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RESEARCH ARTICLE

Enhanced erythropoiesis in mice exposed to low environmental temperature

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SUMMARY

Hematopoietic responses to environmental factors are not fully characterized. Polycythemia has been reported during exposure to low temperatures in ectothermic animals. The relationship between the causes of polycythemia and erythropoiesis during low temperature exposure is not fully understood. In this study, we exposed C57BL/6 mice to 5°C and monitored the blood cell counts and erythropoiesis. The hematocrit level increased from 45.6 to 52.2% after 14 days. Likewise, the hemoglobin concentration, initially 15.1 g dl^{-1}, rose to 16.0 g dl^{-1}. The reticulocyte production index significantly increased from 4 to 8% after 7 days. We examined the anatomy and cell composition of the spleens of the mice. On day 5, the spleens were ~6 mg g^{-1} of body mass, which was twofold greater than the spleens on day 0. Flow cytometry showed fourfold more proerythroblasts on day 5, compared with day 0. Additionally, the number of late-stage mature erythroblasts increased on day 14. Erythropoietin mRNA levels increased in the kidneys, and hypoxia-inducible genes were enhanced in the kidney. Our findings indicated that low ambient temperature is a novel erythropoietic stress, which induces polycythemia by enhanced erythropoiesis.

Key words: low temperature, erythropoiesis, polycythemia, mouse.

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INTRODUCTION

The ability to adapt to environmental stress is essential for survival. Temperature is a known environmental stress, and many studies have shown a wide range of physiological responses to changes in temperature. Ectothermic animals (reptiles, amphibians and fish) do not have the ability to thermoregulate, and decreases in body temperature occur passively when the animals are in a low temperature environment (Salt, 1949). Endothermic animals (birds and mammals) have mechanisms to keep the body temperature constant, independent of the environmental temperature over a certain range. Upon the initial exposure to low temperature, endothermic animals exhibit peripheral vasoconstriction aimed at reducing heat loss. Active thermogenesis occurs by means of periodic shivering if heat dissipation exceeds metabolic heat generation (Gordon, 1990). During prolonged exposure to low temperature, non-shivering thermogenesis is enhanced. Additional metabolic heat generation is accompanied by increased oxygen consumption (Gordon, 1990).

There have been several reports of anemia induced by low temperature exposure in ectothermic animals, including leotard frogs (*Rana pipiens*), European frogs (*Rana esculenta*) and zebrafish (*Danio rerio*) (Cline and Waldmann, 1962; Sinha, 1983; Kulkeaw et al., 2010). We also determined the anemia induced by low temperature exposure in the African clawed frog, *Xenopus laevis* (Maekawa et al., 2012). We observed enhanced hepatic destruction of erythrocytes immediately after exposure to low temperature. Despite enhanced erythropoiesis in response to low temperature, newly produced erythrocytes were confined to the liver (i.e. erythropoietic organ), so that erythrocyte counts remained low during the cold period. Hematopoietic responses to environmental temperature have also been reported in endothermic animals. Tavassoli et al. showed that hematopoiesis capability was retained in the tail vertebrae of newborn rats by transposing the tail into the warmer environment of the abdomen (Tavassoli et al., 1979). In rats and chickens acclimated to low temperature, an increase in the number of erythrocytes was observed (Deveci et al., 2001; Bláhová et al., 2007). The mechanisms remain unknown, however. Erythrocytes are responsible for transporting oxygen to tissue and are essential for the survival of almost all vertebrate animals. Therefore, the mechanisms of erythropoiesis responding to low environmental temperature are one of the vital keys to understanding animal diversity.

Peripheral erythrocyte counts are tightly regulated to respond to tissue oxygen tension. The glycohormone erythropoietin (EPO) is a principal regulator of erythropoiesis (Krantz, 2000). In mammals, EPO is produced in the fetal liver and the adult kidney, and primarily stimulates the proliferation of erythrocyte progenitor cells in the bone marrow (Zanjani et al., 1977; Dame et al., 1998). *Epo* gene transcription is markedly induced in response to hypoxia. Hypoxia-inducible factors (HIFs) are required for *Epo* transcriptional activation in response to hypoxia (Wang and Semenza, 1995; Scortegagna et al., 2005). Erythrocytes differentiate from multi-potential hematopoietic stem cells through a series of intermediates. The first committed erythrocyte precursors are the burst-forming unit erythrocytes (BFU-E), which differentiate into colony-forming unit erythrocytes (CFU-E) and subsequently into proerythroblasts. These late erythrocyte progenitor cells, which are responsive to EPO, proliferate and differentiate further through the basophilic, polychromatic and orthochromatic erythroblast stages. The nucleus shrinks and is finally shed as the cells become reticulocytes before

becoming mature erythrocytes (Gregory and Eaves, 1977; Gregory and Eaves, 1978; Sawada et al., 1987; Broudy et al., 1991). During erythroblast differentiation, the cells lose the expression of transferrin receptor (CD71) and gain the expression of erythroid specific Ter119 antigen, associated with murine glycophorin A (Kina et al., 2000; Lok and Ponka, 2000). Therefore, these cell surface makers can be used to flow cytometric identification of different erythroblast stages in murine hematopoietic tissue (Socolovsky et al., 2001). In mammals, erythrocytes are produced predominantly in the bone marrow. In addition, splenic erythropoiesis has been well described in rodents. During erythropoietic stress, the spleen is the primary organ of erythropoiesis (Hara and Ogawa, 1976; Kam et al., 1999).

The objective of our study was to investigate whether polycythemia induced by low temperature exposure caused enhanced erythropoiesis in ectothermic animal. In the current study, we induced polycythemia by low temperature exposure in mice, and investigated the erythropoiesis under thermogenic conditions.

MATERIALS AND METHODS

Animals

C57BL/6J male mice (6–7 weeks old) were purchased from CLEA Japan (Tokyo, Japan). It was previously described that the body temperature of C57BL/6J mice was kept constant at low temperature exposure (Guerra et al., 1998). Three mice were housed in each plastic cage and maintained in a light- (12 h:12 h light:dark) and temperature-controlled (22°C) conventional culture room. They had free access to laboratory pelleted mouse food (Oriental Yeast, Tokyo, Japan) and water. For low temperature studies, plastic cages containing three mice were maintained in an incubator (Bio Multi Incubator, NK System, Osaka, Japan) at 5°C. All experiments were conducted according to the Regulations for Animal Experimentation at Waseda University.

Blood cell analysis

Peripheral blood (20–30 μl) was obtained from the orbit. Blood samples were anti-coagulated with EDTA-2Na (Dojindo Laboratories, Kumamoto, Japan) for cell counts and cytological analysis. Hematological parameters were assessed using an automated hematological analyzer Sysmex F-820 (Sysmex, Kobe, Japan). Reticulocytes were detected by staining with thiazole orange (Sigma-Aldrich, St Louis, MO, USA). A stock solution (1 mg ml^{-1}) of the dye in methanol was prepared (stored at –20°C). A 1:10,000 dilution of the stock solution in Dulbecco's modified phosphate-buffered saline (DPBS) containing 2 mmol l^{-1} EDTA-2Na was prepared. One microliter of whole blood was mixed with 0.2 ml of the diluted dye solution and incubated for 30 min at room temperature. The samples were analyzed using flow cytometry (Cytomics FC500 MPL flow cytometry system, Beckman Coulter, Fullerton, CA, USA) to determine the percentage of reticulocytes. The reticulocyte production index (RPI) was calculated as: [reticulocyte count (%) × mouse hematocrit (%)]/45 (%) (standard value of mouse hematocrit).

CFU-E colony forming assay

Erythrocytes of bone marrow and spleen were lysed with red blood cell lysing buffer (Sigma-Aldrich). Nucleated cells were cultured in a mixture containing 0.9% methylcellulose (Shin-Etsu Kagaku, Tokyo, Japan), 1% BSA (Wako, Osaka, Japan) after deionized treatment using Bio-Rex MSZ 501(D) Resin (Bio-Rad, Hercules, CA, USA), α-medium (Gibco, Invitrogen, Tokyo, Japan), 20% fetal bovine serum (Sigma-Aldrich), 1×10^{-4} mol l^{-1} mercaptoethanol, 1 U ml^{-1} human recombinant erythropoietin (Epoetin alfa; Kyowa Hakko Kirin, Tokyo, Japan). Plates were incubated at 37°C in an incubator containing 5% CO_2 in air. CFU-E colony formation was monitored at 60 h.

Histology and immunohistochemistry

Mice were quickly killed by cervical dislocation, and the spleens were collected. Spleen pieces were fixed for 24 h at 4°C in 10% formalin in DPBS, dehydrated using a graded series of ethanol, embedded in Paraplast (Kendall-Tyco Healthcare, Mansfield, MA, USA) and sectioned at a thickness of 5 μm at room temperature. The sections were stained with hematoxylin and eosin (HE). For immunohistochemistry, non-specific antibody binding was blocked by incubating the slides in 5% normal goat serum (NGS) in 15 mmol l^{-1} Tris-HCl, 0.15 mol l^{-1} NaCl, pH 7.5 (Tris-buffered saline, TBS), for 60 min at room temperature. The sections were subsequently incubated with biotin-conjugated anti-Ter119 solution (BD Pharmingen, CA, USA; diluted 1:500) in TBS containing 0.5% NGS overnight at 4°C. After antibody incubation, sections were washed three times with TBS for 5 min. The sections were later incubated for 60 min with horseradish peroxidase-conjugated streptavidin (Bio-Rad), and the color was developed with diaminobenzidine for 5–10 min. The preparations were examined by light microscopy (model BX51; Olympus, Tokyo, Japan).

Flow cytometric analysis

The analysis of the erythroblast population subsets was performed as previously described (Socolovsky et al., 2001). Freshly isolated spleen or bone marrow cells were strained through a 40 μm strainer in the presence of DPBS containing 2% fetal calf serum and 2 mmol l^{-1} EDTA-2Na. Cells were immunostained for 20 min at 4°C in the presence of 1 μg ml^{-1} biotin-conjugated anti-Ter119 (BD Pharmingen), and 1 μg ml^{-1} fluorescein isothiocyanate-conjugated rat anti-mouse CD71 (Biolegend, CA, USA) followed by avidin-allophycocyanin (BD Pharmingen). The cells were analyzed using a Cytomics FC500 MPL flow cytometry system (Beckman Coulter).

Real-time reverse transcription polymerase chain reaction

RNA was isolated from the kidneys using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan). Quantification PCR was carried out with the SYBR Green system using StepOnePlus (Applied Biosystems, Foster City, CA, USA). The target amounts were normalized to the *Hprt1* gene. The mean threshold cycle was used to determine the relative expression levels. The primers used for the real-time RT-PCR study were designed using Primer3 software (Rozen and Skaletsky, 2000). The sequences of the primers were as follows: *Hypoxanthine guanine phosphoribosyl transferase 1*-forward, 5′-TGCTGCTTTTACTCTCCTTGCTAC-3′; *Hypoxanthine guanine phosphoribosyl transferase 1*-reverse, 5′-TCTTCCACCTCCAT-TCTTTTCC-3′; *Erythropoietin*-forward, 5′-TGTTGTTGGATA-TGCCCTTG-3′; *Erythropoietin*-reverse, 5′-GCGCTCATCTTAGG-CTTTGT-3′; *Hypoxia-inducible factor-1α*-forward, 5′-CAGA-ATGGAACGGAGCAAAAG-3′; *Hypoxia-inducible factor-1*-reverse, 5′-GCTGTGGTAATCCACTCTCATCC-3′; *Glucose transporter type 1*-forward, 5′-ACATGGAACCACCGCTACG-3′; *Glucose transporter type 1*-reverse, 5′-AGTTCCGCCTGCCAAAG-3′; *Lactate dehydrogenase A*-forward, 5′-AATGAAGGACTT-GGCGGATG-3′; *Lactate dehydrogenase A*-reverse, 5′-GAT GTT CAC GTT TCG CTG GA-3′; *Phosphoglycerate kinase 1*-forward, 5′-GGAAGCGGGTCGTGATGA-3′; *Phosphoglycerate kinase 1*-reverse, 5′-GCC TTG ATC CTT TGG TTG TTT G-3′; *Vascular*

endothelial growth factor-forward, 5′-GTACCCCGACGAGA-TAGAGT-3′; and *Vascular endothelial growth factor*-reverse, 5′-ATGATCTGCATGGTGATGTTG-3′.

Statistics

Error bars represent the standard error of the mean (s.e.m.) in all figures. Comparisons between two groups were performed using Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Polycythemia is induced by low environmental temperature in mice

Mice were exposed to a 5°C environment for 56 days (Fig. 1). The blood hematocrit levels (Hct) gradually increased from 45.6 to 52.2% by day 14 and remained high at day 28 (Fig. 1A). Thereafter, the Hct increased to 59.0% from days 35 to 42. The blood hemoglobin levels (Hb) also increased from 15.1 to 16.0 g dl^{-1} by day 14 and to 17.5 g dl^{-1} by day 49 (Fig. 1B). Additionally, RPI increased from 3.4 to 9.0% at day 7 and gradually declined from day 14 to day 28 (Fig. 1C). Thereafter, a moderate increase of RPI was observed by day 42. Although it is possible that the increase in the RPI was caused by the continuous blood collection, we also observed an increase in the RPI in mice that were exposed to low temperature but did not undergo blood collection before the experimental day (Fig. 2D). The leukocyte counts decreased after day 7 (1.02×10^{10} to 0.85×10^{10} cells l^{-1}) and gradually increased and returned to normal thereafter (Fig. 1E). The platelet counts gradually decreased from 1.08×10^{12} to 0.95×10^{12} cells l^{-1} by day 14 and later returned to normal (Fig. 1F). The mean corpuscular erythrocyte volume (MCV) and the mean corpuscular hemoglobin content (MCH) were significantly increased on days 14 and 42 (Table 1). In contrast, the mean corpuscular hemoglobin concentration was significantly decreased on days 14 and 42 compared with day 0. These results indicate that the number of peripheral erythrocytes increased after day 14 in mice exposed to low temperature. Furthermore, the high values for the RPI and the MCV suggest that the rate of erythrocyte production was enhanced.

Splenomegaly with increased erythroid cells after exposure to low temperature

We examined whether erythropoiesis enhanced at an early stage of low temperature exposure. The enlargement of the spleen was observed after 5 days of exposure to low temperature (5.9 mg g^{-1} body mass on day 5 compared with 3.1 mg g^{-1} body mass on day 0), and the spleen subsequently returned to the normal size (Fig. 2A,F). Around the same time, body mass of mice decreased (Fig. 2E). The data indicated that the decrease in body mass was not a direct cause of the increase in spleen mass. Indeed, the spleens of mice exposed to low temperature exhibited massive splenomegaly (Fig. 2A). Immunohistochemical analysis using a glycophorin-A-associated antibody for Ter119, a marker of erythroid cells (Kina et al., 2000), indicated that the number of erythroid cells increased in the red pulp on day 5 (Fig. 2B). Flow cytometric analysis showed that the spleens of mice on day 5 contained 38% more Ter119-positive cells (66% of total) than day 0 spleens (28% of total) (Fig. 2C,G). Thereafter, the number of Ter119-positive cells in the spleen declined to normal. These results indicate that the splenomegaly induced by exposure to low temperature was caused by an increased number of erythroid cells.

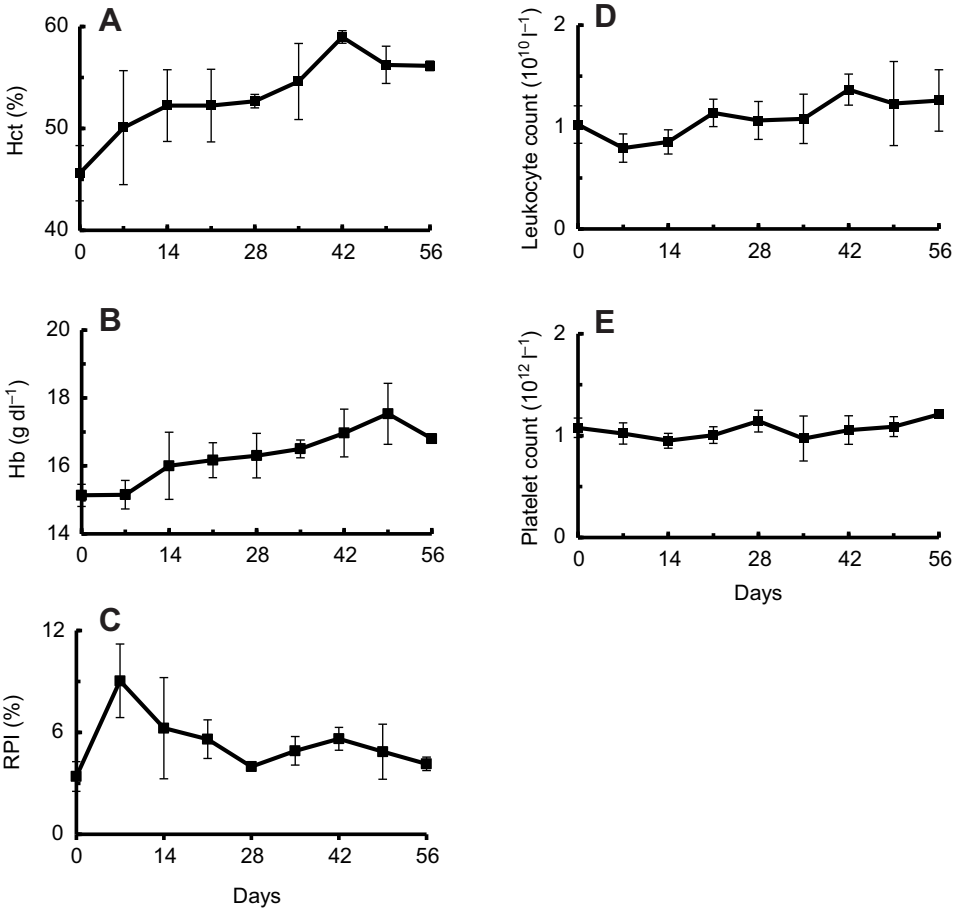

Fig. 1. Blood cell parameters during low temperature (5°C) exposure in C57BL/6J mice. Mice were exposed to 5°C for 56 days, and blood samples were collected once per week: (A) hematocrit (Hct); (B) hemoglobin (Hb); (C) reticulocyte production index (RPI); (D) leukocyte counts; (E) platelet counts. Values represent the means ± s.e.m. of six mice.

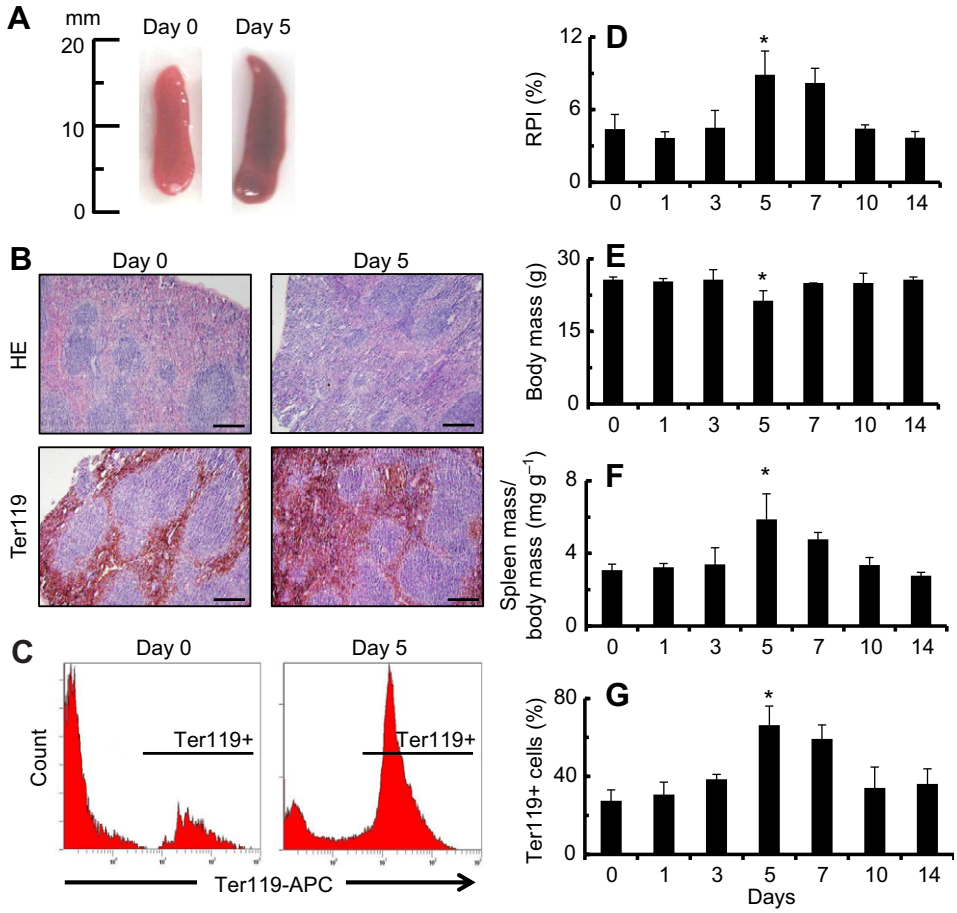

Fig. 2. Splenomegaly and increase in splenic erythroid cells in mice after exposure to low temperature. (A) Photos of spleens from mice on days 0 and 5 of exposure to low temperature. (B) HE-stained splenic sections (upper) and Ter119-stained (brown) splenic sections (bottom; blue is counterstaining with hematoxylin). Original magnification is ×40 for all panels. The scale bar represents 200 μm. (C) Representative flow cytometric quantification of Ter119-positive splenocytes from mice on days 0 and 5. (D–G) The graphs show RPI (D), body mass (E), spleen mass (F) and the percentage of Ter119-positive cells among the total splenocytes (G) of mice after low temperature exposure. Values represent the means ± s.e.m. of three mice per time point (*P<0.05 compared with day 0).

Enhanced erythropoiesis after exposure to low temperature

Next, we used flow cytometry to determine which erythropoietic organ, the spleen or the bone marrow, exhibited increased expansion of erythroblasts. Erythroblast maturation proceeds from the proerythroblast stage (CD71 high, Ter119 mid) to the basophilic erythroblast stage (CD71 high, Ter119 high) to the chromatophilic erythroblast stage (CD71 mid, Ter119 high) to the orthochromatic erythroblast stage (CD71 low, Ter119 high) (Fig. 3A). Mice were killed on days 0, 1, 3, 5, 7, 10 and 14 of exposure to low temperature, and the erythroblasts were analyzed by flow cytometry (Fig. 3B). The proportion of proerythroblasts in the spleen increased greatly by day 5 of exposure to low temperature (36% on day 5 compared with 3.8% on day 0). When corrected for spleen size, this change reflects a 4.5-fold increase in the splenic proerythroblast mass on day 5 compared with day 0. Thereafter, the percentage of proerythroblasts decreased to normal by day 14. The number of basophilic erythroblasts was higher than baseline on day 5 and day 7 and subsequently returned to normal on day 10 and day 14. Meanwhile, the number of late-stage mature erythroblasts (chromatophilic and orthochromatic erythroblasts) decreased on day 7 and later increased on days 10 and 14. Analysis of the bone marrow indicated that the number of proerythroblasts moderately increased after low temperature exposure and thereafter remained at a high level for 14 days. We examined the number of earlier erythrocyte progenitor CFU-E by using *in vitro* colony-forming assay (Fig. 3C). CFU-Es were increased in the spleen on day 3 after exposure to low temperature. In the bone marrow, the number of CFU-Es was increased on day 3 and then maintained at a high value until day 7, compared with day 0. These results showed that splenic erythropoiesis was transiently enhanced after exposure to low temperature. The number of proerythroblasts and CFU-Es in the bone marrow was moderately increased and remained high during low temperature exposure.

Increased renal *Epo* mRNA expression after exposure to low temperature

We investigated whether the level of *Epo* mRNA expression in the kidney was increased after exposure to low temperature by RT-PCR.

Table 1. Hematological parameters at low temperature

Group	RBC (×10^{12} l^{-1})	Hb (g dl^{-1})	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g dl^{-1})
Day 0	9.76±0.51	15.1±0.3	45.6±2.7	46.8±3.1	15.5±0.7	33.3±1.6
Day 14	9.90±0.69	16.0±0.9*	52.2±3.5**	52.9±3.2*	16.2±0.5*	30.7±1.5*
Day 42	10.7±0.19*	17.0±0.7*	59.0±0.6**	54.3±2.9*	16.4±0.6*	30.3±2.7*

Values represent the means ± s.e.m. of six mice per time point. RBC, erythrocytes; Hb, hemoglobin; Hct, hematocrit; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. *P<0.05 compared with day 0; **P<0.01 compared with day 0.

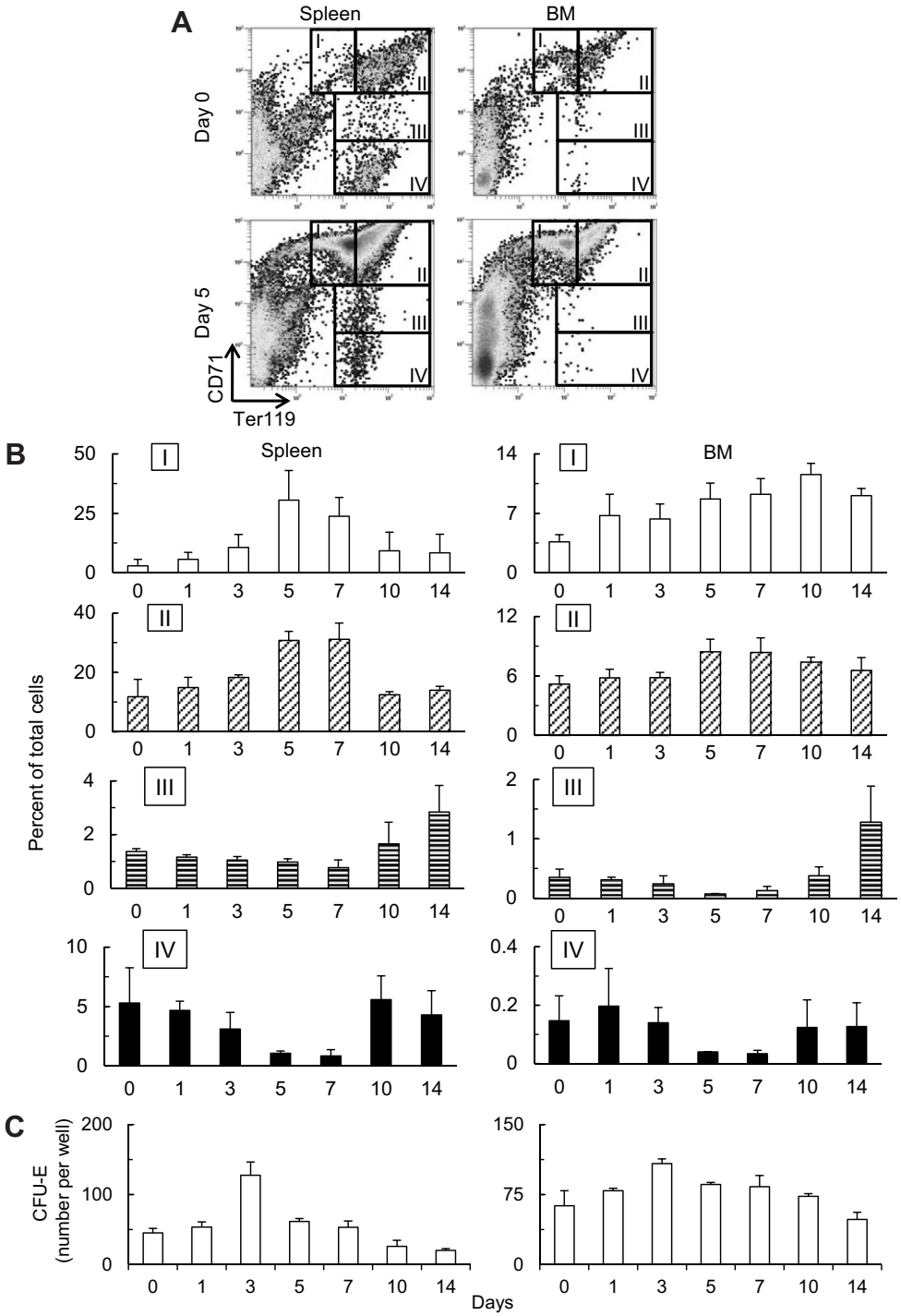

Fig. 3. Erythrocyte progenitor counts in the spleen and bone marrow. (A) Flow cytometric analysis of Ter119 and CD71 expression in splenic and bone marrow (BM) cells on days 0 and 5 after exposure to low temperature. I, proerythroblasts (CD71 high, Ter119 mid); II, basophilic erythroblasts (CD71 high, Ter119 high); III, chromatophilic erythroblasts (CD71 mid, Ter119 high); IV, orthochromatic erythroblasts (CD71 low, Ter119 high). (B) Percentage of each type of erythroblast in the spleen and BM. Values represent the means ± s.e.m. of three mice per time point. (C) Colony-forming unit-erythroid (CFU-E) colony-forming assay in the spleen and BM. The numbers of CFU-Es represent the means ± s.e.m. of three mice per time point.

The *Epo* mRNA levels in the kidney were elevated at day 1 after exposure to low temperature. Thereafter, *Epo* mRNA levels decreased at days 2 and 3. At day 4, *Epo* mRNA levels significantly increased 21-fold compared with day 0 (Fig. 4A). The transcription of *Epo* mRNA is tightly regulated by hypoxia-inducible factors. Hypoxia-inducible factor-1 (HIF-1) controls the expression of a number of hypoxia-related genes, including *Epo*, under low-oxygen conditions. *Hif-1a* mRNA levels increased in the kidney after exposure to low temperature, especially at day 4 (2.6-fold compared with day 0) (Fig. 4B). In contrast, the *Hif-2a* mRNA levels did not increase after exposure to low temperature (data not shown). Finally, we analyzed the mRNA expression of the HIF-1 target genes *Glut1*, *Pgk1*, *Ldha* and *Vegf* in the kidney. The mRNAs of all of these genes increased in the kidney on day 4 after exposure to low temperature (Fig. 4C). These results suggest that erythropoiesis was enhanced, explaining the high level of *Epo* mRNA expression in the kidney. The cause of the enhanced expression of *Epo* mRNA and of the mRNAs of other hypoxia-related genes in the kidney appeared to involve *Hif-1a* mRNA accumulation in the kidney. In addition, the level of oxygen tension in the kidney was decreased after low temperature exposure.

DISCUSSION

The elevation of the erythrocyte counts is induced by low temperature exposure in rats and birds (Deveci et al., 2001; Bláhová

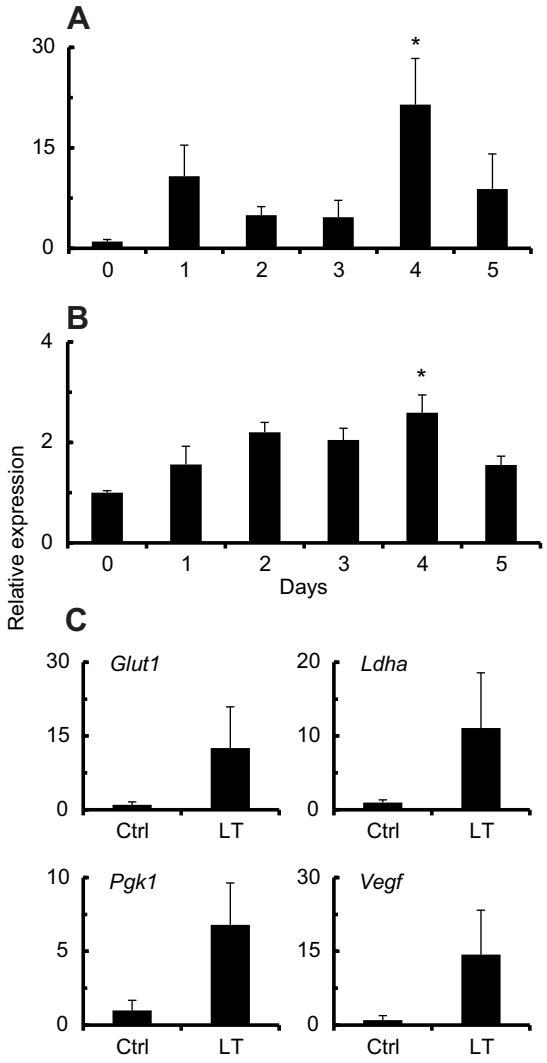

Fig. 4. Real-time PCR analysis of *Epo* and hypoxia-related gene mRNA expression in the kidney. (A) *Epo* mRNA levels were determined in the kidney after exposure to low temperature. Values represent the mean ± s.e.m. (*N*=3, **P*<0.05 compared with day 0). (B) *Hif-1a* mRNA levels were determined in the kidney after exposure to low temperature. Values represent means ± s.e.m. (*N*=3, **P*<0.05 compared with day 0). (C) mRNA levels of the HIF-1 target genes *Glut1*, *Ldha*, *Pgk1* and *Vegf* were determined in the kidney on day 0 (Ctrl) and day 4 (LT) after exposure to low temperature. Values represent means ± s.e.m. (*N*=3). *P*-values compared with day 0: *Glut1*, *P*=0.15; *Ldha*, *P*=0.04; *Pgk1*, *P*=0.05; *Vegf*, *P*=0.10.

et al., 2007). In mice in this study, elevated erythrocyte counts probably effected to increase oxygen supply to peripheral tissues for heat production in endothermic animals (Fig. 1, Table 1). In hypoxic mice, increased tissue oxygen supply is accomplished by decreasing the affinity of hemoglobin for oxygen (right-shifted oxygen dissociation curve) (Penney and Thomas, 1975). As tissue CO_2 tension increased by high metabolic rate during low temperature exposure, tissue oxygen supply could be increased by lower affinity of hemoglobin for oxygen in tissues.

In this study, we described significantly enhanced erythropoiesis induced by exposure to low temperature in mice. These findings indicate a fundamental role for erythropoiesis in responding to low environmental temperature. Under conditions of erythropoietic stress, such as hypoxia and acute hemolytic anemia caused by phenylhydrazine, the number of splenic erythroid cells increases with splenomegaly (Hara and Ogawa, 1976; Kam et al., 1999). It has been reported that enhanced splenic erythropoiesis after erythropoietic stress is essential for the initial increments in erythrocyte counts. In splenectomized mice, an increase of peripheral erythrocyte counts delayed in response to chronic hypoxia, compared with normal mice (Markoe et al., 1973). Low temperature exposure increased the proportions of splenic proerythroblasts by ~10-fold by day 5. The proportions of splenic CFU-Es also increased by day 3 after low temperature exposure. Meanwhile, the number of proerythroblasts and CFU-Es in the bone marrow was moderately elevated compared with the proportions of splenic proerythroblasts and CFU-Es and remained high during the low temperature exposure period (Fig. 3). These data suggest that the spleen was an immediate source of new erythrocytes after low temperature exposure. A moderate and constant high number of erythroblasts in the bone marrow might contribute to sustaining the elevated erythrocyte counts during low temperature exposure.

The principal factor regulating erythropoiesis is EPO (Krantz, 2000). Our data showed that *Epo* mRNA expression in the kidney was enhanced from day 1 after low temperature exposure (Fig. 4). In mice treated with EPO, the number of CFU-Es increases in the spleen and bone marrow (Hara and Ogawa, 1977). Therefore, it is suggested that the numbers of erythroid progenitors in the spleen and bone marrow increased in response to elevated circulating EPO level caused by enhanced renal EPO expression. The regulation of erythropoiesis and the red blood cell mass rely on the modulation of *Epo* gene expression in response to tissue oxygen tension (Fandrey and Bunn, 1993). The transcription factor HIF proteins mediate the hypoxia-induced transcription of oxygen-regulated genes, including *Epo* (Wang and Semenza, 1995). HIF-1 target genes are oxygen sensitive, partly because HIF-1α is rapidly degraded in normoxia but stable in hypoxia to promote transcriptions of the target genes (Bunn et al., 1998). We found that the expressions of *Hif-1a* and HIF-1 target genes were enhanced in the kidney at day 4 after exposed to low temperature (Fig. 4B,C). Although we did not determine HIF-1a protein level, our results suggested that elevated expression of HIF-1 target genes after low temperature exposure is induced by activation of HIF-1α protein. It is also suggested that enhanced *Hif-1a* gene expression and stabilization of HIF-1α protein is caused by lower renal oxygen tension. Lower oxygen tension would be caused by the high oxygen consumption necessary to produce heat during low temperature exposure. We did not detect enhanced expression of *Epo* mRNA or of the mRNAs of oxygen-regulated genes in the liver during low temperature exposure (data not shown). In rats exposed to low environmental temperature, hepatic blood flow increased, whereas renal blood flow did not change (Adán et al., 1994). It has been reported that the skin is a primary co-ordinator of the systemic hypoxic response and modulates cutaneous blood flow to regulate renal and hepatic EPO synthesis in mice (Boutin et al., 2008). In our model, it is suggested that hypoxia and the enhancement of *Epo* gene expression in the liver did not occur because of the increase in hepatic blood flow. Although *Hif1-a* mRNA levels did not increased at day 1 after exposure to low temperature, renal *Epo* mRNA levels moderately increased at day 1 (Fig. 4A,B). Thyroid hormone (TH) plays an important role in the control of thermogenesis. During exposure to low ambient temperature, an increase of serum TH level and subsequent enhancement of heat production were reported previously (Rand et al., 1952; Kassenaar et al., 1956; Kassenaar et al., 1959; Gregerman, 1963; Silva, 2006). Additionally, it has been reported that TH enhances hypoxic-induced *Epo* expression *in vitro*

(Fandrey et al., 1994). In connection with hematopoiesis, earlier studies demonstrated that TH stimulates the proliferation and differentiation of erythrocyte progenitors *in vivo* and *in vitro* (Golde et al., 1977; Dainiak et al., 1978). From these reports and our results, it would appear that TH triggers renal *Epo* gene expression after low temperature exposure, and erythropoiesis is subsequently enhanced by EPO.

In the ectothermic animal, crucian carp (*Carassius carassius*), hypoxia-induced HIF activity increases during low temperature exposure (Rissanen et al., 2006). Conversely, hypoxia-induced HIF-1α protein activity is suppressed in mice during hypothermic conditions (Tanaka et al., 2010). In view of these facts, the mechanism of HIF activation might be different from mice under the thermogenic conditions in the current study. Although the physiological responses to low environmental temperature display a diversity of mechanism among ectothermic, endothermic and hypothermic animals, it is suggested that HIF plays an important role in adaptation to low environmental temperature.

In the rat model, the cause of polycythemia was previously suggested to be a decreased rate of erythrocyte destruction, rather than increased erythropoiesis in rats exposed to 5°C for 8 weeks, based on the lower reticulocyte counts (Deveci et al., 2001). Although erythropoiesis was not examined in rats in the acute phase after low temperature exposure, we hypothesized that the initially increased erythrocyte counts in rats after exposure to low temperature are induced by enhanced erythropoiesis. In our study, the reticulocyte counts were not lower on day 56 after low temperature exposure in mice compared with day 0 (Fig. 1C). Therefore, high erythrocyte counts in rats are maintained by a lower rate of erythrocyte destruction, unlike in mice. Several studies have demonstrated that anemia can be induced by low temperature exposure in ectothermic animals. We also used *Xenopus laevis* in a model of hypothermia and investigated the causes of anemia induced by low temperature exposure (Maekawa et al., 2012). We found that hypothermic anemia is associated with hepatic erythrocyte destruction and that prolonged anemia during low temperature exposure is concomitant with newly produced erythrocytes being confined to the liver in *Xenopus laevis*. This erythropoietic mechanism is different from that observed in the mouse model used in this study. Therefore, erythropoietic systems differ with respect to function between ectothermic and endothermic animals after low temperature exposure, and these systems may be critical for vertebrates to adapt to low temperature environments.

In conclusion, the present study demonstrated that the peripheral erythrocyte counts increased in mice during exposure to low temperature. Furthermore, provisional splenomegaly with increased erythrocyte progenitors is caused by enhanced *Epo* gene expression. These results indicated that low ambient temperature is a novel cause of erythropoietic stress.

LIST OF SYMBOLS AND ABBREVIATIONS

CFU-E	colony-forming unit-erythroid
EPO	erythropoietin
Hb	hemoglobin
Hct	hematocrit
HIF	hypoxia-inducible factor
MCH	mean corpuscular hemoglobin content
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular erythrocyte volume
RPI	reticulocyte production index

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