SE of each control (a) and experimental group (b).  
315 \*Significant difference between pre- and post-intervention tests.

316

317 **References**

- 318 Abramson DI, Kahn A, Tuck S, Jr., Turman GA, Rejal H, Fleischer CJ (1958) Relationship between a  
319 range of tissue temperature and local oxygen uptake in the human forearm. I. Changes  
320 observed under resting conditions. *J Clin Invest* 37 (7):1031-1038. doi:10.1172/JCI103684
- 321 Bae KA, An NY, Kwon YW, Kim C, Yoon CS, Park SC, Kim CK (2003) Muscle fibre size and  
322 capillarity in Korean diving women. *Acta Physiol Scand* 179 (2):167-172.  
323 doi:10.1046/j.1365-201X.2003.01185.x
- 324 Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. *J Gen Physiol*  
325 50 (6):Suppl:197-218
- 326 Bennett AF (1985) Temperature and muscle. *J Exp Biol* 115:333-344
- 327 Bruton JD, Aydin J, Yamada T, Shabalina IG, Ivarsson N, Zhang SJ, Wada M, Tavi P, Nedergaard J,  
328 Katz A, Westerblad H (2010) Increased fatigue resistance linked to Ca<sup>2+</sup>-stimulated  
329 mitochondrial biogenesis in muscle fibres of cold-acclimated mice. *J Physiol* 588 (Pt  
330 21):4275-4288. doi:10.1113/jphysiol.2010.198598
- 331 Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance.  
332 *Physiol Rev* 84 (1):277-359. doi:10.1152/physrev.00015.2003
- 333 Crow MT, Kushmerick MJ (1982) Chemical energetics of slow- and fast-twitch muscles of the mouse.  
334 *J Gen Physiol* 79 (1):147-166
- 335 Deveci D, Egginton S (2002) Differing mechanisms of cold-induced changes in capillary supply in m.  
336 tibialis anterior of rats and hamsters. *J Exp Biol* 205 (Pt 6):829-840
- 337 Krstrup P, Secher NH, Relu MU, Hellsten Y, Soderlund K, Bangsbo J (2008) Neuromuscular  
338 blockade of slow twitch muscle fibres elevates muscle oxygen uptake and energy turnover  
339 during submaximal exercise in humans. *J Physiol* 586 (Pt 24):6037-6048.  
340 doi:10.1113/jphysiol.2008.158162

341 Meyer CW, Willershauser M, Jastroch M, Rourke BC, Fromme T, Oelkrug R, Heldmaier G,  
342 Klingenspor M (2010) Adaptive thermogenesis and thermal conductance in wild-type and  
343 UCP1-KO mice. *Am J Physiol Regul Integr Comp Physiol* 299 (5):R1396-1406.  
344 doi:10.1152/ajpregu.00021.2009

345 Mineo PM, Cassell EA, Roberts ME, Schaeffer PJ (2012) Chronic cold acclimation increases  
346 thermogenic capacity, non-shivering thermogenesis and muscle citrate synthase activity in  
347 both wild-type and brown adipose tissue deficient mice. *Comp Biochem Physiol A Mol Integr*  
348 *Physiol* 161 (4):395-400. doi:10.1016/j.cbpa.2011.12.012

349 Nishimura T, Motoi M, Egashira Y, Choi D, Aoyagi K, Watanuki S (2015) Seasonal variation of  
350 non-shivering thermogenesis (NST) during mild cold exposure. *J Physiol Anthropol* 34 (1):11.  
351 doi:10.1186/s40101-015-0051-9

352 Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, Iwanaga T,  
353 Miyagawa M, Kameya T, Nakada K, Kawai Y, Tsujisaki M (2009) High incidence of  
354 metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure  
355 and adiposity. *Diabetes* 58 (7):1526-1531. doi:10.2337/db09-0530

356 Sillau AH, Aquin L, Lechner AJ, Bui MV, Banchemo N (1980) Increased capillary supply in skeletal  
357 muscle of guinea pigs acclimated to cold. *Respir Physiol* 42 (3):233-245

358 Suzuki J, Gao M, Ohinata H, Kuroshima A, Koyama T (1997) Chronic cold exposure stimulates  
359 microvascular remodeling preferentially in oxidative muscles in rats. *Jpn J Physiol* 47  
360 (6):513-520. doi:10.2170/jjphysiol.47.513

361 Thorsson O, Lilja B, Ahlgren L, Hemdal B, Westlin N (1985) The effect of local cold application on  
362 intramuscular blood flow at rest and after running. *Med Sci Sports Exerc* 17 (6):710-713

363 van Beekvelt MCP, Colier WNJM, Wevers RA, van Engelen BGM (2001) Performance of  
364 near-infrared spectroscopy in measuring local O<sub>2</sub> consumption and blood flow in skeletal

365 muscle. *J Appl Physiol* (1985) 90:515-519

366 van Beekvelt MCP, van Engelen BGM, Wevers RA, Colier WJNM (2002) In vivo quantitative  
367 near-infrared spectroscopy in skeletal muscle during incremental isometric handgrip exercise.  
368 *Clin Physiol Funct Imaging* 22:210-217. doi:10.1046/j.1475-097X.2002.00420

369 van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, Vosselman MJ, Hansen J, Jorgensen JA,  
370 Wu J, Mottaghy FM, Schrauwen P, van Marken Lichtenbelt WD (2013) Cold acclimation  
371 recruits human brown fat and increases nonshivering thermogenesis. *J Clin Invest* 123  
372 (8):3395-3403. doi:10.1172/JCI68993

373 van Marken Lichtenbelt WD, Daanen HA (2003) Cold-induced metabolism. *Curr Opin Clin Nutr*  
374 *Metab Care* 6 (4):469-475. doi:10.1097/01.mco.0000078992.96795.5f

375 van Marken Lichtenbelt WD, Schrauwen P (2011) Implications of nonshivering thermogenesis for  
376 energy balance regulation in humans. *Am J Physiol Regul Integr Comp Physiol* 301  
377 (2):R285-296. doi:10.1152/ajpregu.00652.2010

378 van Marken Lichtenbelt WD, Vanhommel JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy  
379 ND, Schrauwen P, Teule GJ (2009) Cold-activated brown adipose tissue in healthy men. *N*  
380 *Engl J Med* 360 (15):1500-1508. doi:10.1056/NEJMoa0808718

381 Vybiral S, Lesna I, Jansky L, Zeman V (2000) Thermoregulation in winter swimmers and  
382 physiological significance of human catecholamine thermogenesis. *Exp Physiol* 85  
383 (3):321-326

384 Wakabayashi H, Oksa J, Tipton MJ (2015) Exercise performance in acute and chronic cold exposure. *J*  
385 *Phys Fitness Sports Med* 4 (2):177-185. doi:10.7600/jpfs.4.177

386 Walters TJ, Constable SH (1993) Intermittent cold exposure causes a muscle-specific shift in the fiber  
387 type composition in rats. *J Appl Physiol* (1985) 75 (1):264-267

388 Wijers SL, Schrauwen P, Saris WH, van Marken Lichtenbelt WD (2008) Human skeletal muscle

389 mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. PLoS One  
390 3 (3):e1777. doi:10.1371/journal.pone.0001777

391 Wijers SL, Schrauwen P, van Baak MA, Saris WH, van Marken Lichtenbelt WD (2011)  
392 Beta-adrenergic receptor blockade does not inhibit cold-induced thermogenesis in humans:  
393 possible involvement of brown adipose tissue. J Clin Endocrinol Metab 96 (4):E598-605.  
394 doi:10.1210/jc.2010-1957

395 Yamakage M, Namiki A (2003) Deep temperature monitoring using a zero-heat-flow method. J Anesth  
396 17 (2):108-115. doi:10.1007/s005400300026

397 Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, Kawai Y, Iwanaga T, Saito M (2013)  
398 Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest 123  
399 (8):3404-3408. doi:10.1172/JCI67803

All Figures were created using MS Excel 2010.

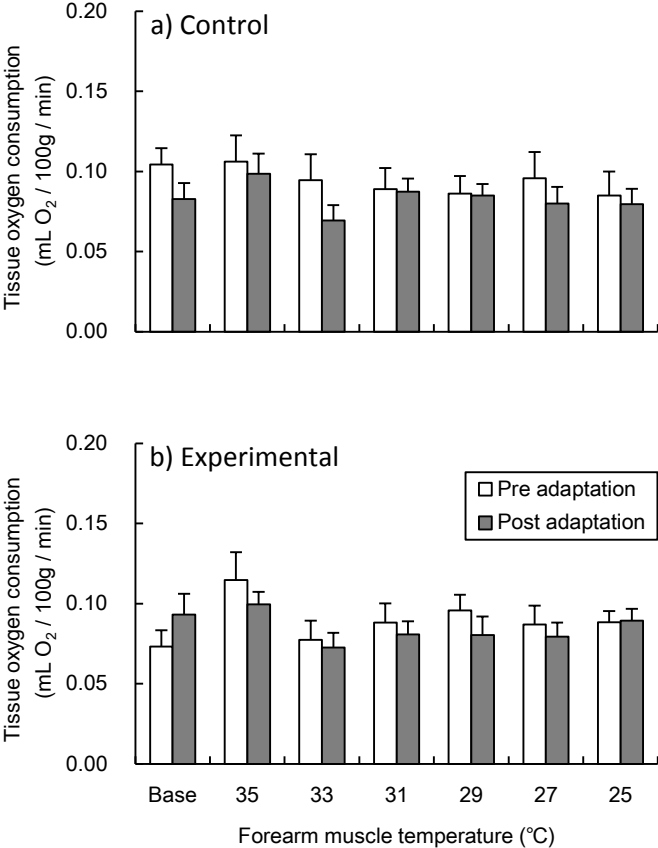


Fig. 1

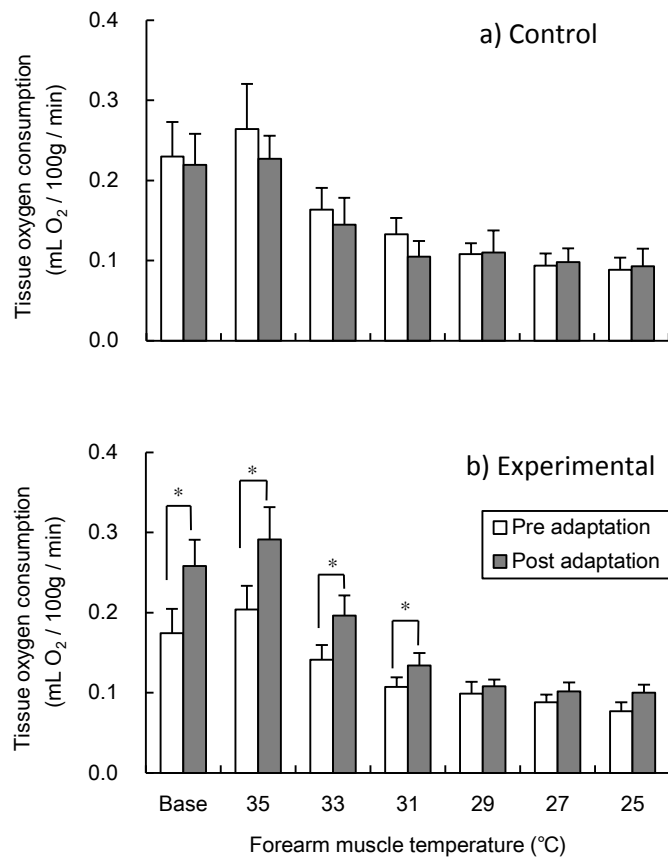


Fig. 2

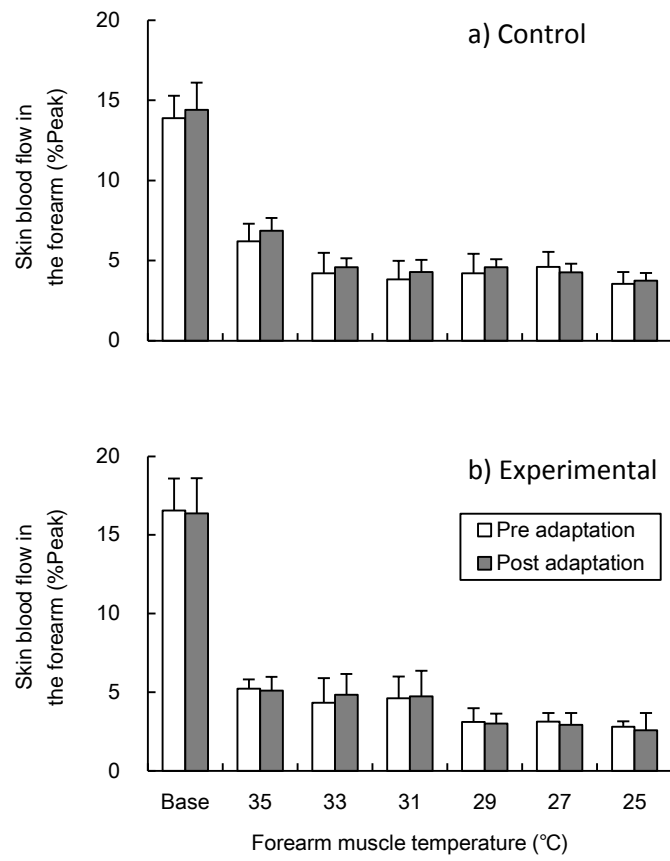


Fig. 3



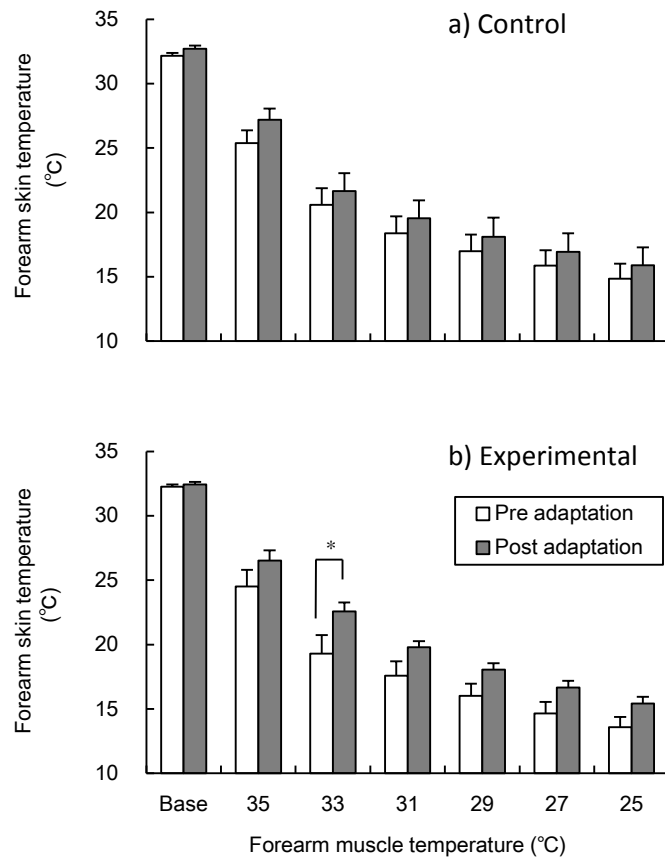


Fig. 4