

Activation of the hypoxia-inducible factor pathway induced by prolyl hydroxylase domain 2 deficiency enhances the effect of running training in mice

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Abstract

Aims: Hypoxic response mediated by hypoxia-inducible factor (HIF) seems to contribute to the benefit of endurance training. To verify the direct contribution of HIF activation to running training without exposure to atmospheric hypoxia, we used prolyl hydroxylase domain 2 (PHD2) conditional knockout mice (cKO), which exhibit HIF activation independent of oxygen concentration, and we examined their maximal exercise capacity before and after 4 weeks of treadmill exercise training.

Methods: *Phd2^{fl/fl}* mice ($n = 26$) and *Phd2* cKO mice ($n = 24$) were randomly divided into two groups, trained and untrained, and were subjected to maximal running test before and after a 4-week treadmill-training regimen.

Results: Prolyl hydroxylase domain 2 deficiency resulted in HIF- α protein accumulation. *Phd2* cKO mice exhibited marked increases in haematocrit values and haemoglobin concentrations, as well as an increase in the capillary number in the skeletal muscle. The 4-week training elicited an increase in the capillary-to-fibre (C/F) ratio and succinyl dehydrogenase activity of the skeletal muscle. Importantly, trained *Phd2* cKO mice showed a significantly greater improvement in running time than trained control mice ($P < 0.05$). Collectively, these data suggest that the combination of training and the activation of the HIF pathway are important for maximizing the effect of running training.

Conclusion: We conclude that the activation of the HIF pathway induced by PHD2 deficiency enhances the effect of running training.

Keywords altitude training, hypoxic response, training effect, treadmill.

Exposing the body to low oxygen (O₂) concentrations in the atmosphere elicits a series of adaptive reactions. Hypoxia-inducible factor (HIF), which is known as a master regulator of the hypoxic response, transcriptionally regulates the expression of several target genes

(Epstein *et al.* 2001, Wenger 2002), including erythropoietin (*Epo*) (Semenza *et al.* 1991) and vascular endothelial growth factor (*Vegf*) (Forsythe *et al.* 1996, Semenza 2002). These adaptations to lower atmospheric O₂ concentration below 15.4% seemed to

contribute to the benefit of altitude training (Levine & Stray-Gundersen 1997); however, the veracity of this benefit is still controversial, and studies ruling out the placebo effect are suggested to be required (Lundby *et al.* 2012). One of the ways to rule out the placebo effect is to show the effect of biological processes mediated by the HIF pathway on the improvement in exercise performance after exercise training.

Hypoxia-inducible factor consists of an oxygen-regulated HIF- α subunit (HIF-1 α , 2 α and 3 α) and a constitutively stable HIF- β subunit (Maltepe *et al.* 1997). These heterodimers activate the transcription of numerous hypoxia-inducible genes (Wang & Semenza 1993, Semenza *et al.* 1994, Wang *et al.* 1995, Jiang *et al.* 1996, Takeda *et al.* 2014). Under normoxic conditions, the HIF- α subunit undergoes hydroxylation by the prolyl hydroxylase domain protein (PHD), and it is rapidly subjected to ubiquitination followed by proteasomal degradation (Epstein *et al.* 2001, Ivan *et al.* 2001, Jaakkola *et al.* 2001). There are at least three PHD isoforms in mammals (PHD1, 2 and 3), with PHD2 being the main isoform responsible for regulating HIF- α degradation (Berra *et al.* 2003). The hydroxylase activity of PHDs is suppressed under hypoxic conditions; thus, under hypoxic conditions, stabilized HIF- α accumulates, heterodimerizes with HIF- β and transactivates hypoxia-responsive genes by binding to hypoxia response elements in the promoter or enhancer regions of these genes (Wang & Semenza 1993, Semenza *et al.* 1994, Jiang *et al.* 1996, Hu *et al.* 2003, Kaelin 2005). Thus, the activities of PHDs act as a switch that regulates the stabilization of HIF and the subsequent hypoxic response.

Interestingly, previous studies (Takeda *et al.* 2007, Minamishima *et al.* 2008) showed that *Phd2* deficiency stabilizes HIF-1 α and consequently elicits a series of HIF-dependent transactivation without exposure to atmospheric hypoxia. The phenotype of *Phd2*-deficient mice, including high haematocrit, was already described (Takeda *et al.* 2007, Minamishima *et al.* 2008); however, the contribution of the stabilization of HIF induced by *Phd2* deficiency to exercise capacity is still unknown.

Additionally, while physiological changes induced by the activation of the HIF pathway may improve oxygen delivery (Bunn 2013, Zhang *et al.* 2014), an excessive elevation of haematocrit values may be associated with hypertension and thromboembolism, resulting in severe or even fatal complications (Bertinieri *et al.* 1998, Vogel *et al.* 2003).

Overall, we believed that the *Phd2*-deficient mouse model enabled us to elucidate the influence of the activated HIF pathway on the exercise training effect. We therefore hypothesized that the stabilization of HIF

and the subsequent haematopoiesis and angiogenesis induced by *Phd2* deficiency contribute to the improvement in exercise training effect. To examine this hypothesis, we investigated the maximal exercise capacity of *Phd2*-deficient mice before and after a 4-week regimen of exercise training.

Methods

Mouse strains and crosses

All experiments were conducted in accordance with the regulations of the Standards for Human Care and Use of Laboratory Animals of Tohoku University (Permit Number: 2015ikokumikae-007, 2015ikodo-008).

Because constitutive knockout of *Phd2* is embryonically lethal (Takeda *et al.* 2006), we used an oestrogen receptor (ER) agonist-induced *Phd2* knockout mouse using the Cre-loxP system. *Phd2/flox* mice (a gift from Dr G.H. Fong) were mated with *Rosa26/Cre-ER(T2)* mice to generate *Phd2* conditional knockout (cKO) mice (*Phd2^{fl/fl}/Rosa26^{CreERT2}*; *Phd2* cKO) by referring to a previous report (Takeda *et al.* 2007). To delete the floxed *Phd2* exon 2, the ER agonist tamoxifen (Sigma, St. Louis, MO, USA) was delivered to 8- to 12-week-old male *Phd2* cKO mice by intraperitoneal injection (10 mg mL⁻¹ in corn oil, 20 mg kg⁻¹ day⁻¹ for five consecutive days). Littermate male *Phd2^{fl/fl}* mice lacking Cre were used as controls and were similarly treated with tamoxifen. For genotyping, DNA was isolated from tails. The sequences of primers are presented in Table 1.

Running capacity assessment and exercise training

The experimental design is shown in Figure 1. To determine the exercise capacity, a running test using a rodent treadmill was performed 2 weeks after tamoxifen treatment as a baseline before training. Before the test, the mice ran for 5 min at 10 m min⁻¹ as a warm-up. During the first 30 min, the speed was set at 10 m min⁻¹ and was increased by 2 m min⁻¹ every 15 min. Throughout the warm-up and test running, the treadmill was set without slopes. The mice were encouraged to run by tail stimulation with a soft brush and a low-voltage power grid. Exhaustion was determined to be the point at which the animal would not resume running for 15 s despite gentle brushing on the tail and a mild electrical foot shock.

After the test, the mice (26 control mice and 24 *Phd2* cKO mice) were randomly divided into two groups: trained and untrained. The trained group was subjected to 4-week treadmill training. Briefly, the mice in the trained group were trained for 30 min at a speed of 18 m min⁻¹ with a fixed slope of 5° for

Table 1 The primer sequences

	Forward	Reverse
<i>Phd2</i>	5'-CAAATGGAGATGGAAGATGC-3'	5'-TCAACTCGAGCTGGAAACC-3'
<i>Cre</i>	5'-GATATCTCACGTAAGTACGG-3'	5'-TGACCAGAGTCATCCTTAGC-3'
<i>Hif-1α</i>	5'-CAAGATCTCGGCGAAGCAA-3'	5'-GGTGAGCCTCATAACAGAAGCTTT-3'
<i>Hif-2α</i>	5'-CAACCTGCAGCCTCAGTGTATC-3'	5'-CACCACGTCGTTCTTCTCGAT-3'
<i>MT-CO2</i>	5'-ACGAAATCAACAACCCCGTA-3'	5'-GGCAGAACGACTCGGTTATC-3'
<i>Glut1</i>	5'-CTTCATTGTGGGCATGTGCTTC-3'	5'-AGGTTCCGGCCTTTGGTCTCAG-3'
<i>Cox5a</i>	5'-TGCGAGCATGTAGACCGTTAAAT-3'	5'-GAGGTCCTGCTTTGTCCTTAACA-3'
<i>β-actin</i>	5'-CTGGGTATGGAATCCTGTGG-3'	5'-GTACTTGCCTCAGGAGGAG-3'

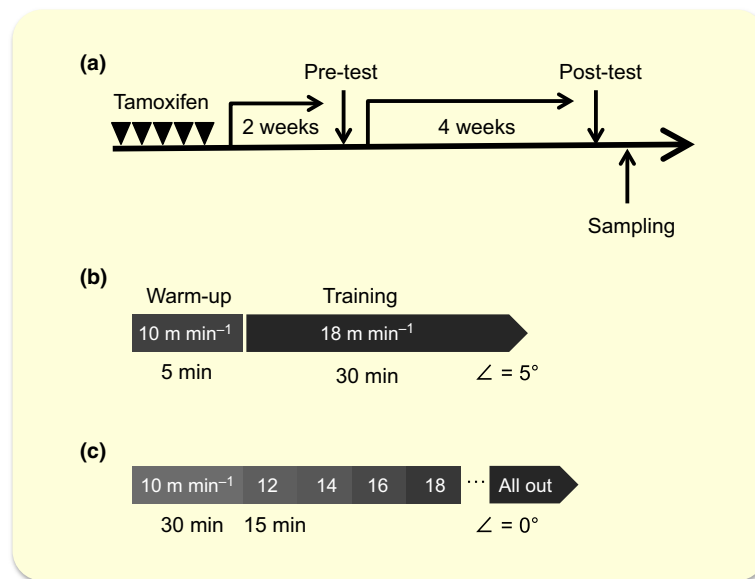


Figure 1 Experimental design. (a) A schematic representation of the study protocol. Two weeks after tamoxifen administration, a treadmill pre-test was performed to determine the base line of exercise capacity. After 4 weeks of trained or untrained period, post-test was performed. (b) The protocol for the 4-week training. The mice in the trained group were trained for 30 min at a speed of 18 m min⁻¹ with a fixed slope of 5° for 5 days per week. (c) The protocol for the maximal exercise test. Before the test, the mice ran for 5 min at 10 m min⁻¹ as a warm-up. During the first 30 min, the speed was set at 10 m min⁻¹ and was increased by 2 m min⁻¹ every 15 min. Throughout the warm-up and test running, the treadmill was set without slopes.

5 days per week. The mice that were reluctant to run were humanely stimulated by a soft brush on the tail. All mice in the trained group ran equal distances throughout 4-week study. After 4 weeks, all mice of both groups were tested again for their maximal exercise capacity. All mice were killed by cervical dislocation under anaesthesia 2 days post-test, and their organs were subsequently harvested for analysis.

Blood analysis

Whole blood was collected 6 weeks after tamoxifen administration from the control and *Phd2* cKO mice via the buccal vein under anaesthesia. All blood components were analysed using Celltac Alpha (Nihon Kohden, Tokyo, Japan). After centrifugation, the supernatant was stored at -20 °C until further

examination. Plasma erythropoietin (EPO), vascular endothelial growth factor (VEGF) and nitric oxide (NO) metabolite levels were measured using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. NO is a gaseous free radical with a short half-life of a few seconds or less *in vivo*. Therefore, the levels of more stable NO metabolites, nitrite (NO₂⁻) and nitrate (NO₃⁻), were evaluated for an indirect measurement of NO in biological fluids (Wennmalm *et al.* 1992).

RNA extraction and quantitative real-time RT-PCR analysis

The total RNA from the tibialis anterior (TA), soleus muscle and kidney samples were isolated with the

RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) and used for cDNA synthesis with a QuantiTect reverse transcription kit (Qiagen). Quantitative real-time RT-PCR (qPCR) was then performed on 15 ng cDNA equivalents using Fast SYBR Green PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA) on a StepOnePlus PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The sequences of the primers are presented in Table 1. Gene expression levels were normalized to β -actin.

Western blot analysis

Total protein extracts from the heart, kidney and skeletal muscle (e.g. TA) were prepared as previously described (Shin *et al.* 2015), and protein concentrations were measured using BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Immediately after tissue sampling, nuclear extractions from the excised skeletal muscle (gastrocnemius muscle) and the excised kidney were performed as previously described (Moslehi *et al.* 2010). The protein concentrations of the nuclear extracts were measured using a Pierce 660 nm Protein Assay reagent (Thermo Fisher Scientific), and the samples were immediately used for Western blotting or stored at -20°C for further analyses. The aliquots for Western blotting analyses were separated by 5–20% gradient sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were transferred onto polyvinylidene fluoride membranes. All samples were transferred onto the same membrane for each protein. Specific bands were detected with the following primary antibodies: rabbit polyclonal anti-HIF-1 α (NB100-479, 1 : 1000; Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti-HIF-2 α (NB100-122, 1 : 1000; Novus Biologicals), rabbit polyclonal anti-PHD2 (#4835, 1 : 1000; Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118, 1 : 2000; Cell Signaling Technology) and mouse monoclonal anti- α -tubulin (#3873, 1 : 2000; Cell Signaling Technology). The bound antibody was detected with secondary antibodies (7074S, 1 : 5000; Cell Signaling Technology; sc-2005, 1 : 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by the ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

Histological analysis of the skeletal muscle

To determine the capillary number, gastrocnemius muscle samples were harvested from the mice of both genotypes. The muscle samples were mounted on a

cork disk. They were then frozen and stored. The middle portion of the frozen muscles was cut into 8- μm sections for histological analysis. The immunostaining was conducted using primary antibodies (CD31, anti-rat 1 : 30; BD Pharmingen, San Diego, CA, USA, and laminin 1 : 1000, anti-rabbit; Sigma) and secondary antibodies (Alexa Fluor 488 anti-rat, 1 : 800 and Alexa Fluor 555 anti-rabbit, 1 : 800; Life Technologies). The samples were fixed for 20 min in cold (4°C) 4% paraformaldehyde and washed in ice-cold PBS three times for 5 min. The samples were blocked for 1 h in PBS with 0.3% Triton X-100 and 10% goat serum prior to primary antibody incubation in a blocking buffer (1 : 300) overnight at 4°C . After washing three times for 5 min with a wash buffer, slides were incubated with the secondary antibody in the blocking buffer (1 : 400) for 1 h at room temperature. Following the secondary incubation, the slides were washed three times for 5 min and mounted in a Vectashield mounting medium (Vector Labs, Burlingame, CA, USA) with DAPI.

The stained muscle sections were visualized under a microscope (20 \times magnification) (C2+; Nikon, Tokyo, Japan) and were analysed using NIS elements (Nikon) and IMAGEJ software version 1.48u4 (National Institutes of Health, Bethesda, MD, USA). CD31-positive cells and the number of fibres were counted in randomly selected at least 10 separate fields per sample from the gastrocnemius muscle. Then, capillary-to-fibre ratio was calculated.

The section of the gastrocnemius muscle was also stained for succinate dehydrogenase (SDH), which is one of the representative oxidative enzymes (Ackrell 2002, Tanji & Bonilla 2008). Sections were first allowed to dry at room temperature and were then incubated in a solution containing nitro blue tetrazolium (0.5 mg mL^{-1}), sodium succinate (50 mM) and phosphate buffer (50 mM) for 50 min at 37°C . Cross-sections were then washed three times with distilled water and then cover-slipped using the glycerol-based mounting medium. For the quantification of SDH-positive fibres, 500- μm square compartments were serially allocated along the surface of SDH-stained section of gastrocnemius muscle to cover the whole outer segment of lateral gastrocnemius. To cover the outer segment of lateral gastrocnemius muscle, 8–13 compartments in average were allocated. All SDH-positive fibres within the allocated compartments were counted and divided by the compartment covered area to calculate the number of SDH-positive fibres per mm^2 in outer segment of lateral gastrocnemius, where the effect of training on the increase in SDH-positive fibres was apparently remarkable at this segment as compared to the other part of gastrocnemius muscle.

Statistical analysis

Data are presented as the mean \pm SE. Groups were compared by an unpaired Student's *t*-test or a two-way ANOVA using the IBM SPSS Statistics v.19.0 (IBM Corp, Armonk, NY, USA) software package.

The unpaired Student's *t*-test was performed to compare the band intensities of each protein. The body and tissue weights, the results of ELISA, the quantitative data of staining and gene expressions were analysed by the two-way ANOVA [training (untrained or trained) \times genotype (control or *Phd2* cKO)]. The running time was compared with the two-way repeated-measures ANOVA [time (pre or post) \times genotype (control or *Phd2* cKO)]. *P*-values below 0.05 were considered significant.

Results

Phd2 expression after tamoxifen administration

Western blotting was performed to determine the expression of PHD2 protein. The representative Western blot images for each tissue are shown in Figure 2a. PHD2 protein expression was markedly lower than control in all the tissues examined. The quantified results of the TA muscle are demonstrated in

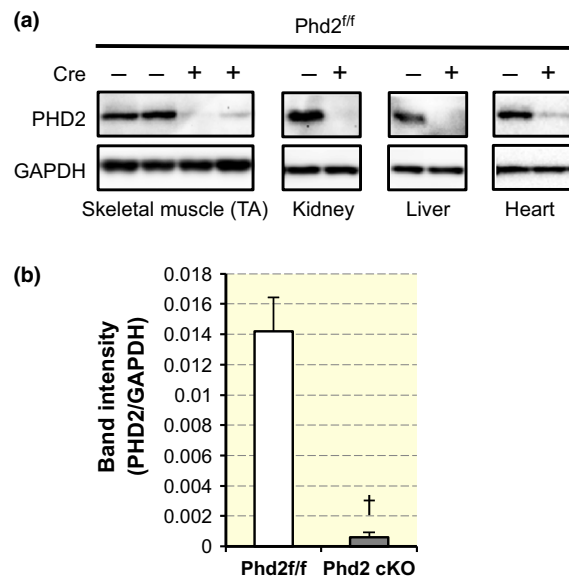


Figure 2 Phenotypes of *Phd2^{fl/fl}/ROSA26^{CreERT2}* mice. (a) Representative Western blots of PHD2 in the tibialis anterior (TA) muscle, kidney, liver and heart. (b) Quantification of band intensity of PHD2/GAPDH in TA ($n = 4$ per group). Values are shown as means \pm SEM. $^{\dagger}P < 0.01$ compared with control using Student's *t*-test.

Figure 2b with a statistical significance determined by Student's *t*-test ($P < 0.01$).

Body weight and skeletal muscle weight

Body weight and skeletal muscle weight/body weight are presented in Table 2. The body weights of the control and *Phd2* cKO mice were comparable ($F_{1,43} = 0.52$, $P = 0.475$ for interaction). Similarly, there was no statistical significance observed for both the interaction and the main effect of genotype and training in the soleus muscle/body weight ($F_{1,43} = 2.17$, $P = 0.148$ for interaction). On the other hand, the gastrocnemius weight/body weight of *Phd2* cKO mice was significantly lower than that of control mice ($F_{1,43} = 7.404$, $P < 0.01$), without significant interaction of training ($F_{1,43} = 0.279$, $P = 0.60$ for interaction). The heart weight/body weight of *Phd2* cKO mice was significantly higher than that of control mice ($F_{1,40} = 7.153$, $P < 0.05$), but there was no significant interaction of training ($F_{1,40} = 0.00$, $P = 0.99$ for interaction).

HIF-dependent adaptive responses

HIF-1 α protein, the key factor in the hypoxic response, was stabilized in the gastrocnemius muscle in the *Phd2* cKO mice compared with that in the gastrocnemius muscle in the control mice (Fig. 3a,b). Similarly, an accumulation of HIF-1 α protein in the kidney was observed as previously reported (Takeda *et al.* 2008; Fig. 3a). Additionally, HIF-2 α protein accumulated in the kidney of the *Phd2* cKO mice (Fig. 3c,d). On the other hand, the expression levels of *Hif-1 α* mRNA in the TA muscle (Fig. 3e) and *Hif-2 α* mRNA in the kidney (Fig. 3f) were not different between the control and *Phd2* cKO mice.

Next, we performed an enzyme-linked immunosorbent assay to determine the plasma levels of HIF-1- and HIF-2-dependent EPO (Fig. 4a) and VEGF (Fig. 4b). *Phd2* deficiency mice showed a markedly high plasma level of EPO ($F_{1,14} = 1567.156$, $P < 0.01$) and VEGF ($F_{1,14} = 2121.371$, $P < 0.01$).

Changes in blood components

To confirm whether the *Phd2* cKO mice exhibited changes in blood components, their haematological parameters were compared (Table 3).

Phd2 cKO mice showed markedly higher red blood cell volume, haematocrit and haemoglobin compared with control mice (RBC, $F_{1,20} = 218.499$, $P < 0.01$; haematocrit, $F_{1,12} = 939.13$, $P < 0.01$; haemoglobin, $F_{1,12} = 518.695$, $P < 0.01$). However, training did not affect the blood profile (RBC, $F_{1,20} = 0.728$,

Table 2 Body and organ weight

	<i>Phd2^{ff}</i>		<i>Phd2</i> cKO	
	Untrained <i>n</i> = 11	Trained <i>n</i> = 13	Untrained <i>n</i> = 11	Trained <i>n</i> = 12
Body weight (g)	27.0 ± 0.63	25.1 ± 0.86	25.4 ± 0.9	24.7 ± 1.0
GC/BW (mg g ⁻¹) [†]	5.85 ± 0.06	5.95 ± 0.32	5.41 ± 0.08	5.30 ± 0.13
SOL/BW (mg g ⁻¹)	0.30 ± 0.01	0.32 ± 0.02	0.31 ± 0.01	0.28 ± 0.01
	<i>n</i> = 8	<i>n</i> = 13	<i>n</i> = 11	<i>n</i> = 12
HW/BW (mg g ⁻¹) [*]	5.38 ± 0.19	5.78 ± 0.28	6.31 ± 0.28	6.72 ± 0.47

BW, body weight; GC, gastrocnemius; SOL, soleus; HW, heart weight.

Values are shown as means ± SEM.

[†]The main effect of genotype on GC/BW ratio was significant ($F_{1,43} = 7.404$, $P < 0.01$), but there was no significant interaction of training ($F_{1,43} = 0.279$, $P = 0.60$ for interaction).

^{*}The main effect of genotype on HW/BW ratio was significant ($F_{1,40} = 7.153$, $P < 0.05$), but there was no significant interaction of training ($F_{1,40} = 0.00$, $P = 0.99$ for interaction).

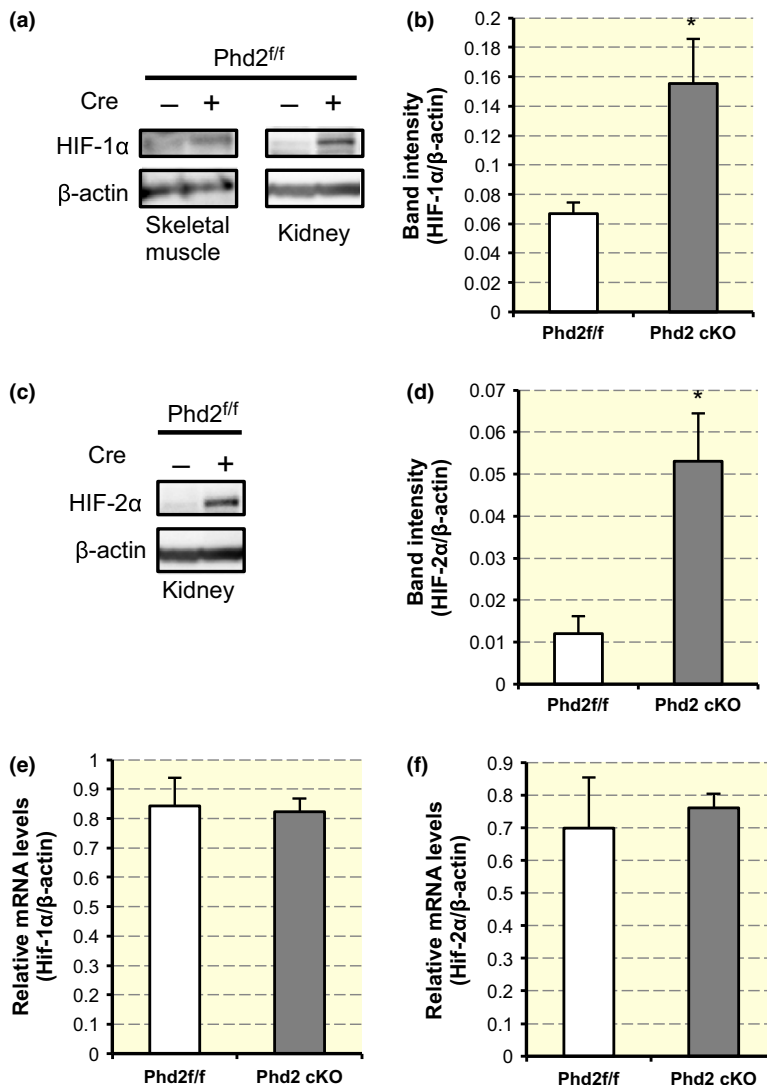
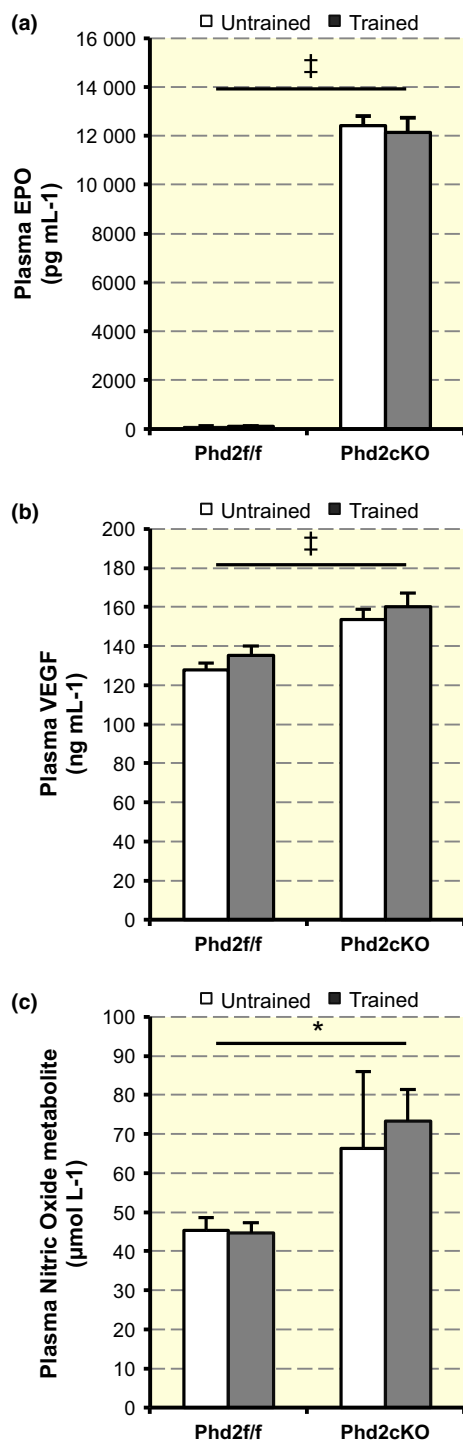


Figure 3 Stabilization of HIF- α proteins in *Phd2* cKO mice. (a) Anti-HIF-1 α and anti- β -actin (loading control) Western blots of nuclear lysates from the kidney and gastrocnemius (GC). (b) Quantification of band intensity of HIF-1 α / β -actin in GC ($n = 4$ per group). *Statistically significant differences compared with the control values using Student's *t*-test ($P < 0.05$). (c) Anti-HIF-2 α and anti- β -actin Western blots of nuclear lysates from the kidney. (d) Quantification of band intensity of HIF-2 α / β -actin in kidney ($n = 3$ per group). *Statistically significant difference compared with the control values using Student's *t*-test ($P < 0.05$). (e) The expression of *Hif-1 α* mRNA in the tibialis anterior (TA) muscle ($n = 3$ per group) ($P = \text{n.s.}$, Student's *t*-test). (f) The expression of *Hif-2 α* mRNA in the kidney ($n = 3$ per group) ($P = \text{n.s.}$, Student's *t*-test).



$P = 0.404$ for interaction; haemoglobin, $F_{1,12} = 0.141$, $P = 0.714$ for interaction; haematocrit, $F_{1,12} = 0.313$, $P = 0.586$ for interaction).

Circulating NO metabolite level

A previous report (Ruschitzka *et al.* 2000) showed that endothelial NO maintains normotension and

Figure 4 Upregulation of plasma EPO, VEGF and nitric oxide (NO) metabolite level. (a) Plasma levels of EPO ($n = 4-5$ per group). A two-way ANOVA [training (untrained or trained) \times genotype (control or *Phd2* cKO)] showed a statistically significant main effect of genotype in the EPO levels ($F_{1,14} = 1567.156$, $^{\dagger}P < 0.001$). There was no significant interaction between training and genotype ($F_{1,14} = 0.23$, $P = 0.639$). Values are shown as means \pm SEM. (b) Plasma levels of VEGF-A ($n = 4-5$ per group). There was a statistically significant main effect of genotype in the VEGF levels ($F_{1,14} = 21.371$, $^{\dagger}P < 0.001$), but there was no significant interaction between training and genotype ($F_{1,13} = 0.08$, $P = 0.929$). Values are shown as means \pm SEM. (c) Total NO metabolite level in circulation ($n = 4-5$ per group). There was a statistically significant main effect of genotype in the plasma NO metabolite level ($F_{1,13} = 5.682$, $^*P < 0.05$), but there was no significant interaction between training and genotype ($F_{1,13} = 0.14$, $P = 0.714$). Values are shown as means \pm SEM.

critically determines the *in vivo* survival under conditions of increased haematocrit values under EPO over-expression. We, therefore, speculated that the NO level would be increased in *Phd2* cKO mice. The plasma NO metabolite level in the *Phd2* cKO mice was significantly higher than that in the control mice ($F_{1,13} = 5.682$, $P < 0.05$) (Fig. 4c), but there was no significant interaction between training and genotype ($F_{1,13} = 0.14$, $P = 0.714$ for interaction). There was no significant effect of training on plasma NO metabolite level ($F_{1,13} = 0.096$, $P = 0.762$).

Capillary number in the skeletal muscle

To determine the capillary number, we co-stained the gastrocnemius muscle sections with CD31, an endothelial cell-specific marker, DAPI and laminin (Fig. 5). The number of CD31-positive cells per field was counted (Fig. 6a) and a two-way ANOVA [training (untrained or trained) \times genotype (control or *Phd2* cKO)] was performed. The capillary number was significantly increased in *Phd2* cKO mice as compared to control mice ($F_{1,8} = 16.053$, $P < 0.05$) (Fig. 6a) irrespective of training status ($F_{1,8} = 0.158$, $P = 0.701$ for interaction). Similarly, the fibre number per field was significantly increased in *Phd2* cKO mice as compared to control mice ($F_{1,8} = 27.268$, $P < 0.01$), irrespective of training status ($F_{1,8} = 0.07$, $P = 0.934$ for interaction) (Fig. 6b). Next, we calculated the capillary-to-fibre (C/F) ratio. The C/F ratio was significantly increased by training ($F_{1,8} = 8.407$, $P < 0.05$), but genotype did not affect the C/F ratio ($F_{1,8} = 0.098$, $P = 0.762$ for interaction) (Fig. 6c).

Table 3 Haematological variables

	<i>Phd2^{fl/fl}</i>		<i>Phd2</i> cKO	
	Untrained	Trained	Untrained	Trained
RBC (10^6 per mm^3) [†]	917.8 ± 28.4	875.2 ± 39.3	1561.3 ± 72.9	1597.5 ± 29.6
Hct (%) [†]	42.4 ± 1.5	44.0 ± 0.6	74.5 ± 0.6	74.9 ± 1.2
Hb (g dL^{-1}) [†]	13.7 ± 0.4	14.1 ± 0.3	21.4 ± 0.1	21.6 ± 0.4

RBC, red blood cell; Hct, haematocrit; Hb, haemoglobin.

Values are shown as means ± SEM.

[†]The main effects of genotype were significant (RBC, $F_{1,20} = 218.499$, $P < 0.01$; haemoglobin, $F_{1,12} = 518.695$, $P < 0.01$; haematocrit, $F_{1,12} = 939.13$, $P < 0.01$).

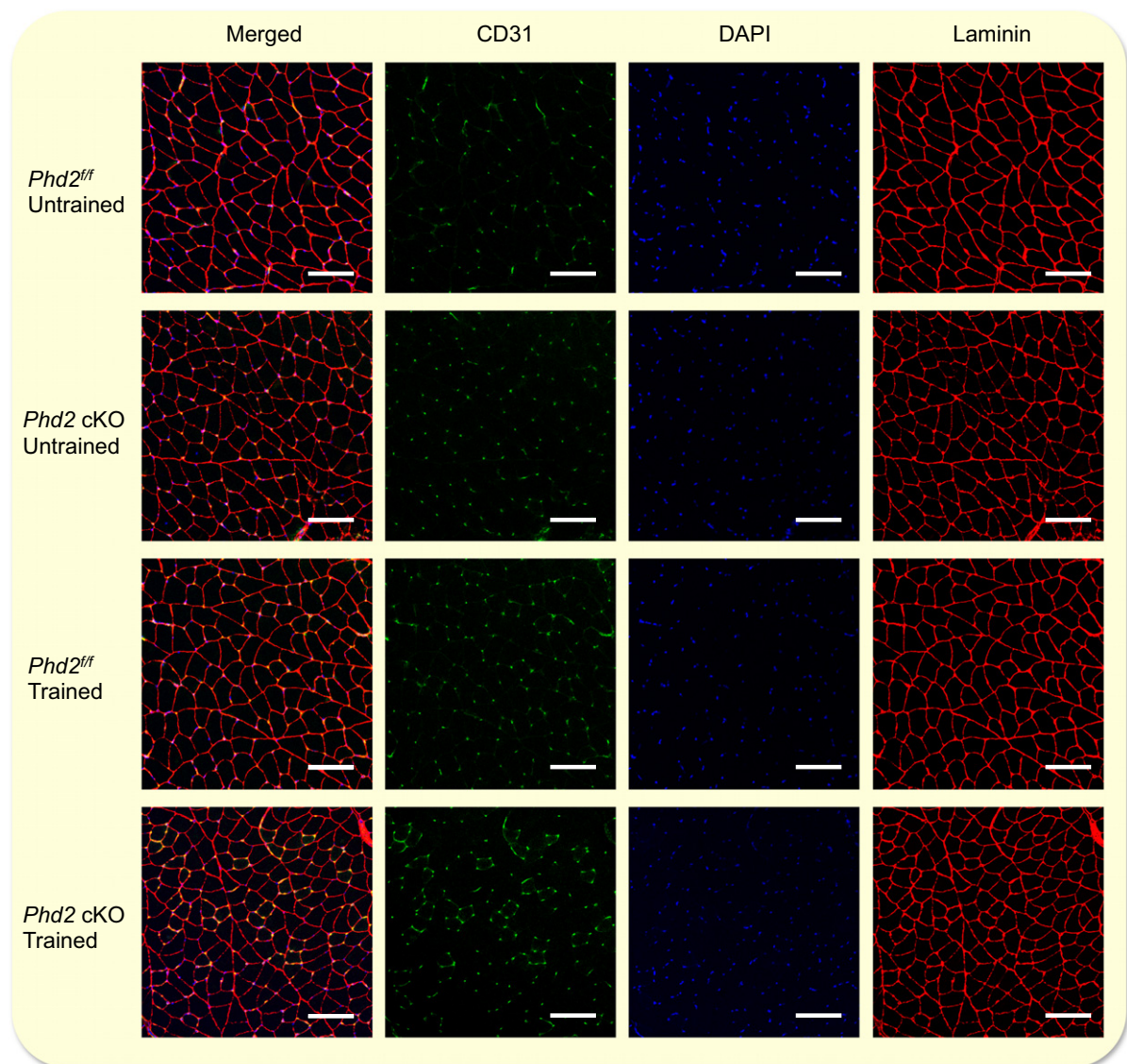


Figure 5 Comparison of CD31 expression and number of fibres in the skeletal muscle between control and *Phd2* cKO mice. Co-staining of the gastrocnemius muscle with CD31, DAPI and laminin. Scale bar: 100 μm .

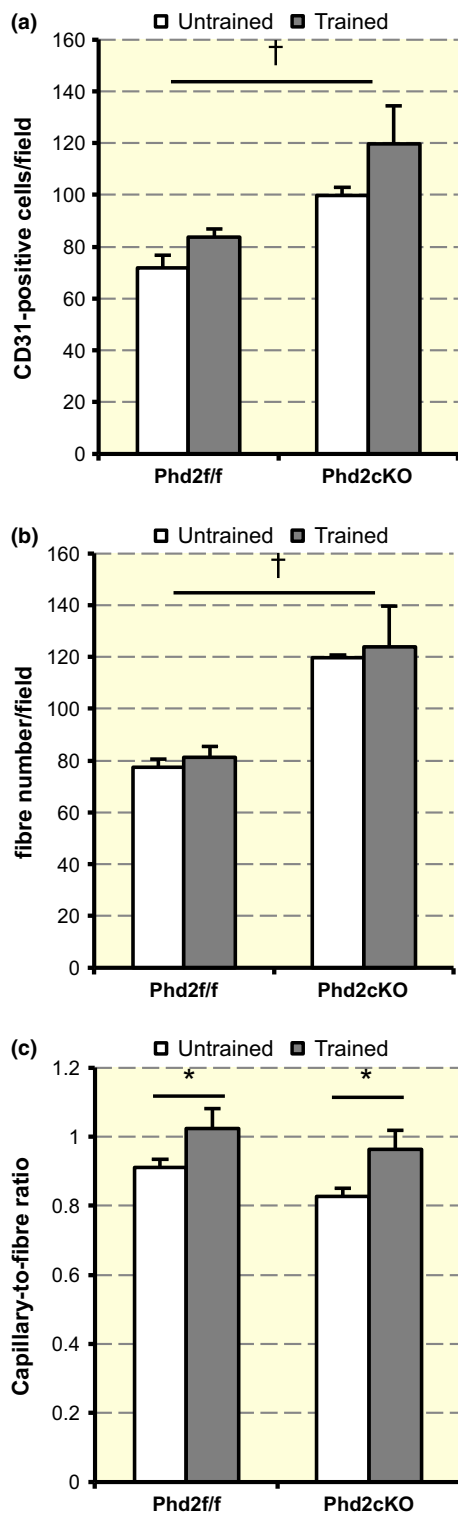


Figure 6 Quantification of Figure 5. (a) Quantitative results of CD31-positive cells per field. A two-way ANOVA [training (untrained or trained) \times genotype (control or *Phd2* cKO)] showed a statistically significant main effect of genotype on the capillary number ($F_{1,8} = 16.053$, $\dagger P < 0.01$), but there was no significant interaction between training and genotype ($F_{1,8} = 0.158$, $P = 0.701$). Values are shown as means \pm SEM. (b) Quantitative results of number of fibres per field. There was a significant main effect of genotype ($F_{1,8} = 27.268$, $\dagger P < 0.01$), but there was no significant interaction between training and genotype ($F_{1,8} = 0.07$, $P = 0.934$). Values are shown as means \pm SEM. (c) Quantitative results of the C/F ratio. There was a statistically significant main effect of training on the C/F ratio ($F_{1,8} = 8.407$, $*P < 0.05$), but there was no significant interaction between training and genotype ($F_{1,8} = 0.098$, $P = 0.762$). Values are shown as means \pm SEM.

SDH-positive fibres were particularly spread into the outer segment of the lateral gastrocnemius muscle of the trained *Phd2* cKO mice ($F_{1,8} = 12.553$, $P < 0.01$ for interaction) (Fig. 7a,b).

Gene expression in skeletal muscle

As the skeletal muscle is a major determinant of aerobic exercise capacity (Wisloff *et al.* 2005, Park *et al.* 2009), we examined the expression of some metabolic and mitochondrial genes in the soleus muscle. The expression of *Glut1*, which is one of the target genes of HIF, was upregulated in untrained *Phd2* cKO mice, but not in trained *Phd2* cKO mice ($F_{1,11} = 7.224$, $P < 0.05$ for interaction) (Fig. 8a). Although there was no significant interaction between training and genotype ($F_{1,10} = 0.801$, $P = 0.39$ for interaction), the expression of *MT-CO2* expression was lower in untrained *Phd2* cKO mice as compared to untrained control mice ($F_{1,10} = 5.286$, $P < 0.05$). The level of *MT-CO2* expression was upregulated to a similar level after training in both genotypes ($F_{1,10} = 6.625$, $P < 0.05$). There was no interaction and main effect found for *Cox5a* ($F_{1,8} = 2.534$, $P = 0.15$ for interaction) (Fig. 8c).

Maximal exercise capacity

To examine whether *Phd2* deficiency improved the effect of treadmill training, maximal exercise tests were performed before and after the 4-week period with or without exercise training (for both the trained and untrained groups) (Fig. 9a,b). Although untrained mice showed a slight increase in running time ($F_{1,23} = 34.0$, $P < 0.01$), the levels of improvement in the running time of control and *Phd2* cKO mice were similar ($F_{1,23} = 3.157$, $P = 0.09$ for interaction)

SDH staining in skeletal muscle

Staining for SDH activity revealed an apparent increase in SDH-positive fibres in the gastrocnemius muscles of the trained groups of both genotypes.

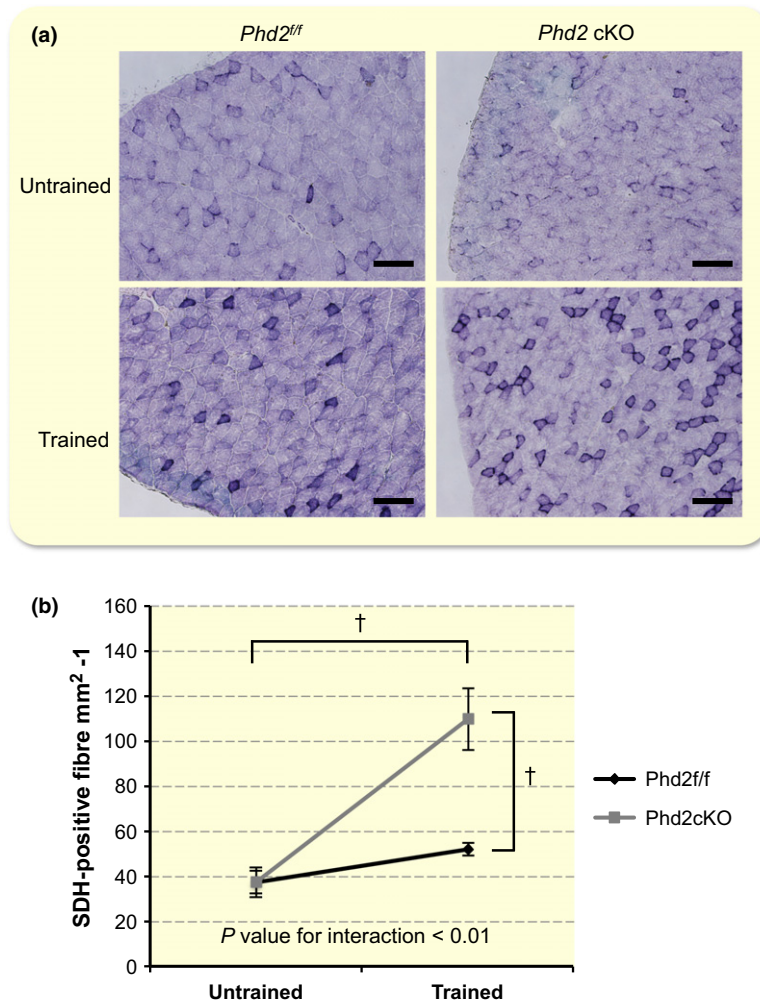


Figure 7 Succinyl dehydrogenase (SDH) staining of the gastrocnemius muscle. (a) Representative SDH staining of the lateral gastrocnemius muscle. Scale bar: 100 μm . (b) Quantitative results of SDH staining. A two-way ANOVA [training (untrained or trained) \times genotype (control or *Phd2* cKO)] showed a significant interaction between training and genotype ($F_{1,8} = 12.553$, $P < 0.01$ for interaction). The main effect of training ($F_{1,8} = 28.590$, $\dagger P < 0.01$) and genotype ($F_{1,8} = 12.661$, $\dagger P < 0.01$) was significant. Values are shown as means \pm SEM.

(Fig. 9a). In contrast, a significant interaction between time and genotype was found in the trained group ($F_{1,23} = 9.353$, $P < 0.01$ for the interaction) (Fig. 9b). The main effect of time ($F_{1,23} = 118.746$, $P < 0.01$) and the main effect of genotype were both significant ($F_{1,23} = 4.802$, $P < 0.05$).

Discussion

The results of this study suggest that the activation of the HIF pathway induced by PHD2 deficiency led to an improved effect of the 4-week regimen of treadmill training. The upregulation of EPO induced by stabilized HIF was accompanied by a notable increase in the haemoglobin concentration and haematocrit value, as previously reported (Minamishima *et al.* 2008). An increase in capillary and fibre number in the skeletal muscle was also observed as a physiological reaction induced in the *Phd2* cKO mice in response to HIF stabilization. The 4-week running training elicited significant increases in the C/F ratio and oxidative capacity of the skeletal muscle in the trained groups in both

genotypes. Most importantly, the increase in the running time after training was longer in the *Phd2* cKO mice by a factor of approx. 1.57, compared with that in the control mice. These data suggest that PHD2 deficiency improved the effect of running training through the activation of HIF pathway and training-induced improvement in oxidative capacity without being exposed to low atmospheric oxygen concentration.

The tamoxifen-treated *Phd2^{fl/fl}/Rosa26^{CreERT2}* mice exhibited PHD2 deficiency and the accumulation of HIF- α protein subunits. However, there was no significant difference in the mRNA levels of *Hif-1 α* in the TA muscle and *Hif-2 α* in the kidney between the control and *Phd2* cKO mice, as previously reported (Takeda *et al.* 2006, 2007). We previously found that there was no significant difference in the mRNA levels of *Hif-1 α* in the gastrocnemius muscle between control and *Phd2* cKO mice (Shin *et al.* 2015). These results suggest that the accumulation of HIF-1 α and HIF-2 α proteins depends on the decrease in PHD2 expression rather than on the expression levels of *Hif-1 α* and *Hif-2 α* mRNA.

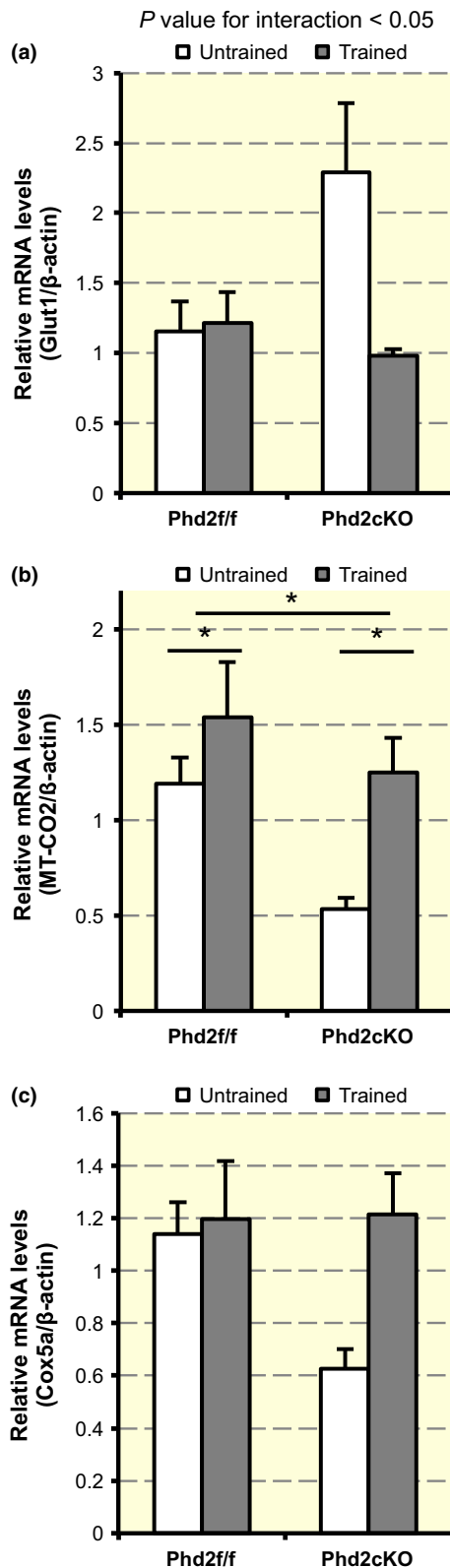


Figure 8 Gene expressions in the skeletal muscle. (a) The expression of *Glut1* mRNA ($n = 3\text{--}4$ per group) in the soleus muscle. A two-way ANOVA exhibited a significant interaction between genotype and time ($F_{1,11} = 7.224$, $P < 0.05$). Values are shown as means \pm SEM. (b) The expression of MT-CO2 mRNA ($n = 3$ per group) in the soleus muscle. There was a significant main effect of genotype ($F_{1,10} = 5.286$, $*P < 0.05$) and training ($F_{1,10} = 6.625$, $*P < 0.05$). Values are shown as means \pm SEM. (c) The expression of *Cox5a* mRNA ($n = 3$ per group) in the soleus muscle. There was no interaction and main effect ($F_{1,8} = 2.534$, $P = 0.15$ for interaction). Values are shown as means \pm SEM.

expectations, none of the mice experienced ill health, despite the fact that some *Phd2* cKO mice had haematocrit values over 75%. Moreover, the *Phd2* cKO mice that had experienced this high haematocrit value were able to perform the running exercise without a break. NO-mediated vasodilatation may explain this phenomenon. A previous report showed that erythrocytotic mice neither developed hypertension nor displayed a change in heart rate or cardiac output, despite having haematocrit values of 80–90% in mice overexpressing EPO (Ruschitzka *et al.* 2000). They suggested that enhanced eNOS activity must have been due to increased blood viscosity that potentiates shear forces. NO-mediated vasodilatation is one of the adaptive mechanisms to combat excessive erythrocytosis in mice that overexpress EPO, which protects the animals from cardiovascular complications including thromboembolism and hypertension (Ruschitzka *et al.* 2000). Additionally, iNOS is a hypoxia-inducible gene because hypoxia-responsive element is present in the iNOS promoter (Melillo *et al.* 1995). Therefore, increased NO production in *Phd2* cKO could also be mediated by inflammatory cells expressing iNOS like macrophages, which needs further investigation.

Previously, Minamishima *et al.* (2008) proposed that *Phd2^{ff}; beta-actin-Cre-ER* mice, is a model of cardiomyopathy because they exhibited marked hypertrophy of cardiomyocytes and a wider space between myocardial fibres. Similarly, the heart weight of the *Phd2* cKO mice was greater than that of the control mice, but they were able to run with no difficulty throughout the 4-week training period. These data suggest that the increase in heart weight induced by *Phd2* deficiency had little effect on either the running time or the improvement in running time.

Phd2 deficiency increased not only the capillary number but also the number of myofibres per field in the skeletal muscle. Therefore, the *Phd2* deficiency *per se* may not be sufficient to increase the C/F ratio. The increased C/F ratio, supposed to improve O₂ flux to the muscle fibres, was elicited by training, which could

We predicted that excessive erythrocytosis might give some deleterious effect such as thromboembolism on the *Phd2* cKO mice. However, contrary to our

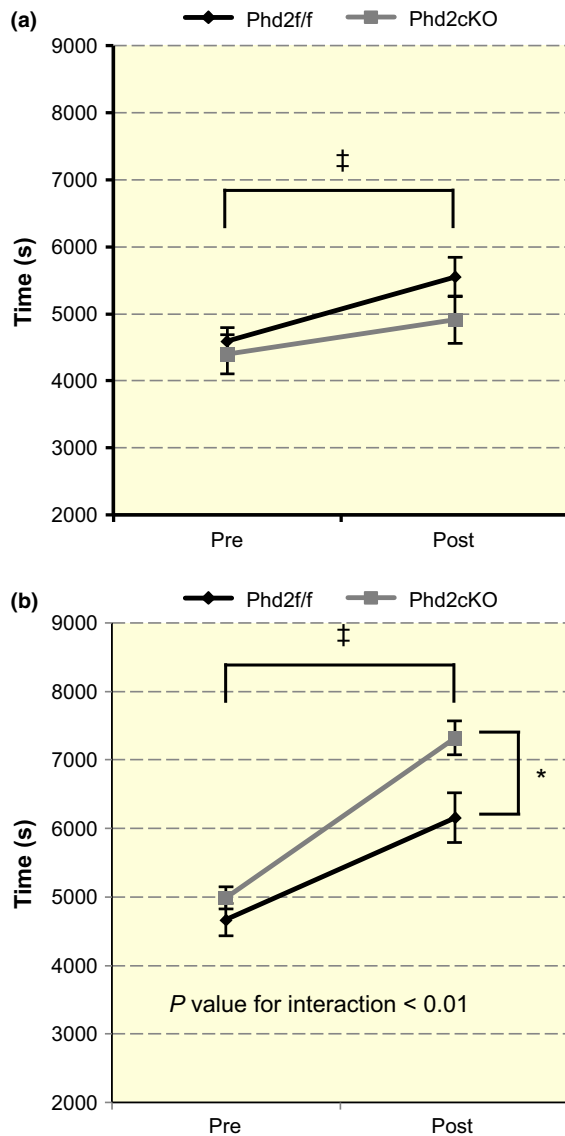


Figure 9 Running times of the control and *Phd2* cKO mice. (a) The running time before and after the 4-week period is shown for the untrained groups ($n = 12\text{--}14$ per group). A two-way ANOVA [time (pre or post) \times genotype (control or *Phd2* cKO)] showed a significant main effect of time ($F_{1,23} = 34.0$, $^{\dagger}P < 0.001$). There was no significant interaction between time and genotype in the untrained group ($F_{1,23} = 3.157$, $P = 0.09$). Values are shown as means \pm SEM. (b) The running time before and after training in the trained mice. A significant interaction between time and genotype was found ($F_{1,23} = 9.353$, $P < 0.01$). The main effect of time ($F_{1,23} = 118.746$, $^{\dagger}P < 0.001$) and genotype was significant ($F_{1,23} = 4.802$, $^*P < 0.05$). Values are shown as means \pm SEM.

contribute to the improvement in the running time for both genotypes. Therefore, angiogenesis induced by *Phd2* deficiency may be further complemented by an additional training-induced increase in the capillary number.

Although type I myofibres were increased in the gastrocnemius muscles in *Phd2* cKO mice without training as we previously reported (Shin *et al.* 2015), there was no marked increase in the SDH-positive fibres in *Phd2* cKO mice before or without training. An increase in the SDH activity was only observed following the exercise training. It is possible that the fibre type transition, as determined by myosin heavy chain I expression, does not necessarily accompany SDH or oxidative enzymatic activity. The increased slow fibres in *Phd2* cKO mice may require endurance training to gain an improved oxidative capacity.

Furthermore, previous reports have suggested that HIF-1 signalling limits oxidative metabolism in the skeletal muscle and that exercise training may prevent this process (Lundby *et al.* 2006, Mason *et al.* 2007). In accordance with these reports, we observed a downregulation in oxidative metabolism, as demonstrated by higher *Glut1* gene expression, and reduced *MT-CO2* gene expression in the untrained *Phd2* cKO mice. This suppression of oxidative metabolism may reduce the capacity to use oxygen, even when its availability is enhanced by increased haemoglobin concentration and haematocrit values. Conversely, the increase in *Glut1* gene expression was suppressed and the reduction in *MT-CO2* gene expression was prevented in the trained *Phd2* cKO mice. These data suggest that the 4-week training regimen counteracted the suppression of oxidative metabolism in the skeletal muscle by the HIF signalling pathway.

Thus, the 4-week running training regimen may have contributed to an improved oxygen utilization of the skeletal muscle in *Phd2* cKO mice. From the reasons above, we suggest that a combination of adequate HIF activation (for oxygen availability) and valid running training (for oxygen utilization) is necessary to elicit a robust endurance training effect.

There was a mild improvement in the running time without training in both groups. Because the improvement was independent of genotypes, it is possible that this improvement in both genotypes was due to the growth of mice.

Interestingly, it has been reported that Chuvash polycythaemia (CP) patients can have haematocrit values over 60%, which is comparable to those of *Phd2* cKO mice in this study. The patients' exercise capacity, however, is reported to be lower compared with healthy controls, similar to *Phd2* cKO mice without training (Formenti *et al.* 2010). They suggested that CP patients are limited in their exercise capacity because their muscles had limited oxygen utilization, even though they may have superior oxygen availability. Although there is no observation as to whether CP patients exhibit a higher adaptability to endurance training, an appropriate protocol of exercise may

enhance their exercise capacity like in *Phd2*-deficient mice, which needs to be cautiously investigated. The risk of high blood pressure or thromboembolism may be determined by the NO level, as well as the risk of altitude training or hypoxic training for athletes, which again needs to be carefully determined in further investigations.

In summary, we demonstrated the influence of the HIF pathway activated by PHD2 deficiency on running training without exposure to atmospheric hypoxia. As determined by the combination of marked phenotypes and exercise training, the *Phd2*-deficient mice exhibited a significantly higher gain of running time. We conclude that the activation of the HIF pathway enhances the effects of endurance running training without exposure to atmospheric hypoxia.

Conflict of interest

There are no conflict of interests to declare.

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