Markers of glycemic control in the mouse: comparisons of 6-h- and overnight-fasted blood glucoses to Hb A\textsubscript{1c}

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Blood glucose varies widely together with food intake during a typical day; thus fasting blood glucose has been used to assess daily averaged level of blood glucose (1, 1a, 10, 16, 17, 31). In humans, overnight-fasted blood glucose is routinely used to diagnose diabetes mellitus and evaluate glycemic control because it correlates with glycosylated hemoglobin (GlyHb) (1a, 1b, 5). Overnight fasting has also been adopted for determination of blood glucose in mice (4, 35). Recent studies have raised concerns regarding the potential physiological stress imposed by long-term fasting in mice (3, 6). Thus, the Animal Models of Diabetes Complications Consortium (www.amdcc.org) recommends a short-term (i.e., 6-h) fasting protocol for blood glucose determination in mice (6). However, the effectiveness with which short-term fasting glucose reflects glycemic control over the course of a day remains to be validated.

Measurement of GlyHb provides a critical parameter for assessing long-term glycemic control and predicting the incidence of diabetic complications in diabetic patients (1, 1a). In humans, glycosylation of NH\textsubscript{2}-terminal valine of the hemoglobin \beta-chain is known as hemoglobin A\textsubscript{1c} (Hb A\textsubscript{1c}) and represents the major form of GlyHb (7). Several methods for determining GlyHb exist, including ion-exchange high-performance liquid chromatography (HPLC), boronate affinity HPLC (23), and an antibody-based approach (2). Ion-exchange HPLC determines GlyHb on the basis of altered Hb charge caused by linkage with glucose molecules, whereas the boronate affinity HPLC measures GlyHb by the presence of coplanar vicinal hydroxyl and/or carbonyl groups of hexoses (23). It has been demonstrated in humans that the boronate affinity HPLC approach determines total GlyHb with less interference from nonglycosylated hemoglobin compounds (23, 24).

Clinically, an immunochrometry-based approach that detects Hb A\textsubscript{1c} using a monoclonal antibody against human Hb A\textsubscript{1c} has been widely used (e.g., DCA 2000 analyzer) (2, 13). Advantages of this technique include the requirement for only a few microliters of blood, making it suitable for determining Hb A\textsubscript{1c} in mice and the rapid simplicity of measurement (18, 36). Although it has been demonstrated in humans that Hb A\textsubscript{1c} determined using DCA 2000 analyzer correlates with GlyHb measured using ion-exchange HPLC (2), a similar relationship in mice remains to be established. Validation of the relationship between Hb A\textsubscript{1c}, GlyHb, and glucose control in mice remains to be established but is critical for studies of diabetic complications in this species. Therefore, we undertook the present studies to characterize the relationship between fasting blood glucose and Hb A\textsubscript{1c} in mice.

MATERIALS AND METHODS

Materials. Inbred C57BL/6J, DBA/2J, and KK/HJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee of Vanderbilt University, and the animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Generating diabetic mice. Diabetes was induced using a low-dose streptozotocin protocol (40 mg \textsuperscript{k}g\textsuperscript{-1}·day\textsuperscript{-1} for DBA/2J and 50 mg \textsuperscript{k}g\textsuperscript{-1}·day\textsuperscript{-1} for C57BL/6J and KK/HJ mice, made freshly in 0.1 mol/l citrate buffer, pH 4.0, ip injection daily for 5 consecutive days), as described previously (25). The induction of diabetes mellitus was defined as a blood glucose level measured using a B-Glucose Ana-
Innovative Methodology

BLOOD GLUCOSE AND Hb A1c IN MICE

A method for implantation of the subcutaneous glucose sensor in mice was established as described below. Briefly, a diabetic or control inbred mouse was restrained in a 50-ml centrifuge tube with air holes drilled in it. The fur on the lower back was shaved and the skin sterilized with an iodine pad, followed by washing with a 70% ethanol pad. The glucose sensor was inserted subcutaneously and fixed to skin using tissue glue (3M Vetbond Tissue Adhesive 1469SB). The mouse was then housed individually in a cage and allowed free access to water and food. The glucose signal was recorded by the glucose monitor placed outside the cage via a cable tethered to the mouse. Three to four measurements of blood glucose were performed during the daytime by puncturing saphenous vein as described previously (26), and the blood glucose values were input into the monitor to calibrate the glucose level. To minimize the effect of sensor implantation on blood glucose, the data recorded by the glucose sensor in the first 5 h were discarded.

Correlation between glucose levels in subcutaneous interstitium and blood. The validity of the CGMS has been established by studies in humans indicating that the interstitial glucose is proportional to blood glucose (27, 28). In the present studies, we examined the relationship between glucose levels in these two compartments in mice. To do this, we injected a 20% of glucose solution (1.5 g/kg body wt) intraperitoneally in mice implanted with the glucose sensor. Blood glucose concentration determined via a saphenous vein bleed and the electrical signal recorded using the glucose sensor were examined at several time points following glucose injection. A significant correlation between blood glucose level and the sensor current (n = 10, R² = 0.762, P < 0.05) was observed, indicating the interstitial glucose sensitively reflects blood glucose in inbred mice.

Determination of Hb A1c. Mouse immunoreactive Hb A1c was determined using DCA 2000 analyzer (Bayer, Elkhart, IN). This system automatically measures both Hb A1c and total hemoglobin in 2 wk of the streptozotocin injection, and hyperglycemia persisted for the duration of the experimental period. All diabetic mice included in the present studies were diabetic for ≥8 wk.

Continuous monitoring of blood glucose in conscious mice. To definitely determine daily average blood glucose level in mice, we used the Continuous Glucose Monitoring System (CGMS; Medtronic MiniMed, Northridge, CA) to continuously measure blood glucose for 24 h. This system utilizes a subcutaneous glucose sensor such that interstitial fluid glucose reacts with the sensor’s glucose oxidase layer, producing an electrical current that is proportional to glucose concentrations (21, 28). The electrical signal was collected every 10 s. These signals were averaged and saved to memory of the cable-tethered glucose monitor every 5 min. The data stored in the monitor were periodically downloaded into a computer for analysis. A correlation between interstitial glucose and blood glucose levels has previously been demonstrated in several species, including mice and humans (19, 27, 28). In the present studies, we adapted this system to mice.


Continuous blood glucose profile in inbred mice. Continuous 24-h monitoring of interstitial fluid glucose levels using the MiniMed CGMS in diabetic and control C57BL/6J, DBA/2J, and KK/HJ mice (Fig. 1) provides an estimate of daily blood glucose profile in each strain of mouse. Figure 2 shows representative blood glucose profiles in control and diabetic inbred mice. Diabetic mice exhibited significantly higher levels of blood glucose than controls. Approximately 30 μl of blood was collected via a saphenous vein in conscious mouse. Ten microliters of blood was used to measure Hb A1c. The remaining 20 μl of the blood was sent to Case Western Reserve University via an overnight service to determine total GlyHb (see below). Preliminary studies showed that there was no difference in Hb A1c levels determined in freshly collected blood and that which had been stored for 1 day (data not shown).

Determination of total GlyHb. Total GlyHb in mouse blood was determined using boronate affinity HPLC (Bio-Rad Variant Hemoglobin Testing Systems; Bio-Rad Laboratories, Hercules, CA). Sample hemolsate is passed over the boronate column, and glycated hemoglobin is preferentially bound due to the formation of a stable complex between the coplanar cis-diol group of glycated hemoglobin and the immobilized 3-amino phenylboronic acid in the affinity cartridge. The nonglycated hemoglobin is eluted from the column with subsequent displacement of the glyculated hemoglobins using a separate buffer. The separated hemoglobins pass through the flow cell of the filter photometer, where changes in the absorbance are measured at 415 nm. A secondary filter at 690 nm corrects for matrix effects caused by mixing buffers of different ionic strength.

Statistical analysis. All data are expressed as means ± SE. One-way ANOVA followed by a Bonferroni post hoc test was used to compare the difference among three groups, and two-tailed t-test was used for comparison of data between two groups. The level of statistical significance was assigned at P < 0.05 by convention.

RESULTS

Continuous blood glucose profile in inbred mice. Continuous 24-h monitoring of interstitial fluid glucose levels using the MiniMed CGMS in diabetic and control C57BL/6J, DBA/2J, and KK/HJ mice (Fig. 1) provides an estimate of daily blood glucose profile in each strain of mouse. Figure 2 shows representative blood glucose profiles in control and diabetic inbred mice. Diabetic mice exhibited significantly higher levels of blood glucose...
and greater glucose excursions than nondiabetic controls (mean standard deviations of daily blood glucose in diabetic vs. control KK/HJ, DBA/2J, and C57BL/6J mice were 63.7 vs. 32.0, 67.4 vs. 17.4, and 79.9 vs. 16.4 mg/dl, respectively; \( P < 0.005 \) in all 3 strains). Among the controls, KK/HJ mice, an inbred strain developing spontaneous glucose intolerance (33), exhibited higher daily averaged blood glucose levels than nondiabetic DBA/2J mice (Table 1).

**Correlation between daily averaged blood glucose and Hb A\(_{1c}\).** To study whether the daily averaged blood glucose correlates with glycosylated hemoglobin in mice, as previously shown in humans (1), we measured Hb A\(_{1c}\) using the DCA 2000 analyzer in mice undergoing continuous glucose monitoring. Daily averaged blood glucose was tightly correlated with Hb A\(_{1c}\) in all three strains of mice with \( R^2 \) values of 0.90, 0.95, and 0.99 in KK/HJ (\( n = 10 \)), C57BL/6J (\( n = 10 \)), and DBA/2J mice (\( n = 11 \)), respectively (\( P < 0.05 \) in all 3 strains).

**Blood glucose level following 6 h vs. overnight fasting.** To define the optimal fasting time for determining blood glucose in mice, we measured saphenous venous glucose following a 6-h fast in diabetic and control C57BL/6J, DBA/2J, and KK/HJ mice. One week later, the blood glucose was measured again in the same mice following an overnight fast. The fasting

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**Table 1. Daily averaged blood glucose levels in control and diabetic inbred mice**

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>DBA/2J</th>
<th>KK/HJ</th>
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<tbody>
<tr>
<td>Control</td>
<td>158.3 ± 2.49 (5)</td>
<td>131.7 ± 6.12 (8)</td>
<td>171.2 ± 14.0 (9)*</td>
</tr>
<tr>
<td>Diabetes</td>
<td>626.8 ± 16.2 (5)†</td>
<td>498.4 ± 60.0 (5)†</td>
<td>463.1 ± 30.7 (4)†</td>
</tr>
</tbody>
</table>

Values are means ± SE. The mean was calculated using averaged daily glucose level of each mouse, and the numbers inside parentheses represent the mouse numbers. *\( P < 0.05 \) vs. value in control DBA/2J; †\( P < 0.0001 \) vs. values in respective control mice.
blood glucose was then compared with the daily averaged blood glucose determined with the use of an implanted interstitial glucose sensor in strain- and age-matched mice. Overnight fasting resulted in significantly lower blood glucose in all groups of mice except diabetic C57BL/6J mice (overnight fasting vs. daily averaged blood glucose in control and diabetic C57BL/6J: 109.5 ± 3.2 vs. 158.3 ± 2.5 and 445.8 ± 60.5 vs. 626.8 ± 16.5 mg/dl, respectively; in control and diabetic KK/HJ mice: 91.5 ± 2.5 vs. 171.2 ± 14.0 and 243.2 ± 16.3 vs. 463.1 ± 30.7 mg/dl, respectively; in control and diabetic DBA/2J mice: 73.5 ± 2.9 vs. 131.7 ± 6.1 and 191.9 ± 57.1 vs. 498.4 ± 60.0 mg/dl, respectively). In contrast, a 6-h fast yielded blood glucose values that were different from the daily averaged MiniMed determined glucose levels.

Six-hour fasting slightly decreased body weight compared with the same mice without a fast (<7%). Overnight fasting resulted in a more marked decrease in body weight (8.0 ± 0.8, 11.0 ± 1.0, and 11.5 ± 1.9% less than 6-h-fasted body weight in control KK/HJ, DBA/2J, and C57BL/6J mice, respectively, and 6.2 ± 0.7, 5.1 ± 2.1, and 8.7 ± 1.1% in diabetic KK/HJ, DBA/2J, and C57BL/6J mice, respectively; P < 0.05 in all groups compared with body weight in 6-h-fasted mice). These data indicate that a 6-h fast imposes less metabolic stress on mice than an overnight fast.

**Correlation between fasting blood glucose and Hb A1c.** The relationship between Hb A1c and 6-h or overnight-fasted blood glucose was also analyzed in C57BL/6J, KK/HJ, and DBA/2J mice. Six-hour-fasted blood glucose correlated more closely with Hb A1c than overnight-fasted blood glucose, although overnight-fasted blood glucose also correlated with Hb A1c (Fig. 3).

**Hb A1c determination in mice.** To validate Hb A1c determined using DCA 2000 as an index of total GlyHb, we measured total GlyHb using a boronate affinity HPLC method on two blood samples collected from the same mouse at the same time. The Hb A1c is comparable with GlyHb in C57BL/6J mice (n = 45) but slightly higher than GlyHb in KK/HJ, DBA/2J, and C57BL/6J mice, respectively, and 6.2 ± 0.7, 5.1 ± 2.1, and 8.7 ± 1.1% in diabetic KK/HJ, DBA/2J, and C57BL/6J mice, respectively; P < 0.05 in all groups compared with body weight in 6-h-fasted mice). Despite this difference, Hb A1c correlates closely with total GlyHb in all strains studied (R² = 0.8823, 0.8765, and 0.8445 in C57BL/6J, KK/HJ, and DBA/2J strains, respectively; P < 0.0001 in all strains). These results indicate that the Hb A1c determined using the DCA 2000 analyzer reflects GlyHb.

**The predictive value of total GlyHb for diabetic nephropathy in inbred mice.** We have demonstrated previously that diabetic KK/HJ and DBA/2J mice develop severe nephropathy, whereas C57BL/6J mice appear to resist diabetic nephropathy (25). In the present study, we examined whether the total GlyHb was different in these three strains when correlated with 6-h fasting blood glucose (total GlyHb/fasting blood glucose). The present studies revealed that KK/HJ and DBA/2J mice, which are susceptible to diabetic nephropathy, exhibit significantly higher GlyHb for any given fasting blood glucose than C57BL/6J mice (Fig. 4).

**DISCUSSION**

Daily averaged blood glucose more closely correlates with Hb A1c than fasted blood glucose in humans (1, 5, 29). Determining the effect of fast duration on blood glucose and the relationship between blood glucose levels and Hb A1c in mice requires the determination of daily averaged blood glucose levels as a reference in this species.

Therefore, we established a method to continuously measure blood glucose level over 24 h in mice using the CGMS. This system determines glucose level in interstitial fluid. A correlation between interstitial fluid and blood glucose levels has been demonstrated in humans and several other species (19, 21, 28). The present studies further demonstrate that this correlation also exists in inbred mice.

Glucose excursions were observed in both control and diabetic mice, but the extent of glucose excursions was significantly greater in diabetic mice than their controls. The glucose excursion appears to be more pronounced during the night, consistent with the nocturnal nature of mice. Glucose excursions were also seen in some mice during the daytime following saphenous vein bleeding for blood glucose determination. Thus, interruption of daytime sleep by blood sampling may lead mice to consume food, contributing to daytime glucose excursions.

Daily averaged glucose levels are strain dependent. Of the three strains of mice studied, KK/HJ mice exhibited the highest level of daily blood glucose, whereas the DBA/2J mice...
had the lowest averaged daily blood glucose. This observation is consistent with previous studies indicating that both KK/HiJ
and C57BL/6J mice develop glucose intolerance (8, 33) and that glucose intolerance in KK/HiJ mice is more severe than in
C57BL/6J mice (33).

Using daily averaged blood glucose as a reference value, we compared the effect of a 6-h vs. overnight fast on mouse blood
glucose. The results indicate that 6-h-fasted blood glucose was
closer to the level of daily averaged blood glucose than blood
glucose following an overnight fast. In addition, body weight
was greater in mice following a 6-h fast than following an
overnight fast, consistent with the notion that a 6-h fast im-
poses less stress than an overnight fast. More importantly,
blood glucose following a 6-h fast correlates with Hb A1c
better than blood glucose following an overnight fast. This
may be explained by the fact that mice are nocturnal feeders, so that
initiation of a fast in the evening could effectively result in a
24-h fast, causing inordinately low blood glucose.

In humans, Hb A1c represents the major form of GlyHb and
correlates with the averaged blood glucose levels over the
preceding 3 mo and the development of diabetic complications
(1, 7). The DCA 2000 system is designed to determine Hb A1c
on the basis of an immunochemical technique that has been
validated against human Hb A1c (2, 13) but has been widely
used in mice (18, 36).

To validate Hb A1c values determined using the DCA 2000
analyzer as accurately reflecting GlyHb in mice, we compared
these values with a boronate affinity HPLC method that
measures total GlyHb on the basis of the presence of coplanar
vicinal hydroxyl and/or carbonyl groups of hexoses (23, 24).
Comparable values for Hb A1c and total GlyHb in C57BL/6J mice indicate that the Hb A1c determined using DCA 2000
analyzer represents a valid determination of GlyHb in this
strain. Considering the small volume of blood required and the
convenience and simplicity of operation, this method has
considerable utility.

Interestingly, the Hb A1c values were consistently lower
than total GlyHb in DBA/2J mice. A difference in Hb A1c
determined using DCA 2000 has been observed previously
(34). This may be explained by the variation in Hb β-chain
alleles in inbred mice (9, 30). For example, DBA/2J appears to
be homozygous for the Hbbd β-chain allele, whereas C57BL/6J
is homozygous for the Hbbb allele (30). The allele variation
may result in changes in antigenicity and/or glycosylation of
hemoglobin. Nevertheless, the close correlations between Hb
A1c and total glycosylated Hb, as well as 6-h-fasted blood
glucose, indicate that the DCA 2000 is a reliable approach for
determination of Hb glycosylation and blood glucose levels in
the strains studied. However, the correlations in other strains
need to be validated prior to using DCA 2000 approach.

Although the boronate affinity HPLC method has been used
as the gold-standard method for GlyHb in humans (23, 24),
direct evidence indicating that this is also true in mice is
lacking. However, agreement of strain susceptibility to diabetic
nephropathy (14, 25) with the ratio of total GlyHb to fasting
blood glucose among the strains of mice studied is consistent
with boronate HPLC-determined total GlyHb providing a
meaningful measurement of GlyHb in mice. The ratio of
GlyHb to blood glucose has been suggested to predict diabetic
glucose in humans to use in conscious mice. The daily
averaged glucose profile and/or fasted blood glucose are more valuable. For long-term
studies, especially pertaining to diabetic complications, glyco-
sylated Hb is advantageous in that it is not subject to moment-
ary changes due to stress or some other acute factor. Thus
establishing the relationship of glycosylated Hb to fasting
glucose will facilitate studies requiring assessment of glucose
control.

In summary, we adapted a method to continuously monitor
blood glucose in humans to use in conscious mice. The daily
averaged blood glucose level determined using the glucose
sensor data correlates closely with Hb A1c. Compared with an
overnight fast, 6-h fasting blood glucose levels correlate more
closely with daily averaged blood glucose levels and Hb A1c.
The present studies also demonstrated that Hb A1c determined
using the DCA 2000 analyzer correlates with total glycosylated
Hb that was determined using a boronate HPLC approach in
the three strains of mice studied. Nevertheless, this relationship
is strain dependent, and Hb A1c values in DBA/2J mice are
relatively lower than total glycosylated hemoglobin compared
with C57BL/6J and KK/HiJ mice. Differences in other strains
may also exist and should be explored on a strain-by-strain
basis. The results of these studies show that Hb A1c measured
using a low-cost, hand-held, automated analyzer (DCA 2000)
provides an accurate means of assessing glycemic control in
the mouse. Use of Hb A1c measurements should permit a more
reliable determination of the contribution of blood glucose
control to diabetic complications in mouse models.

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