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Oxygen Affinity of Hemoglobin Regulates O_2 Consumption, Metabolism, and Physical Activity*

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The oxygen affinity of hemoglobin is critical for gas exchange in the lung and O_2 delivery in peripheral tissues. In the present study, we generated model mice that carry low affinity hemoglobin with the Titusville mutation in the α-globin gene or Presbyterian mutation in the β-globin gene. The mutant mice showed increased O_2 consumption and CO_2 production in tissue metabolism, suggesting enhanced O_2 delivery by mutant Hbs. The histology of muscle showed a phenotypical conversion from a fast glycolytic to fast oxidative type. Surprisingly, mutant mice spontaneously ran twice as far as controls despite mild anemia. The oxygen affinity of hemoglobin may control the basal level of erythropoiesis, tissue O_2 consumption, physical activity, and behavior in mice.

Hemoglobin (Hb),[1] a protein found within erythrocytes, transports oxygen through the vertebrate bloodstream. Hb is a tetrameric protein consisting of α- and β-globin subunits that show a characteristic affinity for oxygen with allosteric effects on various metabolites (1, 2). In the literature, more than 1,000 variants of Hb have been reported (3). Some exhibited an altered oxygen affinity, either higher or lower, while maintaining the stability of Hb. Hb Titusville (Hb^{Titu}) is a low affinity variant of the α-globin chain and is well characterized clinically (4, 5). Hb Presbyterian (Hb^{Pres}) is another low affinity variant of β-globin chain and is well characterized *in vitro* (6–9). Interestingly, individuals with low oxygen affinity Hbs such as Hb^{Titu} or Hb^{Pres} show mild anemia, whereas individuals with high oxygen affinity Hbs such as Hb^{Malmo} or Hb^{Yakima} show symptoms associated with polycythemia (10–13).

In the present study, we generated mutant mice carrying an homologous mutation with Titusville ($Asp^{\alpha 94} \rightarrow Asn$) at the α1 locus or with Presbyterian ($Asn^{\beta 108} \rightarrow Lys$) at the β-major locus of the mouse genome by a targeted knock-in strategy to generate a murine model of the Titusville and Presbyterian hemoglobinopathies. With the targeted knock-in strategy, the autologous locus control region, as well as the erythropoietin enhancer element, can be kept intact without altering the regulation of endogenous gene expression. Therefore, the knock-in α-globin or β-globin allele physiologically reacts to stimuli such as hypoxia-inducible factor 1 and erythropoietin. Thus, the model is physiologically relevant and can be used for *in vivo* physiological analysis of variant Hb. In fact, Titusville heterozygous mice and Presbyterian heterozygous mice both mimic the clinical and laboratory findings of humans with Titusville Hb and Presbyterian Hb, respectively.

We surprisingly found in the present study that Titusville mice, as well as Presbyterian mice, showed enhanced tissue oxygenation, increased O_2 consumption and CO_2 production in tissue metabolism, and an increased running capacity and propensity that resulted in altered behavior with greater physical activity despite mild anemia. Taken together with the human data, the results in the mutant mice implied that Hb determined basic biological parameters such as erythropoiesis, metabolism, physical competence, and behavior.

EXPERIMENTAL PROCEDURES

Generation of Titusville and Presbyterian Mutant Mice—Hb α1 globin gene knock-in mice with the Titusville mutation were obtained by replacing Asp-94 of the α1 globin gene with Asn as described below. The 129-mouse genomic library in λFIXII (Stratagene, CA) was screened with the 372-bp 5′ flanking sequence of the murine α1 globin gene (nucleotides 1–372; GenBank™ accession number V00714) as a probe. Two overlapping clones covered all exons of the gene. The 1.0-kb fragment containing all α1 globin exons was amplified with a *Spe*I/*Afl*II-anchored primer (5′-GGA CTA GTC TTA AGA GAC TCA GGA AGA AAC C-3′) and *Xho*I/*Afl*II-anchored primer (5′-CCT CTA GAC TCG AGC TTA AGG TAG GCA TCC AAT TAT GCT T-3′). The *Spe*I/*Xho*I-restricted α1 globin fragment was mutagenized with a 21-bp mutagenic oligonucleotide (5′-GCT GCG TGT GAA TCC CGT CAA-3′) using the pALTER system (Promega, Madison, Wisconsin). The introduced mutation, D94N, was confirmed by sequencing. The 2.2-kb short homologous fragment was PCR-amplified with a *Xho*I-anchored primer (5′-CCG CTC GAG TCC TTG AGC CAA AGA AGC CA-3′) and *Apa*I/*Sal*I-anchored primer (5′-TTG GGC CCG TCG ACT CTG CCC GCT GGC TGA

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[1] The abbreviations used are: Hb, hemoglobin; Hb^{Titu}, Hb Titusville; Hb^{Pres}, Hb Presbyterian; RP-HPLC, reversed-phase high performance liquid chromatography; $PtiO_2$, tissue O_2; FIO_2, fraction of inspired O_2; $PaCO_2$, partial pressure of arterial CO_2; PaO_2, partial pressure of arterial O_2; SDH, succinate dehydrogenase; wt, wild-type; ATPS, ambient temperature, pressure, saturated.

GCT C-3′). The *Spe*I/*Xho*I-restricted α1 globin fragment and *Xho*I/*Apa*I-restricted short 3′ homologous fragment were sequentially inserted into a *Spe*I/*Apa*I-restricted pBSII SK vector. The long 6.8-kb homologous fragment was amplified with a *Not*I-anchored primer (5′-ATT TGC GGC CGC TGG CAT TCA CAG AGC TCA CCA-3′) and *Spe*I-anchored primer (5′-GGA CTA GTG TCA GAA TCA GAA GTG TCT TGG-3′). The *Not*I/*Spe*I-restricted 5′ long homologous fragment was inserted into the targeting construct. The 1.3-kb MC1neo cassette flanked by loxp sequences was PCR-amplified from pMC1neo-*lox*p vector (14) with a *Sal*I-anchored primer (5′-CGC GTC GAC ATA ACT TCG TAT AAT G-3′) and *Sal*I/*Eco*RI-anchored primer (5′-CGC GTC GAC GAA TTC ATC GAT ACC GGC GAC ATA-3′). The *Sal*I-restricted MC1neo-*lox*p cassette was inserted into the *Xho*I-restricted targeting construct. The resulting construct containing the short and long homologous fragments, the mutagenized α1 globin gene, and the neomycin gene was restricted with *Not*I/*Sal*I and recloned into the targeting vector, pMC1DT-A (B) (Oriental Koubo). The vector was then linearized with *Not*I and used for the electroporation of embryonic stem cells. Genomic DNA from each of 240 G418-resistant embryonic stem clones was digested with *Eco*RI and screened for the homologous recombination by Southern blot analysis using an 800-bp 3′ probe. One embryonic stem clone with the expected homologous recombination was used for generating chimeric mice by the aggregation method as described (15). The chimeric mice were cross-bred with C57BL/6CrSlc (Nihon SLC), and the germline transmission was confirmed by PCR amplification with the primers p1 (5′-TTC CTT GCC TCT GTG AGC-3′) and p2 (5′-TGG GAC CGA GCC ATC TTC-3′) in agouti offspring.

Hb β-globin gene knock-in mice with the Presbyterian mutation were obtained by replacing Asn-108 of the β-globin gene with Lys as described previously (16). The chimeric mice were cross-bred with C57BL/6CrSlc, and the germline transmission was confirmed by PCR amplification with the primers p3 (5′-ACC CAG CGG TAC TTT GAT AGC-3′) and p4 (5′-GCT ACT GAA GCT GTC TAA GGC AAC AGG-3′) in agouti offspring.

Biochemical Analyses of Mutant Hb—Blood samples from mice and humans were collected into EDTA-treated tubes. Erythrocytes were washed in cold 0.85% NaCl, collected by centrifugation, and lysed in 4 volumes of distilled water. The hemolysate was then collected by centrifugation at 15,000 rpm for 30 min. The hemoglobin concentration was determined with a Wako hemoglobin test (Wako Chemicals). The hemolysate was then separated by reversed-phase high performance liquid chromatography (RP-HPLC) using a Develosil ODS 300C4-HG-5 column (4.6 × 150 mm; Nomura Chemicals). Globin peaks were eluted at a flow rate of 1 ml/min with a liner gradient of 36–52% acetonitrile (0.4%/min) in 0.1% trifluoroacetic acid as described previously (16, 17). The absorbance was monitored at 214 nm.

Physiochemical Analysis of Mutant Hb—Oxygen equilibrium studies of washed erythrocytes and Hb solution were carried out using a Hemox analyzer (TCS Products, Southampton, PA) at 37 °C. For examination of the Bohr effect, hemolysates were concentrated with Ultrafree 30,000 (Millipore) and dialyzed in the same 50 mM HEPES buffer, containing 100 mM NaCl, at pH 7.0, 7.4, and 7.8. For examination of the chloride effect, concentrated hemolysates prepared as described above were dialyzed in 50 mM HEPES, pH 7.4, containing a chloride ion concentration of 0, 50, 150, and 500 mM NaCl.

Analysis of Tissue O_2 ($PtiO_2$)—Male wild-type (n = 8), and Presbyterian (n = 9) mice (12 weeks old) were anesthetized with ether for setting in a double-chamber plethysmograph and inserting an O_2 electrode with a thermocouple (Clark-type electrode; Unique Medical) to measure $PtiO_2$. The O_2 electrode was inserted through a guide tube into the left gastrocnemius muscle. The output from the electrode was adjusted to the muscle temperature and continuously displayed on a digital monitor (POG-203; Unique Medical, Japan), being recorded on an analogue tape recorder. The electrode was calibrated with room air before and after each experiment.

Respiratory variables were also measured with a double-chamber plethysmograph as described previously (18). Respiratory frequency (f; breaths/min) was determined as 60/total breath duration. Tidal volume (V_T) was calculated using the equation $V_T = (273 + Tb)/(273 + Tam) \times (760 - PamH_2O)/(760 - PbH_2O) \times 0.5/Vcal \times V_{T_{ATPS}}$, where Tb is rectal temperature (°C); Tam is ambient temperature (°C); $PamH_2O$ and PbH_2O are the water vapor pressures (mmHg) in the ambient air and the alveoli, respectively; and $V_{T_{ATPS}}$ is V_T at ambient temperature, pressure, saturated (ATPS) without calibration (ml). The volume injected into the head chamber was 0.5 (ml ATPS) for calibration, recorded as Vçal (ml ATPS) on the personal computer. Minute lung ventilation ($\dot{V}_E$; ml body temperature, pressure, saturated) was determined as $f \times \dot{V}_T$ and normalized with respect to body weight per 10 g.

Each mouse was allowed to acclimate to the chambers (fraction of inspired O_2; FIO_2 = 0.21) for at least 60 min before the hypoxic gas challenge, and a constant level of baseline $PtiO_2$ was achieved. Subsequently mice were exposed to a hypoxic gas (FIO_2 = 0.15) for 5 min. A gas mixture was delivered from a respiratory gas circuit consisting of flow meters for O_2 and N_2 and a reservoir bottle (2 liters) connected to the head chamber. FIO_2 was altered by mixing O_2 and N_2, being continuously monitored by withdrawing a small fraction of the gas mixture (20 ml/min) with an O_2 and CO_2 analyzer (Respina 1H26; NEC San-ei). $\dot{V}_E$ and $PtiO_2$ were measured at 0, 0.5, 1, 2, 3, 4, and 5 min. $\Delta PtiO_2$ (mmHg) was calculated as the difference between baseline $PtiO_2$ and $PtiO_2$ at each time point.

Blood Gas Analysis—Male wild-type (n = 5), Titusville (n = 5), and Presbyterian (n = 5) mice (12 weeks old) were used. An arterial catheter (BC-1P; Access Technology) was implanted in the left carotid artery under anesthesia (sodium pentobarbital; 25 mg/kg, intraperitoneally) for blood gas analysis. Mice were placed in the plethysmograph for 2 h to recover from the anesthesia and subjected to a hypoxic challenge comparable with that used to obtain $\dot{V}_E$ and $PtiO_2$. Arterial blood (120 μl) was sampled with a heparinized sampling glass tube (MC0020; AVL Scientific Corporation) and immediately analyzed by a blood gas analyzer (OPTI CCA; AVL Scientific Corporation) for pH, partial pressure of arterial CO_2 ($PaCO_2$), and partial pressure of arterial O_2 (PaO_2). Arterial blood was sampled before and at the end of hypoxic gas inhalation.

Metabolism—O_2 consumption (ml standard temperature, pressure, dry), CO_2 production (ml standard temperature, pressure, dry), and respiratory exchange rate were measured during normoxia and hypoxia with an open circuit system (ARCO-1000; ARCO Systems) in male wild-type (n = 4), Titusville (n = 5), and Presbyterian (n = 5) mice. Each mouse was set in a chamber where a steady flow of air was delivered continuously by a vacuum pump for the assessment. The system measured the fractions of O_2, CO_2, and N_2 in the in-flow and out-flow of the chamber with a mass spectrometer and the flow rate with a pneumotachograph. The mouse was placed in the chamber for 60 min to acclimatize to the surroundings before the experiments. The metabolic factors were measured under normoxic conditions and then the hypoxic gas (FIO_2 = 0.15) was delivered from the respiratory gas circuit to the metabolic chamber. Measurements were made during a 5-min steady state period 20 min after the onset of hypoxic gas exposure. O_2 consumption and CO_2 production were normalized with respect to body weight per kg.

Muscle Fiber Type Classification and Succinate Dehydrogenase (SDH) Activity—The right tibialis anterior muscle was removed under sodium pentobarbital anesthesia (50 mg/kg body weight, intraperitoneally). The muscle was placed on cork, stretched to its *in vivo* length, and quickly frozen in isopentane cooled with liquid nitrogen. Serial transverse sections, 10 μm thick, of the mid-belly of the muscle, were cut in a cryostat set at −20 °C. The sections were brought to room temperature and air-dried for 30 min. The sections were stained for ATPase activity following acid (pH 4.5) preincubation for fiber typing. The muscle fibers were classified as type IIA and type IIB (19). SDH activities were used for comparisons among fibers of different types (19). The cross-sectional areas and SDH activities of ~50 fibers from each of a deep (close to the bone), middle (between the deep and superficial), and superficial (near the surface of the muscle) regions of the muscle were determined using a computer-assisted image processing system. These regions were selected for analysis, because the tibialis anterior muscle shows an increasing gradient of fibers having high oxidative enzymatic activity proceeding from the superficial to the deep aspect of the muscle. The sections were digitized as gray scale images and quantified as one of 256 gray levels (20). A gray level value of 0 was equivalent to a 100% transmission of light whereas that of 255 was equivalent to 0% transmission. The mean optical density value within a fiber was determined using a calibration tablet that has 21 steps of gradient density ranges and corresponding diffused density values.

Loaded Running-wheel Protocol—Wild-type (n = 5), Titusville (n = 5), and Presbyterian (n = 5) male mice (15 weeks old) were voluntarily exercised for 28 days using a running wheel apparatus in which distance can be monitored electronically (21). This apparatus includes a standard plastic cage (20.0 × 30.0 × 12.0 cm) and a running wheel (width 5.0 cm, diameter 25.5 cm) attached vertically to a freely rotating shaft inserted into a metal controller box that is supported on a metal base. The running wheel rotates on the shaft whenever the mouse walks or runs in either direction, and the number of revolutions of the running wheel is recorded continuously.

Human Study—Two females, 31 and 29 years old, who were nonsmokers and healthy, were analyzed in this study. Genomic DNA anal-

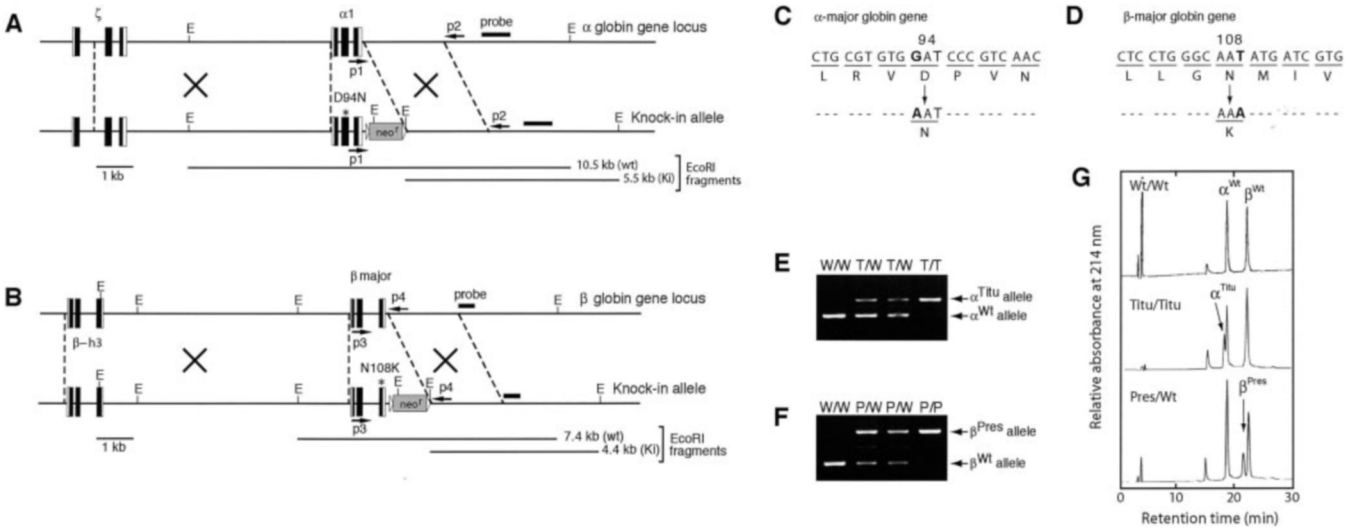

FIG. 1. **Generation of Hb mutant mice.** *A*, strategy used to knock-in the Titusville mutation (D94N) in the murine α1-globin gene locus. Diagrams of the mouse α1-globin locus (*upper*) and predicted knock-in allele (*lower*) are presented. *B*, strategy used to knock-in the Presbyterian mutation (N108K) in the murine β-globin gene major locus. Diagrams of the mouse β-globin major locus (*upper*) and predicted knock-in allele (*lower*) are presented. *E*, *Eco*RI; *neo*^{r}, neomycin resistance gene. *C* and *D*, the introduced mutation replaces α94Asp with Asn in knock-in allele (*C*) and β108Asn with Lys in knock-in allele (*D*). *E*, genotype analysis of Titusville mice by PCR. The wild-type allele of the α^{Wt}-globin gene or knock-in allele of the α^{Titu}-globin gene was amplified by PCR with primers P1 and P2. *F*, genotype analysis of Presbyterian mice by PCR. The wild-type allele of the β^{Wt}-globin gene or knock-in allele of the β^{Pres}-globin gene was amplified by PCR with primers P3 and P4. *G*, RP-HPLC profiles of hemolysate prepared from a wild-type mouse (*Wt/Wt*; *upper*), a Titusville mouse (*Titu/Titu*; *middle*), or a Presbyterian mouse (*Pres/Wt*; *lower*). The peaks of α^{Wt}-globin, α^{Titu}-globin, β^{Wt}-globin, and β^{Pres}-globin are indicated in HPLC profiles. The peak of α^{Titu}-globin was eluted earlier than the peak of α^{Wt}-globin (an *arrow* in the *middle panel*). The peak of β^{Pres}-globin was also eluted earlier than the peak of β^{Wt}-globin (an *arrow* in the *lower panel*).

ysis was approved by the ethical committee of Tokyo Metropolitan Institute of Gerontology, and written informed consent was obtained. Genomic DNAs were prepared from whole blood by GenTLE (Takara, Kyoto, Japan) for PCR. 1,118-bp fragments were amplified using a sense primer (5′-ACC CAG AGG TTC TTT GAG TC-3′) and an antisense primer (5′-TCT GAT AGG CAG CCT GCA CT-3′). The PCR products were isolated and sequenced with a nested sense primer (5′-CTG GGT TAA GGC AAT AGC-3′) by the dye terminator method. Arterial blood gas analysis was carried out with a pH-blood gas analyzer (Bayer medical 860).

RESULTS

Generation of Mutant Mice Expressing Mutant Hb with Altered Oxygen Affinity—To generate mutant mice with a greater capacity to deliver O_2, we first searched for mutant Hbs with altered oxygen affinity in the medical literature. We found that individuals with a variant Hb of higher affinity such as Yakima Hb and Malmo Hb usually manifested polycythemia (10–13) whereas individuals with a variant Hb of lower affinity such as Kansas Hb, Titusville Hb, or Presbyterian Hb showed mild asymptomatic anemia without any medical complications (4–9, 22–24). These medical profiles prompted us to explore the possibility that the variant Hbs with lower affinity improve O_2 delivery to the peripheral tissues in the physiological state. To test this hypothesis, we generated two distinct models, Titusville Hb mice and Presbyterian Hb mice. Titusville Hb is composed of a variant α chain with Asn-94, an amino acid substitution in the α/β interfaces, whereas Presbyterian Hb is composed of a variant β chain with Lys-108 protruding into the central cavity of the Hb molecule. These two hemoglobinopathies thus have distinct mechanisms for altering the affinity of Hb for oxygen but a common clinical phenotype such as anemia, suggesting that the lowered affinity of Hb generally enhances, whereas the raised affinity generally suppresses, O_2 delivery in the peripheral tissues. In addition, Presbyterian Hb confers a novel allosteric effect with the variant Lys residue interacting with the Cl^- ion in the central cavity, but Titusville Hb showed no allosteric effect. Therefore, by means of these two models, we can devise multiple strategies to enhance O_2 delivery either by manipulating α-globin and β-globin or by using a novel allosteric effect.

As schematized in Fig. 1, the homologous recombination in the mouse α-globin or β-globin genome with target vectors replaced the α1 exon or β major exon with a modified α1 carrying Asn-94 (Fig. 1*A*) or β major carrying Lys-108 (Fig. 1*B*), respectively. The intercrossing of heterozygous mice successfully generated fertile homozygous mice. Southern blot analyses (data not shown) and PCR amplification of α-globin or β-globin genomes (Fig. 1, *E* and *F*) confirmed the expected homologous recombination. The homozygous and heterozygous mice were born viable, grew normally, and were fertile. Sequence analyses of PCR products from homozygous mice further confirmed the expected αD94N mutation in Titusville mice (Fig. 1*C*) and βN108K mutation in Presbyterian mice (Fig. 1*D*). To confirm whether the knock-in allele productively expressed the mutated α- or β-chain, we biochemically characterized the hemoglobin prepared from mutant mice. To separate α- and β-globin chains, purified hemoglobins were applied to an RP-HPLC column under acidic conditions. HPLC profiles of Hb prepared from Titusville mice or Presbyterian mice showed double peaks for the α chain or β chain (Fig. 1*G*, *middle* and *lower*). Based on the profiles, we estimated that ~15% of the Hb in the peripheral blood of Titusville mice consists of Hb^{Titu} whereas ~30% of that in Presbyterian mice consists of Hb^{Pres}. The medical literature on individuals with mutant hemoglobinopathies revealed the expression level of Hb^{Titu} to be 34.7% (4) and Hb^{Pres} to be 29.9–41.7% (6–9) in human cases. We therefore characterized heterozygous Titusville mice and heterozygous Presbyterian mice in this study as animal models for variant hemoglobinopathies with lower oxygen affinity. In the peripheral blood, red blood cells of Titusville mice showed normal hemograms whereas Presbyterian mice showed mild anemia (Hb 14.8 ± 0.8 *versus* 12.9 ± 1.1 g/dl, $p < 0.05$; see Table I) without signs of hemolysis (reticulocytes 1.7 ± 0.2% *versus* 1.6 ± 0.7%; see Table I), suggesting that these model mice mimic the human cases well (4–9).

TABLE I
Complete blood cell count of Titusville and Presbyterian mice
Significant differences between wild-type and Presbyterian mice were determined by unpaired Student's *t*-test. Data are the means ± S.E.

	Wild-type	Titusville	Wild-type	Presbyterian
RBC[a] ($\times 10^6$/ml)	9.67 ± 0.35	9.99 ± 0.44	9.71 ± 0.50	9.19 ± 0.77
Hb (g/dl)	15.2 ± 0.6	14.6 ± 0.4	14.8 ± 0.8	12.9 ± 1.1[b]
Ht (%)	56.9 ± 2.1	55.1 ± 1.9	57.0 ± 3.3	50.2 ± 4.0
MCV (fl)	59.0 ± 0.7	55.2 ± 0.8	58.6 ± 2.0	54.6 ± 3.6
MCH (pg)	16.0 ± 0.0	14.7 ± 0.5	15.4 ± 0.6	14.2 ± 0.8
MCHC (g/dl)	27.0 ± 0.0	26.5 ± 0.5	26.0 ± 0.7	25.8 ± 1.8
WBC ($\times 10^3$/ml)	3.36 ± 0.18	2.63 ± 0.42	2.30 ± 0.72	2.67 ± 1.01
Plt ($\times 10^3$/ml)			72.6 ± 28.2	66.2 ± 9.8
Ret (%)	2.2 ± 0.1	2.8 ± 0.7	1.6 ± 0.7	1.7 ± 0.2

[a] RBC, red blood cell; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; WBC, white blood cell; Plt, platelet; Ret, reticulocyte.
[b] $p < 0.05$.

The Mutant Hb Showed Low Affinity for Oxygen in Vitro—To characterize the physiochemical properties of Hb^{Titu}, Hb^{Pres}, and double mutant $Hb^{Titu, Pres}$, we assessed the oxygen dissociation of red blood cells prepared from Titusville mice, Presbyterian mice, and Titusville/Presbyterian double mutant mice. In oxygen dissociation plots, Hb^{Pres} showed a rightward shift in comparison with wild-type Hb (Fig. 2*A*, P_{50} = 43.5 *versus* 47.0 mmHg) whereas Hb^{Titu} and $Hb^{Titu, Pres}$ exhibited even more extensive rightward shifts as shown in Fig. 2 (P_{50} = 66.0 or 72.0, respectively). Analyses of Hill's plot and the Bohr effect, however, indicated that Hb^{Pres} retained all physiochemical properties (Fig. 2, *B* and *C*) whereas Hb^{Titu} or $Hb^{Titu, Pres}$ showed a reduced Hill's coefficient, suggesting that the Titusville, but not Presbyterian, mutation conferred the reduced incorporation of Hb as reported previously in human cases (4, 25). As for *de novo* allosteric effects, we investigated the influence of Cl^-. Interestingly, Cl^- stabilized the deoxy state of Hb^{Pres} in a dose-dependent manner, suggesting that the introduced Lys residue protrudes into the central cavity to bind to Cl^- ion as suggested in the previous model (Fig. 2*D*).

Hb^{Pres} Delivers More Oxygen to Peripheral Tissues under Moderately Hypoxic Conditions—Presbyterian mice were exposed to 15% O_2 for 5 min to investigate the physiological effects of Hb^{Pres} on tissue hypoxia *in vivo*. $\Delta PtiO_2$ and *Ve* values during hypoxia are shown in Fig. 3, *A* and *B*, respectively. After 5 min of hypoxia, the tissue O_2 of Presbyterian mice was significantly retained and sustained a higher level than in wild mice ($p < 0.05$, a two-way analysis of variance for repeated measures). In the course of hypoxia, Presbyterian mice showed a similar decline in tissue O_2 to wild-type mice within 1 min of hypoxia whereas tissue O_2 started to dissociate in the hypoxic phase that followed (Fig. 3*A*). The result suggested that more oxygen is delivered to the tissues in Presbyterian than wild-type mice over a certain range of hypoxic conditions. In fact the benefit of changes in the affinity of Hb^{Pres} *in vitro* is greatest at a PaO_2 concentration of ~50 mmHg as shown in Fig. 2*A*. Therefore, it is reasonable that the beneficial effect of Hb^{Pres} is more remarkable *in vivo* in advanced tissue hypoxia as shown in Fig. 3*A*.

We simultaneously monitored *Ve* to elucidate whether the increased tissue oxygenation was attributable to the increased ventilation of Presbyterian mice or an efficient O_2 delivery by the mutant Hb. The results revealed that Presbyterian mice had a consistent depressed ventilation before and during hypoxia, although they showed a similar pattern of ventilatory responses to wild-type mice, such as the initial hypoxic response and subsequent depression (Fig. 3*B*). The data clearly suggested that in Presbyterian mice, more oxygen was delivered to tissues not by a ventilatory increase but by increased O_2 delivery by Hb^{Pres}.

Presbyterian Mice Showed an Altered Set Point of the Acid-Base Balance—To investigate whether mutant Hbs influence the acid-base balance, we measured the pH, $PaCO_2$, and PaO_2 level of arterial blood in Titusville and Presbyterian mice (Table II). Titusville mice showed a normal PaO_2, $PaCO_2$, and pH in room air and during hypoxia whereas Presbyterian mice showed a low pH associated with an elevated $PaCO_2$ both in room air and under hypoxic conditions (Table II). The results may simply indicate that Presbyterian mice developed chronic respiratory acidosis because of hypoventilation. However, such a pathophysiological explanation is unlikely because, (i) the acidosis is not compensated by metabolic alkalosis (Table II), (ii) Presbyterian mice showed no lung disease causing alveolar hypoventilation, and (iii) they showed a normal PaO_2 level (Table II). Given the normal respiratory functions and reduced ventilation (*Ve*) (Fig. 3*B*), the primary cause of elevated $PaCO_2$ levels in Presbyterian mice may be attributable to central hypoventilation. In this context, we speculate that Presbyterian mice set their acid-base balance to a lower pH and higher $PaCO_2$ by reducing the ventilation. It is still surprising that Presbyterian mice consumed more O_2 and produced more CO_2 in room air, as well as during hypoxia (see below), despite the fact that they manifest signs of hypoventilation (Fig. 3*B*) and mild anemia (Table I). Given the fact that a human with Presbyterian Hb also showed a lower pH and higher $PaCO_2$ level on exercising (see Fig. 6*D*) and that an acid-base imbalance was not observed in Titusville mice, this imbalance may be a Presbyterian-specific phenotype associated with the β108Lys residue. Because primary genetic mutations theoretically confer increased oxygen delivery in peripheral tissues, one explanation for these abnormalities is that Presbyterian mice compensate for tissue hyperoxia caused by Hb^{Pres} by reducing ventilation. Alternatively, Hb^{Pres} may modulate the respiratory center, especially the ventilatory response to CO_2 in the brain of mutant mice, in a Presbyterian-specific manner.

Titusville and Presbyterian Mice Consume More O_2 and Produce More CO_2—To investigate whether enhanced tissue oxygenation alters the basic metabolism of mutant mice, we measured metabolic parameters such as O_2 consumption, CO_2 production, and the respiratory ratio in Titusville and Presbyterian mice (Table III). The metabolic analyses showed that both mutant mice consumed more O_2 and produced more CO_2 in room air and hypoxic conditions (Table III), implying that the low affinity of mutant Hbs drives the mice to consume more O_2 to exclude the excess tissue O_2 delivered by mutant Hbs. The increase in O_2 consumption may then lead to the increased production of CO_2 in the tissue, albeit the respiratory ratio being slightly higher in room air in both mutant mice. This is the first report, to our knowledge, that Hb regulates the basic metabolism of the body by regulating the tissue oxygenation.

Muscle Fiber Distribution and Mitochondrial SDH Activity Were Altered in Titusville and Presbyterian Mice—To clarify

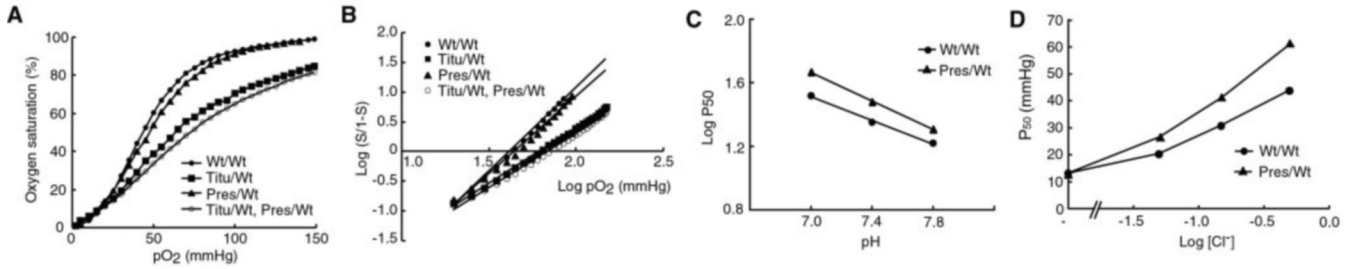

FIG. 2. **Physiological properties of mutant Hb.** *A*, oxygen dissociation curves of red blood cells from wild-type, Titusville, Presbyterian, and double heterozygous Titusville/Presbyterian mice. Mutant red blood cells showed a rightward shift in the oxygen dissociation curve. *B*, Hill's plots of wild-type, Titusville, Presbyterian, and double heterozygous Titusville/Presbyterian red blood cells. Hill's coefficient was conserved in Presbyterian mice but not in Titusville mice or heterozygous Titusville/Presbyterian mice. *C*, Bohr effect of hemolysates from Presbyterian mice and wild-type mice. The Bohr effect was also conserved in Presbyterian mice. *D*, the effect of Cl^- concentration on oxygen dissociation in hemolysates from Presbyterian mice and wild-type mice. An enhanced dose-dependent Cl^- effect was seen in Presbyterian mice.

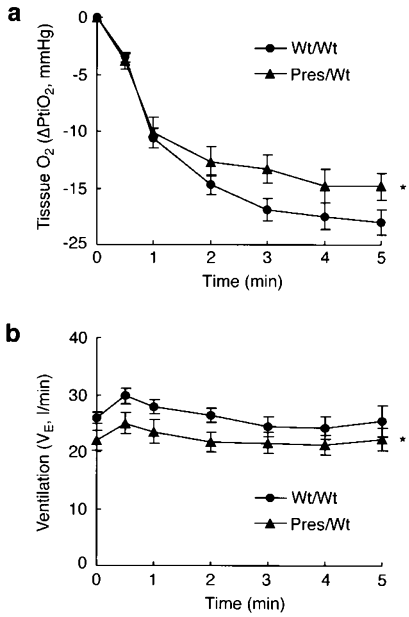

FIG. 3. **Tissue O_2 in Presbyterian mice was a significantly higher level than that in wild-type mice during mild hypoxia.** *A*, tissue O_2 ($\Delta PtiO_2$) response to hypoxia in wild-type (*filled circle*; $n = 8$) and Presbyterian mice (*filled triangle*; $n = 9$). *B*, changes in ventilation (VE) to hypoxia. Data are the mean ± S.E. and were analyzed by a two-way analysis of variance for repeated measures. Statistical interaction between time and group is shown. *, $p < 0.05$.

whether a relationship exists between increased O_2 delivery by Hb^{Pres} and skeletal muscle properties, we determined the fiber type distributions, fiber cross-sectional areas, and fiber mitochondrial SDH activities in tibialis anterior muscle of Presbyterian mice and compared the results with those for wild-type mice. In cross-sections from deep, middle, and superficial regions of the tibialis anterior muscle, Titusville and Presbyterian mice showed no fiber hypertrophy or atrophy, regardless of the muscle region (data not shown). However, on histochemical staining for ATPase activity, both mice showed a higher percentage of type IIA fibers and a lower percentage of type IIB fibers in deep regions of the muscle compared with wild-type mice (Fig. 4, *A* and *D*). The fiber type distribution analyzed in the present study indicated that the tibialis anterior muscle of Titusville mice was composed of 51.4% type IIA and 48.6% type IIB fibers compared with 39.5 and 60.5%, respectively, in the wild-type (Fig. 4*B*), whereas Presbyterian mice contained 49.8% type IIA and 50.2% type IIB fibers compared with 41.0 and 58.9% (Fig. 4*E*). The results indicate that Titusville and Presbyterian mice have a higher ratio of type IIA/IIB fibers than do wild-type mice. Interestingly, a higher ratio favors oxidative energy metabolism in skeletal muscles, supporting the idea that Titusville mice, as well as Presbyterian mice, genetically alternate energy expenditure to favor the high oxidative type of metabolism in muscle. To confirm this hypothesis, we analyzed fiber mitochondrial SDH activity in the same sections (Fig. 4, *C* and *F*). Surprisingly, SDH activities in both type IIA and type IIB fibers were greater in deep regions of the tibialis anterior compared with those of wild-type mice. Type IIB fibers are characterized as being fast contracting, high glycolytic in their enzymatic activity, and easily fatiguable. Because this type of fiber is only supposed to increase SDH activity with physical exercise, it is worth speculating that the genetic alteration in Hb^{Titu} and Hb^{Pres} also converts the propensity of type IIB fibers favoring glycolytic ATP production over oxidative phosphorylation by increasing SDH activity in mitochondria.

Titusville and Presbyterian Mice Spontaneously Run ~2 Times Further on a Running-wheel Apparatus—To clarify whether mutant Hbs influence behavior such as spontaneous physical activity, *i.e.* running, we monitored the running distances of Titusville and Presbyterian mice during a 28-day exercise period. Surprisingly, both mutant mice ran more than twice as far as wild-type mice (Fig. 5). In the initial training phase of the exercise, both wild-type and mutant mice extended their running distances, but the increase was more remarkable in the Titusville and Presbyterian mice (*day 0–14* in Fig. 5). In the second phase of exercise, Titusville mice showed a steady state of daily running with an ~2.5 times longer distance than wild-type mice whereas Presbyterian mice showed an ~2 times longer distance. Mean running distances of Titusville and Presbyterian mice *versus* wild-type mice were 9539 *versus* 4613 m day^{-1} and 7580 *versus* 3732 m day^{-1}, respectively. These results strongly suggested that Titusville and Presbyterian mice have a propensity to run spontaneously with or without a running apparatus. The results also showed an enhanced steady state capacity for running in mutant mice. Taken together with the histochemical findings, Titusville and Presbyterian mice consume more O_2 in skeletal muscles by oxidative phosphorylation. It is therefore speculated that the excessive ATP produced by oxidative phosphorylation with increased SDH activity in mitochondria of mutant mice is consumed by spontaneous running.

A Human with Presbyterian Hb Showed an Altered Ventilatory Response to CO_2 during Exercise—A 29-year old female (*case 2* in Fig. 6, *A* and *B*) inherited the Presbyterian mutation, A for C at nucleotide 1,357 of human β-globin exon 3 (*case 2* in Fig. 6, *A* and *B*), from her father and grandmother whereas her 31-year-old sister did not (*case 1* in Fig. 6, *A* and *B*). The mutation was also confirmed in a chromatographic study in which Presbyterian β-globin (β^{Pres}-globin) was specifically detected in the hemolysate from case 2 but not detected in case 1 (Fig. 6*C*). This mutant peak in HPLC was detected in the hemolysate from the subject's father who carries Hb^{Pres} (data

TABLE II
Blood gas analyses of Titusville and Presbyterian mice
Significant differences between wild-type and Presbyterian mice were determined by unpaired Student's *t*-test. Data are the means ± S.E.

	Wild-type	Titusville	Wild-type	Presbyterian
pH				
Room air	7.42 ± 0.01	7.42 ± 0.03	7.42 ± 0.00	7.34 ± 0.02[a]
Hypoxia	7.45 ± 0.03	7.46 ± 0.03	7.44 ± 0.01	7.40 ± 0.01[b]
$PaCO_2$ (*mmHg*)				
Room air	44.0 ± 0.9	41.9 ± 4.4	39.8 ± 0.6	47.2 ± 1.7[a]
Hypoxia	39.6 ± 6.6	37.5 ± 5.6	39.6 ± 0.1	44.2 ± 0.6[c]
PaO_2 (*mmHg*)				
Room air	85.7 ± 4.2	84.8 ± 6.8	90.6 ± 1.8	90.0 ± 5.1
Hypoxia	56.2 ± 9.0	58.7 ± 11.0	54.8 ± 1.4	60.0 ± 4.5

[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.001$.

TABLE III
Metabolic analyses of Titusville and Presbyterian mice
Significant differences between wild-type and Titusville or Presbyterian mice was determined by unpaired Student's *t*-test. Data are the means ± S.E.

	Wild-type	Titusville	Wild-type	Presbyterian
CO_2 production (*ml/min/kg*)				
Room air	26.0 ± 1.2	35.1 ± 1.0[a]	23.3 ± 1.2	32.3 ± 1.8[a]
Hypoxia	15.8 ± 0.8	20.2 ± 1.6[b]	16.9 ± 0.4	20.5 ± 1.0[b]
O_2 consumption (*ml/min/kg*)				
Room air	34.7 ± 2.0	42.2 ± 2.2[b]	30.8 ± 1.5	40.0 ± 2.6[b]
Hypoxia	24.7 ± 1.2	29.8 ± 2.3[b]	26.5 ± 1.1	30.8 ± 1.1[b]
Respiratory exchange ratio				
Room air	0.75 ± 0.02	0.84 ± 0.03[b]	0.76 ± 0.01	0.81 ± 0.02[b]
Hypoxia	0.64 ± 0.01	0.68 ± 0.01	0.64 ± 0.02	0.66 ± 0.01

[a] $p < 0.01$.
[b] $p < 0.05$.

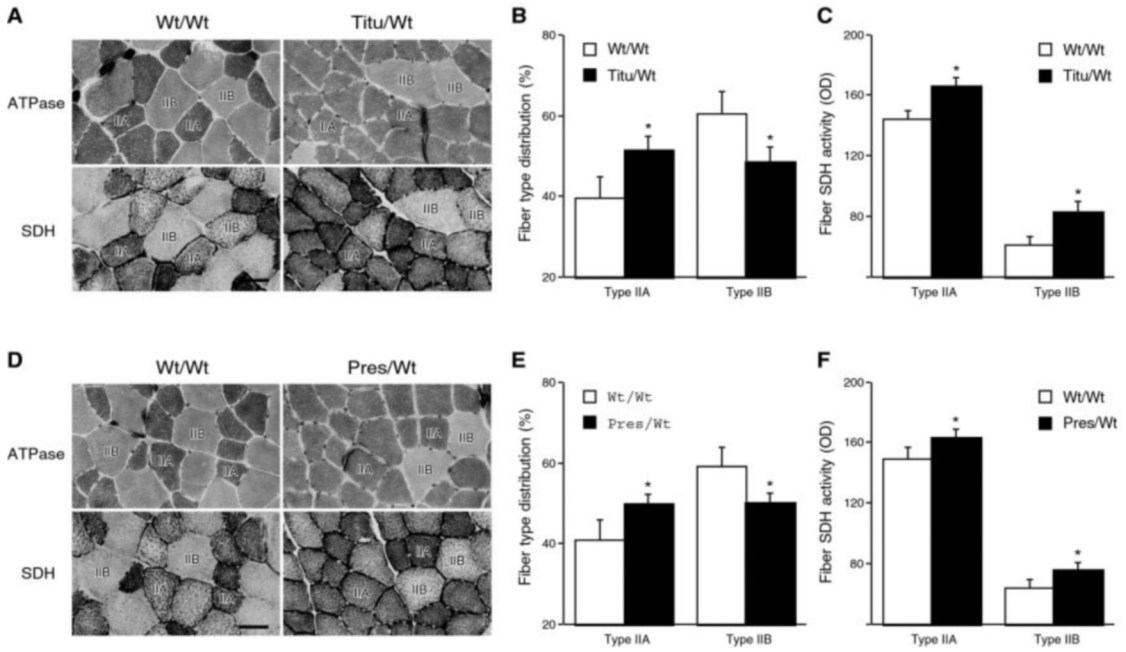

FIG. 4. **Histochemical and enzymological analyses in muscles of mutant mice.** Transverse sections of the deep region in tibialis anterior muscle of Titusville (*A*) and Presbyterian (*D*) mice stained for ATPase activity on preincubation at pH 4.5 (*upper*) and for succinate dehydrogenase (*lower*) activity. *IIA*, type IIA fiber; *IIB*, type IIB fiber. *Scale bars* indicate 50 μm. Shown are fiber type distributions (*B* and *E*) and succinate dehydrogenase activities (*C* and *F*) in tibialis anterior muscle of wild-type and Titusville or Presbyterian mice. Significant differences between wild-type and Titusville or Presbyterian mice are shown (*, $p < 0.001$; unpaired Student's *t* test). Data are means ± S.D. ($n = 5$). *, $p < 0.001$.

not shown). Metabolism and respiration were then assessed in these sisters with a bicycle ergometer. During the exercise, the Presbyterian individual showed a depressed ventilatory response with a respiratory ratio below 1.0 throughout the test whereas the control sister showed a normal ventilatory response (data not shown). Blood gas analysis on moderate exercise (100 watts) showed a severe respiratory acidosis in the younger sister, suggesting that the Presbyterian individual failed to compensate for the metabolic acidosis by inducing ventilation; instead, the depressed ventilation exacerbated the metabolic acidosis (Fig. 6*D*). The dysregulation of ventilatory response on exercise observed in the Presbyterian individual is consistent with the impaired acid-base balance observed in Presbyterian mice (Table II), suggesting a common mechanism

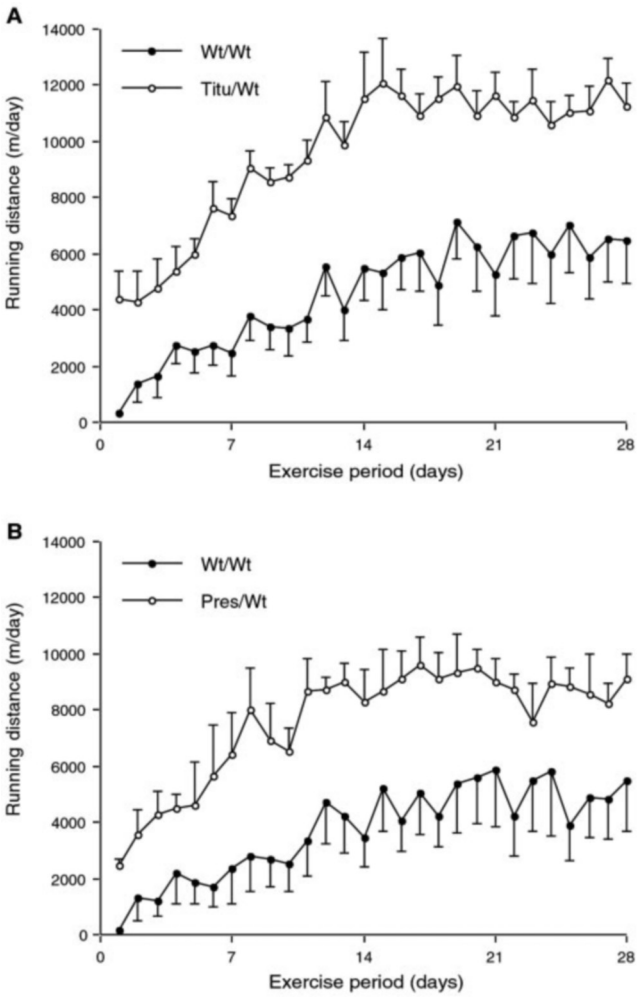

FIG. 5. **Physical activity of mutant mice on the running-wheel apparatus.** Daily running distances of Titusville (*A*) and Presbyterian (*B*) mice during 28 days of exercise on the running wheel apparatus. Data are means ± S.D. ($n = 5$).

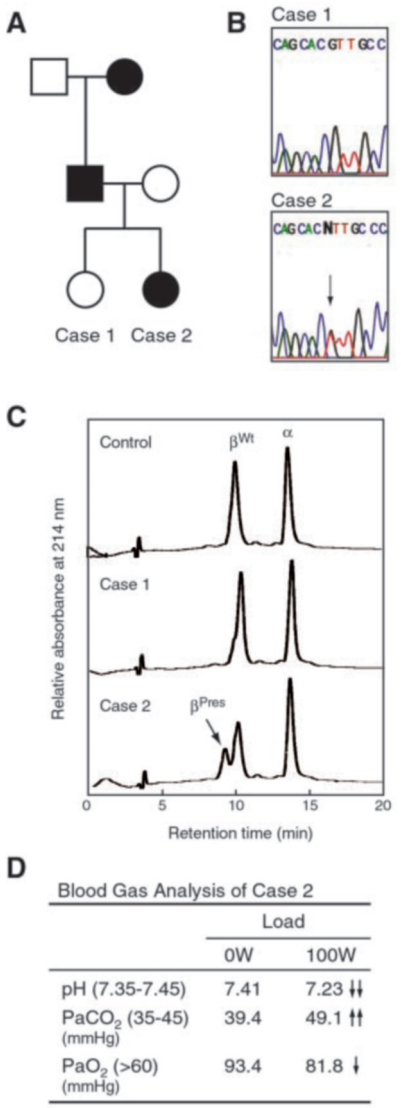

FIG. 6. **Characterization of a patient with Presbyterian Hb.** *A*, a pedigree of Presbyterian Hb. *B*, the mutation (N108K) in the β-globin gene was confirmed by DNA sequencing in case 2. *C*, RP-HPLC profiles of hemolysate prepared from a normal individual (*top*), case 1 (*middle*), and case 2 (*bottom*). The peaks of β^{Pres}-globin, β^{Wt}-globin, and α-globin are indicated in the profiles. The peak of human β^{Pres}-globin was eluted earlier than the peak of β^{Wt}-globin as shown in the profile of Presbyterian mice (Fig. 1*G*). *D*, blood gas analyses for case 2 in a graded exercise test.

of ventilatory dysregulation by the Presbyterian mutation in the β-globin gene between humans and mice.

DISCUSSION

Titusville and Presbyterian Mice May be Gain-of-function Mutations in Mouse and Human—Individuals who carry variant Hbs with low O_2 affinity such as Hb Titusville, Hb Presbyterian, and Hb Kansas (4–9, 22–24) manifest asymptomatic anemia, irrespective of the mutations, whereas individuals who carry Hbs with high O_2 affinity such as Hb Malmo (10, 13) and Hb Yakina (11, 12) generally show polycythemia. It is therefore speculated that Hbs with low oxygen affinity can dissociate more O_2 in the peripheral tissues whereas the other variant Hbs proceed with normal gas exchange in the lung. To test this hypothesis, in the present study, we generated mice carrying mutant Hbs with low O_2 affinity, Hb Titusville as a mutant of α-globin, and Hb Presbyterian as a mutant of β-globin. Using these models, we addressed whether low affinity Hb actually releases more O_2 in the tissue *in vivo* and investigated the various physiological advantages attributed to mutant Hbs *in vivo*. We surprisingly found that Titusville mice, as well as Presbyterian mice, showed enhanced tissue oxygenation, increased O_2 consumption in tissues, and an increased running capacity and propensity that resulted in altered behavior with greater physical activities.

From an evolutional point, the primary structure of Hb is closely associated with the life and behavior of animals. For example, crocodiles and alligators can hold their breath under water for 30 min, because their Hb has an allosteric effect on bicarbonates produced in the tissues (26). Thus, it is intriguing that Titusville and Presbyterian mutations enable mice to run twice as long as wild-type mice. Because running is a vital form of mouse behavior, the increased running ability of mutant mice is obviously a gain-of-function phenotype in the context of animal evolution. It is also noteworthy that this phenotype may be conserved in mouse and human, although they only share 80% amino acid sequence homology in the β-globin locus. Because neither of these gain-of-function mutations (α94Asp → Asn and β108 Asn → Lys) has been accumulated in the genome of mouse or human as a dominant allele, an as-yet unidentified deleterious effect may exist that prevents the mutation from prevailing in the genome.

Titusville and Presbyterian Mice May Compensate for Tissue Hyperoxia through Anemia, Increased Tissue O_2 Consumption,

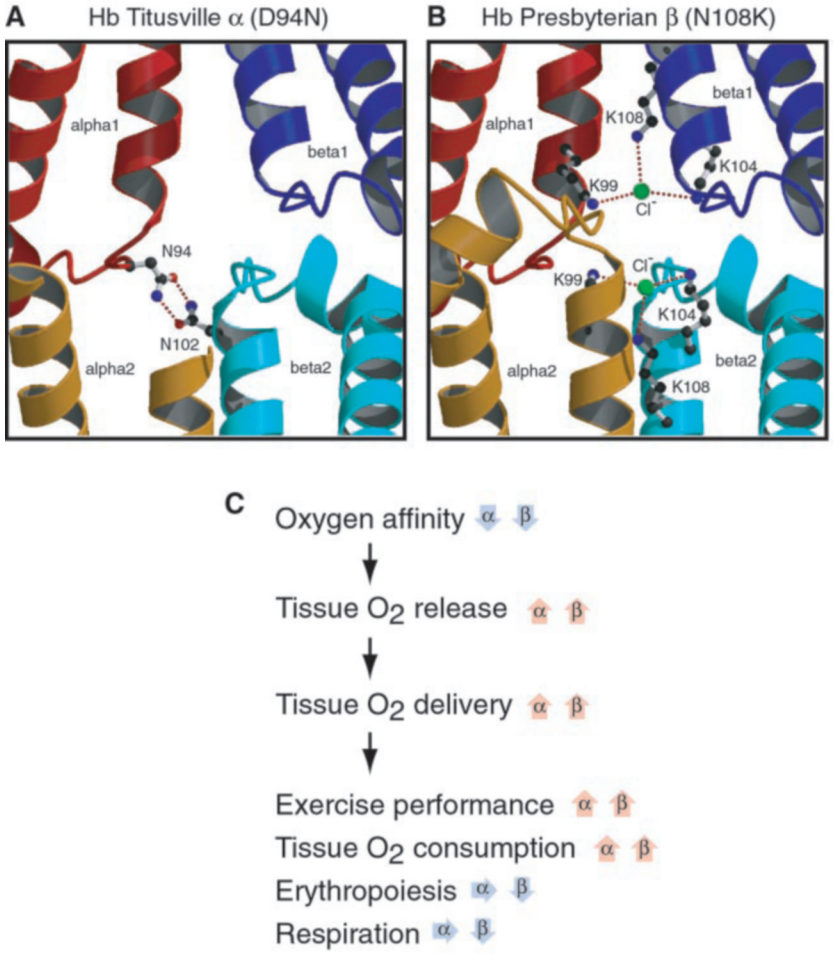

FIG. 7. **Molecular physiology of low affinity Hbs.** *A*, molecular modeling of Hb Titusville α(D94N); *B*, molecular modeling of Hb Presbyterian β(N108K); *C*, physiological implications of low oxygen affinity Hbs.

and Increased Spontaneous Exercise—This is the first report that Hb determines or controls the basal level of erythropoiesis, tissue O_2 consumption, physical activity, and behavior. Although we could not explain all abnormal phenotypes of Titusville and Presbyterian mice at the molecular level, it is obvious that the initial event is an introduced mutation that modulates the affinity of Hb for O_2 as shown in Fig. 7. In Titusville mice, the introduced Asn residue locates at the interface of the $\alpha1\beta2$ subunit as shown in Fig. 7*A*, causing the subunit to be stabilized in a deoxy state. In Presbyterian mice, however, the introduced Lysine residue protrudes into the central cavity to bind metabolites such as a phosphate or a chloride ion as illustrated in Fig. 7*B*, generating a novel allosteric effect that favors the deoxy state. Tissue oxygenation, *i.e.* the supply of oxygen to tissues, is an essential biological reaction on which every animal cell, tissue, and organ is energetically based. Therefore, tissue hypoxia, the lack of oxygen, is the most dangerous insult for an animal and has been investigated extensively in laboratory animals and *in vitro* studies. Tissue hyperoxia, however, has yet to be studied extensively, because no relevant animal model has been available. We presented here the first relevant animal model for the study of tissue hyperoxia.

The primary function of mitochondria is ATP production in the use of oxygen. In this context, the cell depends on mitochondria to generate energy, but at the same time, mitochondria play a biological role in the reduction of oxygen inside the cell. It is thus important to control the redox state in various organelles including mitochondria, because disruption of the cellular redox state can often result in apoptosis in animal cells (27). From this viewpoint, another important function of mitochondria is to regulate the cellular oxygen concentration by producing ATP or heat (28). It is then interesting that SDH activity is up-regulated in both IIA and IIB type fibers of Titusville and Presbyterian mice, suggesting that the primary sequence of alteration in muscle may be the compensatory reaction for the increased consumption of excess oxygen delivered by mutant Hbs.

It is difficult to judge whether the mutant mice run twice as far to consume more oxygen in the muscle or voluntarily as a result of altered behavior. Because the Titusville and Presbyterian mutations may influence the development of the brain after birth, the propensity to run spontaneously may be attributed to the altered behavioral pattern caused by the mutations. Alternatively, an unidentified signal sensing the cellular redox state, tissue oxygenating state, or hyperoxic state in the peripheral tissues may trigger the central nervous system to partake more actively in running than is the case for wild-type mice.

It is also difficult to clarify the molecular mechanisms down-regulating the ventilation in Presbyterian mice. Hypoxia positively drives the ventilation by neuronal signaling via the carotid body (29), whereas hyperoxia may negatively regulate the respiratory center in the central nervous system. Interestingly, the individual with Presbyterian Hb (*case 2* in Fig. 6) showed an impaired hyperoxic suppression by the carotid body,[2] indicating the impaired regulatory mechanism in Presbyterian mice. It is also noteworthy that down-regulation of erythropoiesis is one strategy to compensate for tissue hyperoxia in Pres-

[2] M. Tamaki, M. Izumizaki, Y. Suzuki, T. Shimizu, J. Suzuki, M. Nakamura, K. Ueshima, H. Inoue, M. Iwase, T. Kuriyama, I. Homma, and T. Shirasawa, manuscript in preparation.

byterian mice. Because the amount of hemoglobin contained in the peripheral blood directly correlates with the efficiency of O_2 transport in tissues, one of the determinants of the hemoglobin concentration may be O_2 delivered in the tissues as suggested in this study.

Perspective of Clinical Applications of Presbyterian Hb—Recombinant human Presbyterian Hb has been developed as a blood substitute (25, 30). In the present study, we investigated the physiological advantages of Titusville Hb or Presbyterian Hb *in vivo*, demonstrating that in these mutant mice more oxygen is released under hypoxic conditions. Patients with chronic respiratory failure because of lung diseases show tissue hypoxia. However, O_2 therapy largely restricts a patient's daily life. Titusville Hb or Presbyterian Hb can release more oxygen in the peripheral tissues under hypoxic conditions, suggesting that recombinant Hbs or erythrocytes containing mutant Hbs could improve the symptoms of chronic respiratory failure when transfused or introduced by gene therapy.

Moreover, mutant Hb releases more oxygen in anemic conditions, suggesting that recombinant Hb would also benefit ischemic heart diseases or ischemic cerebrovascular disorders. A synthetic allosteric modifier such as RSR13 that induces a rightward shift in hemoglobin improved cardiac metabolism under ischemic cardiac conditions in experimental animals (31). A synthetic chemical is versatile in clinical situations, in which the temporal supply of oxygen is emergently indicated. Because the allosteric effectors of tissue metabolites such as 2,3-diphosphoglycerate were often increased in ischemic tissues, a variant Hb with a novel allosteric effect such as Presbyterian Hb may be more advantageous for chronic ischemic conditions especially associated with impaired respiratory functions. Further animal experiments should be explored to determine the clinical applications of Titusville and Presbyterian mice.

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