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 a-globin gene or Presbyterian mutation in the b-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), a protein found within erythrocytes, transports oxygen through the vertebrate bloodstream. Hb is a tetrameric protein consisting of a- and b-globin subunits that carry 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 (HbTitu) is a low affinity variant of the a-globin chain and is well characterized clinically (4, 5). Hb Presbyterian (HbPres) is another low affinity variant of b-globin chain and is well characterized in vitro (6–9). Interestingly, individuals with low oxygen affinity Hbs such as HbMalmo or HbYakima show symptoms associated with polycythemia (10–13).

In the present study, we generated mutant mice carrying an homologous mutation with Titusville (Asp$^{108}$ → Asn) at the a locus or with Presbyterian (Asn$^{108}$ → Lys) at the b-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. Thus, the knock-in a-globin or b-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 a1 globin gene knock-in mice with the Titusville mutation were obtained by replacing Asp$^{94}$ of the a1 globin gene with Asn as described below. The 129-mouse genomic library in pPIXII (Stratagene, CA) was screened with the 372-bp 5’ flanking sequence of the murine a1 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 a1 globin exons was amplified with a SpeI/II anchored primer (5’-GGG GCA CTC GTA TTA AAG TAA GTG TGT CAC GCT GTA GGG GAC CAC TCT/3’) and XhoI/II-anchored primer (5’-CTC GGA TGA TGG TGG TAC ACC CTA CCA TCA TGT CAC TGC/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 XhoI-anchored primer (5’-CCG CTC GAG TCC TTG AGC CAA AGA AGC CAA-3’) and ApaI/SalI-anchored primer (5’-TTG GGC CGG TCG ACT CTG CCC GCT GGC TGA-3’).

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Each mouse was allowed to acclimate to the chambers (fraction of inspired O2; PFO2 = 0.21) for at least 60 min before the hypoxic gas challenge, and a constant level of baseline PtiO2 was achieved. Subsequently mice were exposed to a hypoxic gas (PFO2 = 0.15) for 5 min. A gas mixture was delivered from a respiratory gas circuit consisting of flow meters for O2 and N2 and a reservoir bottle (2 liters) connected to the head chamber. PtiO2 was altered by mixing O2 and N2, being continuously monitored by withdrawing a small fraction of the gas mixture (20 ml/min) with an O2 and CO2 analyzer (Respira H216; NEC San-ei). Vc and PtiO2 were measured at 0.5, 1, 2, 3, 4, and 5 min. ΔPtiO2 (mmHg) was calculated as the difference between baseline PtiO2 and PtiO2 at each time point.

**Blood Gas Analysis**—Male wild-type (n = 5), Titusville (n = 5), and Presbyterian (n = 5) mice (ages 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 Vc and PtiO2. Arterial blood (120 μl) was sampled with a heparinized sampling glass tube (MIC0020; AVL Scientific Corporation) and immediately analyzed by a blood gas analyzer (OPTI CCA; AVL Scientific Corporation) for pH, partial pressure of arterial CO2 (PaCO2), and partial pressure of arterial O2 (PaO2). Arterial blood was sampled before and at the end of hypoxic inhalation.

The muscle fibers were classified as type IIA and type IIB (19). SDH 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 step 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 at −20 °C. The sections were washed with phosphate buffered saline (PBS) prior to being dehydrated 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. O2 consumption and CO2 production were normalized with respect to body weight per kg.
products were isolated and sequenced with a nested sense primer (Bayer medical 860).

Arterial blood gas analysis was carried out with a pH-blood gas analyzer. Wt-globin (an arrow in the middle panel). The peak of β\(^{-}\)globin was also eluted earlier than the peak of α\(^{-}\)globin (an arrow in the lower panel).

**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\(^{\beta}\) 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. 1A) or β major carrying Lys-108 (Fig. 1B), 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 d94N mutation in Titusville mice (Fig. 1C) and bN108K mutation in Presbyterian mice (Fig. 1D). 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, 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 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).
Hb Oxygen Affinity Regulates Physical Activity

The Mutant Hb Showed Low Affinity for Oxygen in Vitro—To characterize the physiochemical properties of HbTitu, HbPres, and double mutant HbTitu,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, HbPres showed a rightward shift in comparison with wild-type Hb (Fig. 2A, P50 = 43.5 versus 47.0 mmHg) whereas HbTitu and HbTitu,Pres exhibited even more extensive rightward shifts as shown in Fig. 2 (P50 = 66.0 or 72.0, respectively). Analyses of Hill’s plot and the Bohr effect, however, indicated that HbPres retained all physiochemical properties (Fig. 2, B and C) whereas HbTitu or HbTitu,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 HbPres 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. 2D).

HbPres Delivers More Oxygen to Peripheral Tissues under Moderately Hypoxic Conditions—Presbyterian mice were exposed to 15% O2 for 5 min to investigate the physiochemical effects of HbPres on tissue hypoxia in vivo. ΔPtO2 and Ve values during hypoxia are shown in Fig. 3, A and B, respectively. After 5 min of hypoxia, the tissue O2 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 O2 to wild-type mice within 1 min of hypoxia whereas tissue O2 started to dissociate in the hypoxic phase that followed (Fig. 3A). 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 HbPres in vitro is greatest at a PaO2 concentration of ~50 mmHg as shown in Fig. 2A. Therefore, it is reasonable that the beneficial effect of HbPres is more remarkable in vivo in advanced tissue hypoxia as shown in Fig. 3A.

We simultaneously monitored Ve to elucidate whether the increased tissue oxygenation was attributable to the increased ventilation of Presbyterian mice or an efficient O2 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. 3B). The data clearly suggested that in Presbyterian mice, more oxygen was delivered to tissues not by a ventilatory increase but by increased O2 delivery by HbPres.

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, PaCO2, and PaO2 level of arterial blood in Titusville and Presbyterian mice (Table II). Titusville mice showed a normal PaO2, PaCO2, and pH in room air and during hypoxia whereas Presbyterian mice showed a low pH associated with an elevated PaCO2 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 PaO2 level (Table II). Given the normal respiratory functions and reduced ventilation (Ve) (Fig. 3B), the primary cause of elevated PaCO2 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 PaCO2 by reducing the ventilation. It is still surprising that Presbyterian mice consumed more O2 and produced more CO2 in room air, as well as during hypoxia (see below), despite the fact that they manifest signs of hypoventilation (Fig. 3B) and mild anemia (Table I). Given the fact that a human with Presbyterian Hb also showed a lower pH and higher PaCO2 level on exercising (see Fig. 6D) 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 hypoxia caused by HbPres by reducing ventilation. Alternatively, HbPres may modulate the respiratory center, especially the ventilatory response to CO2 in the brain of mutant mice, in a Presbyterian-specific manner.

Titusville and Presbyterian Mice Consume More O2 and Produce More CO2—To investigate whether enhanced tissue oxygenation alters the basic metabolism of mutant mice, we measured metabolic parameters such as O2 consumption, CO2 production, and the respiratory ratio in Titusville and Presbyterian mice (Table III). The metabolic analyses showed that both mutant mice consumed more O2 and produced more CO2 in room air and hypoxic conditions (Table III), implying that the low affinity of mutant Hbs drives the mice to consume more O2 to exclude the excess tissue O2 delivered by mutant Hbs. The increase in O2 consumption may then lead to the increased production of CO2 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
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 to 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 HbTitu and HbPres 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 (βPres-globin) was specifically detected in the hemolysate from case 2 but not detected in case 1 (Fig. 6C). This mutant peak in HPLC was detected in the hemolysate from the subject’s father who carries HbPres (data
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. 6D). 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.

### 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               | 7.42 ± 0.01 | 7.42 ± 0.03 | 7.42 ± 0.00 | 7.34 ± 0.02* |
| Room air         | 7.45 ± 0.03 | 7.46 ± 0.03 | 7.44 ± 0.01 | 7.40 ± 0.01* |
| PaCO₂ (mmHg)     | 44.0 ± 0.9  | 41.9 ± 4.4  | 39.8 ± 0.6  | 47.2 ± 1.7*  |
| Hypoxia          | 39.6 ± 6.6  | 37.5 ± 5.6  | 39.6 ± 0.1  | 44.2 ± 0.6*  |
| 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   |

* p < 0.01.

**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 |
|------------------|-----------|------------|-----------|--------------|
| CO₂ production (ml/min/kg) | 26.0 ± 1.2 | 35.1 ± 1.0* | 23.3 ± 1.2 | 32.3 ± 1.8*  |
| Room air         | 15.8 ± 0.8 | 20.2 ± 1.6* | 16.9 ± 0.4 | 20.5 ± 1.0*  |
| Hypoxia          | 34.7 ± 2.0 | 42.2 ± 2.2* | 30.8 ± 1.5 | 40.0 ± 2.6*  |
| O₂ consumption (ml/min/kg) | 24.7 ± 1.2 | 29.8 ± 2.3* | 26.3 ± 1.1 | 30.8 ± 1.1*  |
| Room air         | 0.75 ± 0.02 | 0.84 ± 0.03* | 0.76 ± 0.01 | 0.81 ± 0.02* |
| Hypoxia          | 0.64 ± 0.01 | 0.68 ± 0.01 | 0.64 ± 0.02 | 0.66 ± 0.01  |

* p < 0.01.

**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.
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 O₂ 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 O₂ 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 O₂ 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 O₂ affinity, Hb Titusville as a mutant of \( H^\beta_9251 \)-globin, and Hb Presbyterian as a mutant of \( H^\beta_9252 \)-globin. Using these models, we addressed whether low affinity Hb actually releases more O₂ 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 O₂ consumption in tissues, and an increased running capacity and propensity that resulted in altered behavior with greater physical activities.

From an evolutionary 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 \( H^\beta_9252 \)-globin locus. Because neither of these gain-of-function mutations (\( \beta^94 \text{Asp} \rightarrow \text{Asn} \) and \( \beta^108 \text{Asn} \rightarrow \text{Lys} \)) 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 O₂ Consumption,**

![Fig. 5. Physical activity of mutant mice on the running-wheel apparatus.](image1)

**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.](image2)

**Fig. 6.** Characterization of a patient with Presbyterian Hb. A, a pedigree of Presbyterian Hb; B, the mutation (N108K) in the \( \beta \)-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 \( \beta^\text{Pres} \)-globin, \( \beta^\text{Wt} \)-globin, and \( \alpha \)-globin are indicated in the profiles. The peak of human \( \beta^\text{Pres} \)-globin was eluted earlier than the peak of \( \beta^\text{Wt} \)-globin as shown in the profile of Presbyterian mice (Fig. 1G); D, blood gas analyses for case 2 in a graded exercise test.

| Load       | 0W  | 100W |
|------------|-----|------|
| pH (7.35–7.45) | 7.41 | 7.25 |
| PaCO₂ (mmHg)  | 49.4 | 49.1 |
| PaO₂ (mM)     | 93.4 | 81.8 |
and Increased Spontaneous Exercise—This is the first report that Hb determines or controls the basal level of erythropoiesis, tissue O₂ 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 O₂ as shown in Fig. 7. In Titusville mice, the introduced Asn residue locates at the interface of the α1β2 subunit as shown in Fig. 7A, 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. 7B, 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, 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-

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.
byterian mice. Because the amount of hemoglobin contained in the peripheral blood directly correlates with the efficiency of O2 transport in tissues, one of the determinants of the hemoglobin concentration can be O2 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, O2 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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