Decrease in the red cell cofactor 2,3-diphosphoglycerate increases hemoglobin oxygen affinity in the hibernating brown bear *Ursus arctos*

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Revsbech IG, Malte H, Fröbert O, Evans A, Blanc S, Josefsson J, Fago A. Decrease in the red cell cofactor 2,3-diphosphoglycerate increases hemoglobin oxygen affinity in the hibernating brown bear *Ursus arctos*. *Am J Physiol Regul Integr Comp Physiol* 304: R43–R49, 2013. First published November 21, 2012; doi:10.1152/ajpregu.00440.2012.—During winter hibernation, brown bears (*Ursus arctos*) reduce basal O2 consumption rate to ~25% compared with the active state, while body temperature decreases moderately (to ~30°C), suggesting a temperature-independent component in their metabolic depression. To establish whether changes in O2 consumption during hibernation correlate with changes in blood O2 affinity, we took blood samples from the same six individuals of hibernating and nonhibernating free-ranging bears during winter and summer, respectively. A single hemoglobin (Hb) component was detected in all samples, indicating no switch in Hb synthesis. O2-binding curves measured on red blood cell lysates at 30°C and 37°C showed a less temperature-sensitive O2 affinity than in other vertebrates. Furthermore, hemolysates from hibernating bears consistently showed lower cooperativity and higher O2 affinity than their summer counterparts, regardless of the temperature. We found that this increase in O2 affinity was associated with a significant decrease in the red cell Hb-cofactor, 2,3-diphosphoglycerate (DPG) during hibernation to approximately half of the summer value. Experiments performed on purified Hb, to which DPG had been added to match summer and winter levels, confirmed that the low DPG content was the cause of the left shift in the Hb-O2 equilibrium curve during hibernation. Levels of plasma lactate indicated that glycolysis is not upregulated during hibernation and that metabolism is essentially aerobic. Calculations show that the increase in Hb-O2 affinity and decrease in cooperativity resulting from decreased red cell DPG may be crucial in maintaining a fairly constant tissue oxygen tension during hibernation in vivo.

A DISTINCT TRAIT OF MAMMALIAN hibernation is the highly controlled decrease in body temperature and metabolic rate. A hibernating brown bear (*Ursus arctos*) routinely spends 5–7 mo per year in continuous dormancy with no food or water intake, no urination, and no defecation (19, 36). During this time, bears appear to be resistant to loss of muscle mass, strength, or bone density (18, 24, 29, 43, 14, 44). During hibernation bear body temperature is downregulated only slightly, fluctuating from ~37°C to a minimum of 30°C, as found in brown and black bears (*Ursus americanus*) (17, 26, 27, 36, 43), whereas O2 consumption rate is downregulated by 75% (43). In comparison, in most smaller hibernators, such as ground squirrels and marmots, body temperature drops dramatically to values close to ambient temperatures (32, 37, 48), with a consequent strong Q10-induced depression of metabolic rate (where Q10 is the rate coefficient for a 10°C change in temperature). In spite of substantial downregulation of ventilation and heart rate, most hibernators likely experience only slight or no hypoxia and in some ground squirrels arterial O2 tension (P02) is normal during torpor (16). As opposed to smaller hibernators that exhibit periods of spontaneous arousals back to normothermic temperature and metabolic rate (33, 31), bears do not arouse to normothermic temperature during winter but have shallow multiday cyclic (1.6–7.3 days) fluctuations in body temperature (43). Nevertheless, as demonstrated in a recent study (43), bears exhibit a strong active aerobic metabolic depression and a weight-specific metabolic rate similar to that of smaller hibernators (20, 25, 43), and for this reason, they are now recognized as true hibernators, even though they do not show the dramatic drop in body temperature and arousals typical of small hibernators (43). Whether and how the blood O2 transport of bears adapts to a decreased O2 supply to tissues during hibernation is however, still unknown.

Earlier studies have found that in small hibernating mammals, blood O2 affinity increases markedly during hibernation (4, 9, 32). Potentially, blood O2 affinity can be affected in two ways: by structural changes in the blood O2 carrier hemoglobin (Hb) that lead to changes in the protein sensitivity to allosteric cofactors and temperature, or by changes in the concentration of allosteric cofactors inside the red blood cell. Such allosteric cofactors include organic phosphates that bind to the central cavity of the Hb tetramer and decrease O2 affinity by shifting the allosteric T-R equilibrium between the low-affinity (T) and the high-affinity (R) protein conformation toward the T state. In most mammalian Hbs, the main anionic cofactors are the organic phosphate 2,3-diphosphoglycerate (DPG) and Cl⁻, that in bear Hb have a large synergistic effect in regulating O2 binding (11). DPG binds allosterically to the low-affinity T-state conformation of mammalian Hbs and, thereby, decreases Hb-O2 affinity (5). An increase in erythrocytic DPG decreases Hb-O2 affinity, and, conversely, a decrease in DPG increases Hb-O2 affinity.

An increase in blood O2 affinity has been observed in several small hibernating animals during dormancy (4, 34), but has not yet been measured in bears. Although the molecular mechanisms for a hibernation-induced increase in blood O2 affinity have not been much investigated, reductions in DPG levels...
have been found to be involved in the hedgehog (28) and possibly in some hibernating rodents (23, 32).

Here, we report $O_2$ binding curves of red blood cell hemolysates and purified Hb from free-ranging radio-collared brown bears during summer activity and winter hibernation. Curves were measured at temperatures close to the lowest measured body temperature of hibernating bears and the normothermic temperature of nonhibernating bears, 30°C and 37°C, respectively (26, 43), to take into account the effect of temperature on Hb oxygenation. We also examined Hb multiplicity, concentration levels of the allosteric cofactor DPG present in the red cells, and of plasma lactate to evaluate possible differences in glycolytic activity for hibernating and nonhibernating bears.

MATERIALS AND METHODS

Blood sample collection and preparation. Samples of blood were taken from the same six free-ranging 2- to 3-yr-old Eurasian brown bears, Ursus arctos, three females and three males captured during winter hibernation (February: females 35, 57, and 59 kg; males 21, 25 and 58 kg) and summer (June: females 28, 72, and 47 kg; males 27, 51, and 22 kg) in Dalarna county, Sweden, as described previously (17). The bears were immobilized by darting in the den during February 2011 and again by darting from a helicopter during June. Bears were anesthetized as described in detail in a previous study (17). Briefly, in winter, a mixture of tiletamine-zolazepam (1.1 mg/kg, except 2.5 mg/kg in one male bear, 25 kg), medetomidine (0.03 mg/kg) and ketamine (1.3 mg/kg, except 3 mg/kg in one male bear, 25 kg) was used, and in summer, a mixture of tiletamine-zolazepam (4.7 mg/kg) and medetomidine (0.09 mg/kg) was used. Doses were based on body mass and time of year (due to differences in expected metabolism), as previously reported (17). Blood samples were taken within ~20 min from darting. All animal handling and sampling was carried out under approval of the Swedish Ethical Committee on animal research (C212/9). The performed procedure was in compliance with Swedish laws and regulations. In the field, blood samples (~1 ml) were taken from the jugular vein of anesthetized animals into syringes containing 50 µl of 200 mM EDTA as anticoagulant. At the time of sampling, rectal temperature of the bears were 33.8 ± 1.5°C during winter sampling and 39.1 ± 1.4°C during summer sampling. Plasma pH was unchanged (7.25 ± 0.09 for winter bears and 7.25 ± 0.06 for summer bears), and slightly acid, probably due to anesthesia, as previously reported (17). Blood samples were centrifuged with a portable centrifuge in Eppendorf tubes immediately after collection to separate plasma and red blood cells (RBCs). Plasma and RBCs from each individual were immediately frozen on dry ice and shipped to Aarhus University, Denmark.

In the laboratory, samples were processed individually. Aliquots of frozen RBCs were added to 0.2 M HEPES buffer (pH 7.40) at a 1:1 volume ratio, and cell debris was removed by centrifugation (7,000 g, 5 min, 4°C). Levels of oxidized (met) Hb were negligible in all samples, as judged form the absorbance ratios at 577 and 541 nm (A577/A541 > 1).

DPG, hemoglobin, and chloride. DPG concentration was assayed spectrophotometrically in all individual hemolysates using the DPG assay kit (Roche Diagnostics, Mannheim, Germany; cat no. 10 148 334 001), where concentration of DPG in the reaction assay is stoichiometrically coupled to the decrease of NADH to NAD$^+$ that was followed at 340 nm (extinction coefficient 6.22 mM$^{-1}$ cm$^{-1}$) using a Uvikon 923 B double-beam UV/Vis spectrophotometer (Kontron Instruments, Milan, Italy) in 1-cm cuvettes. Deproteinization with 0.6 M perchloric acid and neutralization of the supernatant using 2.5 M K$_2$CO$_3$ was carried out with one tenth of the prescribed volumes. In the protocol used, 100 µl of hemolysate was added to 500 µl ice-cold 0.6 M perchloric acid, mixed and centrifuged (2,000 g, 10 min), 400 µl of the clear supernatant was then neutralized with 50 µl of ice-cold 2.5 M K$_2$CO$_3$, and left on ice for ~70 min. Samples were centrifuged again to spin down precipitate (2,000 g, 5 min, 4°C), and 100 µl of supernatant was used for the spectrophotometric assay following the instructions provided by the manufacturer. One blank served as reference for every 4–6 samples. The reaction was completed after 25 min, whereafter the final absorbance did not change noticeably. The method was validated in control experiments made with fresh human blood and frozen RBCs. These experiments yielded DPG values for human blood (4.25 ± 0.54 mmol/l RBC) equivalent to those reported by the kit manufacturer (4.83 ± 0.15 mmol/l RBC).

To determine the DPG to tetrameric Hb ratios in individual samples, Hb concentration was measured using Drabkin’s method (15, 42). Absorbance was read at 540 nm using a Uvikon 923 B Double Beam UV/Vis spectrophotometer (Kontron instruments; Milan, Italy) and the hemoglobin concentration determined from the cyanmethemoglobin extinction coefficient at 540 nm of 10.99 mM$^{-1}$ cm$^{-1}$ (heme basis) (49).

Hemoglobin multiplicity. To evaluate Hb multiplicity, individual winter and summer hemolysates were analyzed on an isoelectric focusing (IEF; pH range 3–9) polyacrylamide gels (Phastigel GE Healthcare Biosciences AB, Uppsala Sweden). A heme concentration of 200 nM in the samples diluted in milliQ water yielded visible red bands. Adult human hemolysate was used for comparison.

Oxygen binding measurements of hemolysates. $O_2$ equilibrium curves were determined at constant temperatures of 30 and 37°C (± 0.2°C). These temperatures were chosen as they are near to the lowest body temperature measured in hibernating bears and the normal temperature in summer active state, respectively (26, 36, 43). Curves were determined using a modified diffusion chamber, as described previously (40, 45) at a heme concentration of 1 mM in 0.1 M HEPES buffer, pH 7.4. A pH of 7.4 was chosen, as it is close to normal mammalian blood pH. Two HEPES buffer stock solutions (1 M) differing slightly in pH were used to achieve the same final pH of 7.4 of the sample at the two chosen temperatures. For each sample, pH was measured using a Radiometer BMS2 Mk2 microelectrode assembly coupled to a Radiometer PHM64 pH meter. In each experiment, a thin layer (~0.01 mm) of sample was equilibrated with humidified gases of varying $O_2$ tensions supplied by two cascaded Wösthoff (Bochum, Germany) gas-mixing pumps mixing pure (99.998%) $N_2$ and $O_2$ (available on request). The $O_2$ partial pressure required to achieve 50% saturation of the Hb ($P_{50}$) and cooperativity coefficient at 50% saturation ($n_{50}$) were calculated from the zero intercept and slope, respectively, of Hill plots, log$Y/[1-Y]$ vs. log$P_{O_2}$, where $Y$ is fractional saturation. Hill plots were based on at least 4 saturation steps between 0.3 and 0.7.

The temperature dependence of $O_2$ binding expressed as the apparent heat of oxygenation (kcal/mol, 1 kcal = 4.184 kJ/mol) was calculated by the van’t Hoff equation: $\Delta H = -4.57 [T/\delta [T_1 - T_2] \times \Delta \log P_{O_2}/1,000 \, \text{kcal/mol, where } T_1$ and $T_2$ are the absolute temperatures (Kelvin) and $\Delta \log P_{O_2}$ is the corresponding difference in log$P_{O_2}$ at the two temperatures. The $\Delta H$ values presented have been corrected for heat of $O_2$ in solution (~3.0 kcal/mol) (1).

Oxygen binding measurements of purified hemoglobin. Hb was purified from three winter and three summer individual hemolysates. Bear Hb was stripped from DPG by gel filtration by passing the plasma samples, Hb and frozen RBCs. These experiments yielded DPG values for human blood (4.25 ± 0.54 mmol/l RBC) equivalent to those reported by the kit manufacturer (4.83 ± 0.15 mmol/l RBC).
Applying the indicated plasma volumes, the measured lactate values in the lower end of this concentration range (0.1 to 0.5 mM lactate). Lactate was measured using 1 M NaCl. Chloride (Cl⁻) was added to the same final concentration as that measured in the hemolysates (10 mM) using a Sherwood MKII model 926S chloride analyzer (Sherwood Scientific), requiring 5 μl per assessment (measurements were done in duplicates).

Plasma lactate concentration. To assess for changes in anaerobic metabolism, concentration of lactate was determined enzymatically in the plasma of winter and summer blood samples, following a previously described method (38). This method utilizes the stoichiometric conversion of NAD⁺ and lactate to NADH and pyruvate by lactic dehydrogenase (LDH), reaction 1 in the scheme below. The reaction is kept shifted to the right by the constant removal of pyruvate, which reacts with glutamate in the presence of glutamic pyruvate transaminase (GPT), reaction 2, as shown below. Production of NADH (equivalent to the lactate present) is quantified from the increase in absorbance at 340 nm using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

\[
\text{Lactate} + \text{NAD}^+ \leftrightarrow \text{Pyruvate} + \text{NADH} + \text{H}^+ \quad (I)
\]

\[
\text{Pyruvate} + \text{glutamate} \rightarrow \text{alanine} + \alpha - \text{keto glutamate} \quad (2)
\]

The original method of Passonneau and Lowry was slightly modified. Thawed plasma (35–50 μl) was used directly for the assay (1 ml total volume) in 1-cm cuvettes. Final concentrations in the cuvette were: 50 mM 2-amino-2-methylpropanol buffer (ICN Biomedicals, Santa Ana, CA), pH 9.9; 50 mM glutamate (l-glutamic acid monosodium salt; ICN Biomedicals), pH 9.8; 3 mM NAD⁺ (NAD⁺ free acid, grade II, 98%; Roche Diagnostics); 100 μg/ml LDH (from bovine heart, type XVII; Sigma-Aldrich), 100 μg/ml GPT (Roche Diagnostics). Glutamate and NAD⁺ solutions were prepared each day and kept on ice until used. Absorbance of samples was recorded immediately after addition of GPT and again after 30-min incubation at room temperature until absorbance was stable. A blank (containing milliQ water instead of plasma) was run in parallel every 1–4 samples. A standard curve was constructed from 0.1 to 2 mM lactate (sodium l-lactate; Sigma-Aldrich). Greatest linearity was observed in the lower end of this concentration range (0.1 to 0.5 mM lactate). Applying the indicated plasma volumes, the measured lactate values were in the most sensitive range of the calibration curve.

Estimation of venous Po₂. Estimations of the venous O₂ partial pressure (PvO₂) in brown bears during activity and hibernation were carried out applying physiological data obtained in a recent study on black bears (43) that reports matching data for body weight, heart rate, and O₂ consumption during activity and hibernation in the same, undisturbed individuals, under the assumption that the respiratory and cardiovascular physiology of resting brown and black bears is overall the same. We first calculated the mixed venous O₂ concentration (CvO₂) from the Fick equation for hibernating and summer active bears:

\[
CvO_2 = CaO_2 - \frac{V_O_2}{Q} \quad (3)
\]

where CaO₂ is the arterial blood O₂ concentration, VO₂ is the O₂ consumption rate, and Q is the cardiac output (i.e., heart rate × stroke volume). At any given PO₂, O₂ concentration in either arterial or venous blood (C(O₂)) is given by the sum of the physically dissolved O₂ (BO₂, PO₂) and that bound to Hb, which, in turn, is the product of the tetrameric Hb concentration (4CHb) and the fractional O₂ saturation given by the Hill equation, as indicated below:

\[
C_{O_2} = \beta_{O_2} \cdot P_{O_2} + 4C_{Hb} \cdot \frac{P_{O_2}^n}{P_{O_2}^n + P_{50}^n} \quad (4)
\]

where n is Hill’s cooperativity coefficient (n50) and P₅₀ is the half-saturation partial pressure and BO₂ is the physical solubility of O₂ (8). We used n₅₀ and P₅₀ values measured here at 37°C and 30°C for summer and winter samples, respectively (see Table 1 under RESULTS). CaO₂ was first calculated from Eq. 4 assuming an arterial PaO₂ of 100 torr and an intracellular tetramer Hb concentration of 5 mM and using reported values of hematocrit (Hct) of 45 and 56.8% for brown bears measured during summer and winter, respectively (2). O₂ consumption rates (VO₂) of 0.30 and 0.069 ml O₂·g⁻¹·h⁻¹ and heart rates of 55 bpm and 14.4 bpm were taken from previously published values (43) for summer and winter black bears, respectively. Cardiac output was calculated from the heart rate using a stroke volume of 0.06 liter as expected for a 60-kg bear (i.e., the mean weight of the bears used in the study of 43), as described by Schmidt-Nielsen (39). After obtaining CaO₂, the CvO₂ value for summer active and hibernating bears was obtained by Eq. 3, and was then used in Eq. 4 to obtain PvO₂ values. Calculations were performed by iteration using a Mathematica script (Wolfram Research, Champaign, IL).

Statistics. Values are presented as mean ± SD. Paired t-test was used for the statistical comparison between summer and winter individual samples. Comparisons were statistically significant with P < 0.001. One comparison of PvO₂ of hemolysates at summer 37 and 30°C (Table 2) was found to be significant by the Wilcoxon signed rank test, with P = 0.031.

RESULTS

There was no indication of variation in Hb isoforms between summer and winter blood samples, as determined by IEF gels (Fig. 1). Bears expressed a single Hb with an isoelectric point similar to that of human HbA.

Oxygen binding curves of summer and winter hemolysates measured at 30°C and 37°C, and the corresponding changes in P₅₀ are shown in Fig. 2. When measured at the respective physiological temperatures, O₂ affinity of winter hemolysates measured at 30°C was significantly higher (i.e., P₅₀ was significantly lower) than that of summer hemolysates measured at 37°C (Fig. 2, Table 1). Although a significant effect of temperature on P₅₀ values was observed (Fig. 2B), the temperature change alone was not enough to provide the observed shift in the O₂ equilibrium curve (Fig. 2A), and P₅₀ values of winter and summer samples measured at identical temperatures were significantly different (Fig. 2B), suggesting changes in a soluble red cell allosteric cofactor. The cooperativity coefficient n₅₀ was also significantly lower in winter compared with summer (Table 1), regardless of the temperature of measurement, indicating a change in the overall allosteric equilibrium between T and R state. The heat of oxygenation (ΔH) was similar in summer and winter.

| Table 1. O₂ affinity (P₅₀), cooperativity coefficients (n₅₀) measured at 30°C and 37°C and derived heat of oxygenation (ΔH) in brown bear hemolysates during winter and summer |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Summer 37°C     | Summer 30°C     | Winter 37°C     | Winter 30°C     |
| P₅₀, torr                        | 15.4 ± 0.6      | 10.0 ± 0.4      | 11.4 ± 0.8      | 7.3 ± 0.6*      |
| n₅₀                              | 2.4 ± 0.1       | 2.4 ± 0.1       | 1.9 ± 0.2*      | 1.7 ± 0.2*      |
| ΔH, kcal/mol                     | −8.5 ± 0.8      | −8.7 ± 1.5      | −8.7 ± 1.5      | −8.7 ± 1.5      |

*Significant differences (P < 0.001) from summer values (means ± SD); n = 6.

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winter samples, with values of $-8.5 \pm 0.8$ kcal/mol and $-8.7 \pm 1.5$ kcal/mol, respectively.

The red cell hemolysate DPG concentration was significantly lower in winter compared with summer hemolysates (Table 2). O$_2$ equilibria are largely affected by DPG to Hb tetramer ratios rather than by DPG concentrations alone. In the winter samples this ratio was $1:1$, whereas in the summer samples it increased significantly to $\sim 2:1$. Plasma lactate did not vary significantly between winter and summer (Table 2).

To evaluate whether the observed left shift in the O$_2$ equilibrium curve found for the hemolysate of hibernating bears (Fig. 2) was due to a decrease in DPG, we removed endogenous DPG from the hemolysate from three bears, added exogenous DPG to the same DPG:Hb tetramer ratio as in the untreated hemolysate (i.e., 2:1 for summer samples and 1:1 for winter samples) and measured O$_2$ equilibria at the two temperatures. Cl$^-$ was added to the same final concentration as measured in the untreated hemolysates ($10$ mM Cl$^-$). The $P_{50}$ and $n_{50}$ values (means $\pm$ SD, $n = 3$) obtained in these samples were for winter bears $6.6 \pm 0.5$ torr, and $1.8 \pm 0.04$ (30°C), $9.5 \pm 0.9$ torr and $1.7 \pm 0.02$ (37°C), respectively. For summer bears, the same parameters were $9.2 \pm 0.2$ torr and $2.0 \pm 0.18$ (30°C), $12.6 \pm 0.2$ torr, and $2.0 \pm 0.09$ (37°C), respectively.

As shown in Fig. 3, the change in $P_{50}$ between summer and winter samples obtained with purified Hb added to DPG was not significantly different from that obtained with the untreated RBC lysates, at both temperatures (Fig. 3), demonstrating that the change in DPG concentration was responsible for the left-shifted O$_2$ equilibrium curves of hibernating bears reported in Fig. 2.

When assuming similar heart rates and O$_2$ consumption as in undisturbed hibernating and active black bears (43), the O$_2$ tension of mixed venous blood (that approximates that existing in tissues) in hibernating and active brown bears can be

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**Table 2. DPG concentrations and DPG to hemoglobin tetramer molar ratios in red blood cells and plasma lactate concentrations in brown bear hemolysates during summer and winter**

<table>
<thead>
<tr>
<th></th>
<th>RBC DPG, mM</th>
<th>[DPG]/[Hb$\alpha$]</th>
<th>Plasma Lactate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer</td>
<td>4.41 ± 0.50</td>
<td>2.12 ± 0.39</td>
<td>5.81 ± 2.77</td>
</tr>
<tr>
<td>Winter</td>
<td>2.37 ± 0.39*</td>
<td>0.99 ± 0.21*</td>
<td>3.05 ± 1.32</td>
</tr>
</tbody>
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*Significant differences ($P < 0.001$) from summer values (means $\pm$ SD); $n = 6$. RBC, red blood cells; DPG, 2,3-diphosphoglycerate.

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**Fig. 1.** Representative isoelectric focusing gel performed on three individual brown bear summer and winter hemolysates, indicating the presence of a single hemoglobin isoform as in human hemolysate. Identical results were obtained with the remaining individual bear hemolysates (not shown).

**Fig. 2.** A: representative Hb-O$_2$ binding curves from winter and summer hemolysates of one individual brown bear measured at 30°C and 37°C. B: $P_{50}$ values (means $\pm$ SD) for summer and winter hemolysates at 30°C and 37°C ($n = 6$). *Significant differences ($P < 0.001$) in $P_{50}$ between 37°C and 30°C. **Significant differences ($P < 0.001$) between summer and winter. One comparison of $P_{50}$ values (summer 37°C and 30°C, left-hand column) was significant by Wilcoxon signed rank test ($P = 0.031$). Conditions were 1 mM heme, 0.1 M HEPES buffer, pH 7.4.

**Fig. 3.** Changes in $P_{50}$ (torr) for brown bear winter and summer hemolysates ($n = 6$) and for samples of purified Hbs containing the same DPG/Hb tetramer molar ratios ($n = 3$) as in summer and winter hemolysates (Table 2), measured at 30°C and 37°C. NS, not significant.
predicted from the Fick equation when knowing values for $P_{50}$, cooperativity coefficients $n_{50}$ and blood Hb concentration, as described in detail under MATERIALS AND METHODS. Figure 4 shows the predicted $O_2$ equilibrium curves for winter and summer animals along with the estimated arterial and venous $O_2$ saturation and respective $P_{O_2}$ values. In the calculations, $P_{50}$ and $n_{50}$ values were those measured at the physiological temperatures of 37°C and 30°C for summer and winter samples, respectively (Table 1). As evident from Fig. 4, winter $O_2$ content of the blood is considerably elevated because of the increase in Hct occurring during hibernation (2). If the $O_2$ binding curve had remained unchanged during winter (dotted line, Fig. 4), venous $P_{O_2}$ would have been substantially elevated ($\sim 21.8$ torr). However, a left-shift in the Hb-$O_2$ binding curve may avoid this situation and maintain $P_{VO_2}$ of hibernating brown bears relatively unchanged (summer $\sim 14.8$ and winter $\sim 11.5$ torr) (Fig. 4).

DISCUSSION

In this study of free-ranging brown bears, we found a marked left-shift of the $O_2$ equilibrium curve during hibernation, which was associated with an increase in the Hb-$O_2$ affinity and a decrease in cooperativity. We demonstrated that the differences between hibernation and active state can be explained by a decrease in the red cell cofactor DPG, a major allosteric cofactor of Hb, whereas the reduction in body temperature during hibernation alone cannot account for the observed shift in Hb oxygenation. In concert with decreased ventilation and heart rate, a left-shifted $O_2$ equilibrium curve would maintain the necessary $O_2$ supply to tissues under conditions of a prolonged depressed $O_2$ consumption rate, as during hibernation. Such long-term effects are typically mediated by changes in the levels of organic phosphates, from high-altitude mammals (46) to hypoxic fish (47). A similar decrease in DPG levels as found here has been demonstrated in other hibernating mammals, such as the 13-lined ground squirrel, the golden-mantled ground squirrel, the woodchuck, and the hedgehog (7, 23, 28). However, only in the hedgehog has the DPG decrease been demonstrated to be directly coupled with the increase in blood $O_2$ affinity during hibernation (28). By contrast, high $O_2$ consumption rates, as during intense muscle exercise, are typically associated with a transient right shift of the $O_2$ binding curve, as, for example, due to the Bohr effect. In this study, we found that during hibernation, a depressed $O_2$ consumption rate was not compensated for by increased glycolysis, as plasma lactate levels did not increase in winter samples, in agreement with previous measurements (17). Levels of plasma lactate found in winter ($3.05 \pm 1.32$ mM) were comparable to normal human values (22, 30, 41). The higher variation of plasma lactate concentration in the summer samples may reflect a more variable activity of the individual bears at the time of sampling (Table 2). This further confirms that energy metabolism in hibernating bears is essentially aerobic, as also indicated by the use of fatty acids as the primary energy fuel (35, 36).

When in the presence of anionic allosteric effectors, the Hb from brown bear, similar to that from other polar and cold-tolerant mammals, shows a reduced temperature sensitivity (i.e., a less negative $\Delta H$) compared with other mammals, including humans (3, 11, 12, 21), a feature that has been attributed to the presence of an additional allosteric $Cl^-$ binding site on the $\beta$ subunit between residues Lys8$\beta$ and Lys76$\beta$ (11, 12). Limited effect of temperature on Hb-$O_2$ binding facilitates $O_2$ delivery to poorly insulated body parts, including cold extremities in contact with ice, a feature that has been interpreted as an energy-saving adaptive mechanism (10, 13). In bears, the comparatively small decrease in body temperature during hibernation would then cause a slight (albeit significant, Fig. 2B) effect on the $O_2$ binding curve, as shown here by us and earlier by others (11). The calculated heat of oxygenation ($\Delta H$) values ($-8.5 \pm 0.8$ summer and $-8.7 \pm 1.5$ kcal/mol winter) were consistent with previous $\Delta H$ data reported for bear Hb, with values ranging from $-7$ to $-8.5$ kcal/mol (6, 11). Temperature sensitivity of $O_2$ binding to Hb was overall similar in summer and winter hemolysates, indicating that a fall in body temperature alone is necessary but not sufficient for the decrease in $P_{50}$ of hibernating bear hemolysates. We found that a significant part of the observed shift of the $O_2$ equilibrium curve in hibernating bears is due to a decrease in red blood cell DPG levels (Figs. 2 and 3). No indication of switch in Hb expression with synthesis of new isoHb components with a higher $O_2$ affinity was found in the brown bears (Fig. 1).

By binding to a specific site (including His2$\beta$, Lys82$\beta$, and His143$\beta$ in bear Hbs) in the central cavity of the T-state conformation, DPG is a potent allosteric cofactor of brown bear Hb acting synergistically with $Cl^-$ ions (11). The overall effect of a DPG decrease is an increase in the Hb-$O_2$ affinity and a decrease in cooperativity. These effects are explained by a destabilized T state due to a decrease in the DPG to Hb tetramer ratio, with the consequent shift of the T-R equilibrium toward the high-affinity R state. We demonstrated that DPG is the allosteric cofactor responsible for the affinity changes, as these were reproducible when purified Hb and DPG were mixed in exactly the same ratios as found in summer and winter samples. In addition, higher DPG to Hb

Fig. 4. Predicted hemolysate $O_2$ equilibrium curves of summer and winter brown bears with the estimated arterial and mixed venous point plotted on the curves (●). Curves are calculated from the Fick’s equation using mean values of $P_{50}$ and $n_{50}$ measured in summer hemolysates at 37°C ($P_{50} = 15.4$ torr, $n_{50} = 2.4$) and winter hemolysates at 30°C ($P_{50} = 7.3$ torr, $n_{50} = 1.7$) (Table 1), using summer and winter Hcts of 45% and 56.8%, respectively (2). Dotted line indicates the theoretical curve having high winter Hct (56.8%) and $P_{50}$ and $n_{50}$ values of summer hemolysate, thus causing an increased $P_{VO_2}$ (∫). Other details are described in the MATERIALS AND METHODS section.
tetrameter ratios in summer bears (~2:1) than in humans (~1:1) may indicate some differences in the regulation of DPG as a red blood cell metabolite.

Calculations show that the hibernation-associated elevation in blood O2 affinity and decrease in cooperativity of O2 binding may be crucial for maintaining mixed venous PO2 at a physiologically reasonable level in spite of the strong elevation of Hct (2) (Fig. 4). In the case of an unaltered O2 binding curve, the high Hct would have elevated winter PV02 substantially (Fig. 4) and cause a potentially detrimental increase in dissolved O2. The same conclusion is reached when applying physiological data from hibernating and active brown bears under anesthesia (17), although the sedative and disturbance-induced elevation, particularly in the heart rate, poses limitations to their use. Interestingly, P50 values calculated from the whole blood saturation data of anesthetized winter and summer bears (19.7 and 32.1 torr, respectively) (17), also show a similar left-shifted O2 binding curve during hibernation and confirm the results here obtained on diluted hemolysates.

In black bears during and after hibernation, evidence has been presented that changes in body temperature and metabolic rate expressed as rate of O2 consumption are to a certain extent independent from each other (43). Bears emerging from their dens show a rapid recovery of their body temperature to normal levels, whereas their basal metabolic rate seems to remain suppressed and recovers only slowly. These findings indicate a strong temperature-independent remodeling of their metabolism during hibernation. Our results in brown bears further indicate that such remodeling may play a role in regulating Hb-O2 binding during hibernation.

Perspectives and Significance

The physiology of brown bear hibernation is inherently intriguing as bears seem less dependent on reductions in body temperature to aid metabolic regulation compared with other (smaller) hibernators, but rather may rely more heavily on active aerobic metabolic suppression. Here, we found indications that a side product of glycolysis, DPG, is substantially downregulated during hibernation, in effect increasing blood O2 affinity substantially. The described changes in O2 affinity and cooperativity of Hb-O2 binding may be crucial for defending internal Po2 during the hibernation period. Clearly, further studies will be needed to identify the mechanisms involved in metabolic suppression in hibernating bears and to establish whether the observed decrease in erythrocyte DPG, besides its role in adapting the blood O2 equilibrium curve, is part of this metabolic remodeling. In this case, regulation of glycolytic enzymes upstream of DPG production by pH, temperature, or other factors could be involved. Such findings could be of relevance to identify pharmacological manipulation of energy metabolism that would preserve tissue integrity in critically ill human patients. Further studies on hibernators such as the brown bear may reveal details of how the substantial metabolic regulation is achieved.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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