Considerations in the Design of Hyperinsulinemic-Euglycemic Clamps in the Conscious Mouse

Julio E. Ayala, Deanna P. Bracy, Owen P. McGuinness, and David H. Wasserman

Despite increased use of the hyperinsulinemic-euglycemic clamp to study insulin action in mice, the effects of experimental parameters on the results obtained have not been addressed. In our studies, we determined the influences of sampling sites, fasting duration, and insulin delivery on results obtained from clamps in conscious mice. Carotid artery and jugular vein catheters were implanted in C57BL/6J mice (n = 6–10/group) fed a normal diet for sampling and infusions. After a 5-day recovery period, mice underwent a 120-min clamp (2.5-mU insulin infusion; ~120–130 mg/dl glucose) while receiving [3-3H]glucose to determine glucose appearance (endoRa) and disappearance (Rg). Sampling large volumes (~100 µl) from the cut tail resulted in elevated catecholamines and basal glucose compared with artery sampling. Catecholamines were not elevated when taking small samples (~5 µl) from the cut tail. Overnight (18-h) fasting resulted in greater loss of total body, lean, and fat masses and hepatic glycogen but resulted in enhanced insulin sensitivity compared with 5-h fasting. Compared with a 16-mU/kg insulin prime, a 300-mU/kg prime resulted in hepatic insulin resistance and slower acquisition of steady-state glucose infusion rates (GIR) after a 5-h fast. The steady-state GIR was expedited after the 300-mU/kg prime in 18-h–fasted mice. The GIR and Ra were increased with increasing insulin infusions (0.8, 2.5, 4, and 20 mU·kg⁻¹·min⁻¹), but endoRa was fully suppressed with doses higher than 0.8 mU·kg⁻¹·min⁻¹. Thus, common variations in experimental factors yield different results and should be considered in designing and interpreting clamps. Diabetes 55:390–397, 2006

The hyperinsulinemic-euglycemic clamp, or insulin clamp, has been referred to as the “gold standard” for measuring insulin sensitivity in vivo. In this procedure, insulin is administered to raise the insulin concentration while glucose is infused to maintain euglycemia. The glucose infusion rate (GIR) needed to maintain euglycemia is a reflection of insulin action. When radioisotopes, such as [3H]glucose, are infused in conjunction with an insulin clamp, endogenous glucose appearance (endoRa) and glucose disappearance (Rg) can be measured, allowing for the differentiation between hepatic and peripheral insulin sensitivity. Given the prominent role of insulin resistance in the development of various metabolic disorders, the insulin clamp is a useful tool in basic and clinical research.

With the development of transgenic technologies in the mouse, the miniaturization of the insulin clamp for use in this animal has been an important advancement. It is thus surprising that in the >10 years since its first use in the mouse, there has been no assessment of the insulin clamp methodology in this animal. A literature search for the period 2003–2004 found 25 publications using the insulin clamp in conscious mice (1–25). In these studies, the various protocols that were used differed in blood sampling sites, insulin and tracer infusion methods, and fasting duration. For example, 44% of these studies described the sampling site as the cut tail, whereas 12% used an arterial catheter. The remaining 44% did not indicate or reference a study indicating a sampling site. In a similar finding, 48% of these studies used mice fasted overnight (for 16–18 h), 40% used mice fasted for 5–6 h, and 12% used other fasting durations. Finally, 40% of these clamp studies were performed without a priming dose of insulin, whereas 48% administered a prime equal to the total insulin infused at a constant rate. The remaining 12% used priming doses ranging between these two extremes. Given this diversity of approaches, it is crucial to determine how experimental parameters influence clamp studies in the mouse to better compare results obtained by different investigators. Using common clamp protocols as guidelines (18,26–28), three parameters were compared: blood sampling sites, fasting duration, and insulin infusion (prime and constant rate) methods.

RESEARCH DESIGN AND METHODS
All procedures were approved by the Vanderbilt Animal Care and Use Committee. Male, 8-week-old C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME) were obtained and placed on a sterilized rodent diet (Harlan Teklad LM-485, #7012; Harlan Teklad, Madison, WI) for 1 week before surgery. Animals were housed under controlled temperature (23°C) and lighting (12 h light:12 h dark) conditions with free access to water and food.

Mice (n = 95) were catheterized at least 5 days before the experiments (29) after being anesthetized with sodium pentobarbital (70 mg/kg body wt). The left common carotid artery was catheterized for sampling using a two-part catheter consisting of PE-10 (inserted into the artery) and silastic (0.025 inner diameter [OD]). The right jugular vein was catheterized for infusions with a silastic catheter (0.025 OD). The free catheter ends were tunneled under the skin to the back of the neck and attached via stainless steel connectors to tubing made of Micro-Renathane (0.033 OD). The tubing was externalized and sealed with stainless steel plugs. In a subset of mice, only a jugular vein catheter was implanted (see COMPARISON OF SAMPLING SITES [below]). Lines were flushed daily with ~50 µl saline containing 200 units/ml heparin and 5 mg/ml ampicillin. Animals were individually housed after surgery, and their body weight was recorded daily. Animals not within 10% of their presurgery weight by postsurgery day 5 were excluded from the study.

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GIR, glucose infusion rate.

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Hyperinsulinemic-euglycemic clamps. This section describes the general protocol used in all experiments. Modifications are described where appropriate in the following sections.

After being fasted for 5 or 18 h, mice were studied in individual 1.44-plastic containers with bedding or a restrainer (552-BSR; Plas-Labs, Lansing, MI) for cut tail sampling. The protocol consisted of a 120-min tracer equilibration period \((t = -120 \text{ to } 0 \text{ min})\) beginning at 8:00 A.M. followed by a 120-min experimental period \((t = 0 \text{ to } 120 \text{ min})\) beginning at 10:00 A.M. A blood sample \((-5 \mu l)\) was obtained at \(t = -120 \text{ min}\) to determine initial glucose levels (HemoCue Meter; HemoCue, Lake Forest, CA). A 5-\(\mu\)Ci bolus of [\(3-\text{H}\)]glucose purified by high-performance liquid chromatography was given at \(t = -120 \text{ min}\) followed by a 0.05 \(\mu\)Ci/min infusion for 2 h. At \(t = -5 \text{ min}\), a blood sample \((-100 \mu l)\) was taken for the assessment of basal glucose and insulin levels and glucose turnover. The insulin clamp was begun at \(t = 0 \text{ min}\) with a primed-continuous infusion of human insulin (16 or 300 \(\mu\)U/kg bolus followed by 2.5 \(\mu\)U \cdot kg \(^{-1}\) \cdot min \(^{-1}\); Humulin R; Eli Lilly, Indianapolis, IN). The [\(3-\text{H}\)]glucose infusion was increased to 0.1 \(\mu\)Ci/min for the remainder of the experiment to minimize changes in specific activity from the equilibration period. Specific activity for individual time points did not vary by >15% from the average specific activity during the last 40 min of the clamp, and the slope of specific activity over time was not significantly different from zero. Euglycemia \((-120 – 130 \text{ mg/dl})\) was maintained during clamps by measuring blood glucose every 10 min starting at \(t = 0 \text{ min}\) and infusing 20% dextrose as necessary. Blood samples (60–200 \(\mu l\)) were taken every 10 min from \(t = 80 \text{ to } 120 \text{ min}\) and processed to determine glucose specific activity. Clamp insulin levels were determined from samples obtained at \(t = 100 \text{ and } 120 \text{ min}\). Additional samples \((-100 \mu l)\) were taken at \(t = -120,-5, \text{ and } 120 \text{ min}\) for the assessment of catecholamines. Mice received saline-washed erythrocytes from donors throughout the experimental period (5–6 \(\mu l\) min) to prevent a fall of >5% hematocrit.

Comparison of sampling sites. In these studies, 18-h–fasted mice were compared using a 300-\(\mu\)U/kg insulin prime. For cut tail sampling, mice were surgically prepared with only a jugular vein catheter. The tip of the tail \((-1 \text{ cm})\) was cut off at \(t = -120 \text{ min}\) in restrained animals, and blood was sampled by squeezing the tail from base to tip. Samples obtained from the cut tail are composed of arterial and venous blood as well as lymph fluid. For arterial sampling, the externalized catheters were tethered to a stainless steel swivel (Instech Laboratories, Plymouth Meeting, PA) with Micro-Renathane tubing. This approach eliminated the need for restraining or any further handling of the mouse. In some studies, samples were obtained from both the cut tail and an arterial catheter in restrained mice.

Comparison of fasting duration. Arterial catheters were used for sampling in these experiments. For the 5-h–fasting studies, food was removed at 5:00 A.M. on the day of the experiment, and for the 18-h–fasting studies, food was removed at 4:00 P.M. on the day before the experiment. Thus, all insulin infusions followed a 16-h fast. Clamps followed a 16-\(\mu\)U/kg insulin prime. Body composition was determined in 5-h– and 18-h–fasted mice using a mq10 NMR analyzer (Bruker Optics, The Woodlands, TX). Liver and gastrocnemius muscle glycogen was determined as previously described (30). Food consumption was determined using a precision scale and continuous computer monitoring.

Comparison of insulin delivery methods. Arterial catheters were used for sampling in these experiments. In one set of experiments, the insulin prime was 16 \(\mu\)U/kg. For cut tail sampling, mice were compared using a 300-\(\mu\)U/kg insulin prime. For cut tail sampling, samples were obtained from both the cut tail and an arterial catheter in restrained mice.

Comparison of fasting duration. Arterial catheters were used for sampling in these experiments. For the 5-h–fasting studies, food was removed at 5:00 A.M. on the day of the experiment, and for the 18-h–fasting studies, food was removed at 4:00 P.M. on the day before the experiment. Thus, all insulin infusions followed a 16-h fast. Clamps followed a 16-\(\mu\)U/kg insulin prime. Body composition was determined in 5-h– and 18-h–fasted mice using a mq10 NMR analyzer (Bruker Optics, The Woodlands, TX). Liver and gastrocnemius muscle glycogen was determined as previously described (30). Food consumption was determined using a precision scale and continuous computer monitoring.

Comparison of insulin delivery methods. Arterial catheters were used for sampling in these experiments. In one set of experiments, the insulin prime was 16 \(\mu\)U/kg. For cut tail sampling, samples were obtained from both the cut tail and an arterial catheter in restrained mice.

Processing of plasma samples. Plasma for [\(3-\text{H}\)]glucose determinations was deproteinized with Ba(OH)\(_2\) and ZnSO\(_4\). For each sample, an aliquot of the supernatant was counted directly and another was dried to remove \(3\text{H}_2\text{O}\). Plasma \(\text{H}_2\text{O}\) was determined as the difference between dried and undried samples. Immunoreactive insulin (32) and catecholamines (33) were determined as previously described.

Calculations. \(R_a\) and \(R_c\) were determined using Steele’s non–steady-state equations (34). Endogenous glucose production (end\(R_g\), given as milligrams per kilogram per minute) was determined by subtracting the GFR from total \(R_g\). Glycolytic rates were estimated from the increment per unit of time of \(\text{H}_2\text{O}\) multiplied by the estimated body water divided by [\(3-\text{H}\)]glucose specific activity. \(\text{H}_2\text{O}\) appearance was determined by linear regression of the measurements at \(t = 80 \text{ to } 120 \text{ min}\). Body water was assumed as 60% of body weight (35).

Statistical analysis. Data are presented as means ± SE. Differences between groups were determined by two-way ANOVA followed by Tukey’s post hoc tests or by the \(t\) test, as appropriate. The significance level was \(P < 0.05\).

RESULTS

Comparison of sampling sites. Of the insulin clamp studies in the mouse published in 2003–2004, 50% specified how blood was obtained. In most of these (80%), samples were obtained from the cut tail. We compared insulin clamps where the blood was obtained from the cut tail with those in which blood was obtained from an indwelling catheter. As shown in Fig. 1, epinephrine and norepinephrine were elevated in cut tail–compared with arterial–sampled mice throughout the study. This is consistent with previous reports of catecholamines in mice where blood was acquired from the cut tail (36). These elevated catecholamines were not due to mouse restraint or tail cutting. Restrained mice that had their tails cut off but were sampled from the artery showed catecholamine levels similar to those in unrestrained, artery–sampled mice (Fig. 1). The elevated catecholamines in the cut tail–sampled group were due to stress from the acquisition of the blood volume \((-100 \mu l)\) required for hormone assessment, which necessitates more manual squeezing of the tail than for smaller blood volumes. In mice sampled from both the artery and cut tail (Fig. 1), the larger volume of blood needed for catecholamine analysis was obtained from the artery, thus requiring less handling than when large samples were taken from the cut tail. The blood volume taken from the cut tail in these mice was \(-5 \mu l\).
Blood glucose during a clamp experiment where blood was acquired from an arterial catheter in unrestrained mice (Fig. 2A). Clamp insulin levels were not different between groups (Table 4). Blood glucose fell with 5-h–fasted mice (Table 3). Clamps were performed using a 16-mU/kg insulin prime, and samples were obtained from an arterial catheter. Clamp insulin levels were not different between groups (Table 4). Blood glucose fell in 18-h– compared with 5-h–fasted mice during the first 40 min of the clamp, after which glucose was similar in both groups (Fig. 4A). The lower initial glucose levels and difficulty in maintaining euglycemia in 18-h–fasted mice was due to a delay in compensating for the increased decrease in arterial glucose (Fig. 2B). The GIR was not different in cut tail– compared with artery-sampled mice during the remainder of the experiment (Fig. 2B). Clamp endoR_g was significantly higher in cut tail– compared with artery-sampled mice (Table 1). However, clamp R_g was also significantly higher in cut tail– compared with artery-sampled mice (Table 1), thus maintaining similar GIR between groups during the clamp.

**Comparison of fasting duration.** The effects of fasting duration on the results from insulin clamps were investigated given the equal distribution of recent studies using 5-h– or 18-h–fasted mice. Because mice are primarily nocturnal feeders, an 18-h overnight fast represents a proportionately larger deprivation of calories. Mice consume ~30% of their daily food intake during the daytime (Fig. 3). Thus, mice are already in a low-consumption mode when an 18-h fast is started (27). The subsequent overnight fast in our studies resulted in a significant loss of body weight (5 vs. 16% in 5-h– and 18-h–fasted mice, respectively) due to a reduction in fat and muscle tissue (Table 2). Overnight fasting also resulted in significantly lower hepatic, but not muscle, glycogen content (Table 2).

Basal glucose and insulin were lower in 18-h– compared with 5-h–fasted mice (Table 3). Clamps were performed using a 16-mU/kg insulin prime, and samples were obtained from an arterial catheter. Clamp insulin levels were not different between groups (Table 4). Blood glucose fell in 18-h– compared with 5-h–fasted mice during the first 40 min of the clamp, after which glucose was similar in both groups (Fig. 4A). The lower initial glucose levels and difficulty in maintaining euglycemia in 18-h–fasted mice was due to a delay in compensating for the increased decrease in arterial glucose (Fig. 2B). The GIR was not different in cut tail– compared with artery-sampled mice during the remainder of the experiment (Fig. 2B). Clamp endoR_g was significantly higher in cut tail– compared with artery-sampled mice (Table 1). However, clamp R_g was also significantly higher in cut tail– compared with artery-sampled mice (Table 1), thus maintaining similar GIR between groups during the clamp.

**FIG. 2.** Comparison of sampling sites, showing blood glucose (A) and glucose infusion rates (B) during a 120-min hyperinsulinemic-euglycemic clamp experiment in chronically catheterized, conscious mice. C57BL/6J mice were fasted for 18 h before the experiment. A 300 mU/kg insulin priming dose was given at the onset of the clamp period. Blood was acquired from the cut tail in restrained mice (●) or an arterial catheter in unrestrained mice (空白). *P < 0.05 vs. artery. **Inset:** Blood glucose during a clamp experiment where blood was acquired from both the cut tail (●) and an arterial catheter (空白) in restrained mice. Arterial or cut tail blood was obtained and measured for plasma [3-18H]glucose as described in research design and methods. Data are means ± SE for 7-12 mice/group.

**TABLE 1.** Insulin clamp characteristics in C57BL/6J mice by sampling method.

<table>
<thead>
<tr>
<th>Sampling Method</th>
<th>Cut Tail</th>
<th>Arterial Catheter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>123 ± 5</td>
<td>129 ± 6</td>
</tr>
<tr>
<td>GIR (mg·kg⁻¹·min⁻¹)</td>
<td>56 ± 5</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>29 ± 3</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>EndoR_d (mg·kg⁻¹·min⁻¹)</td>
<td>5 ± 1</td>
<td>-5 ± 3*</td>
</tr>
<tr>
<td>R_g (mg·kg⁻¹·min⁻¹)</td>
<td>62 ± 7</td>
<td>50 ± 4*</td>
</tr>
</tbody>
</table>

Data are means ± SE and represent the average of five measurements taken during the last 40 min of the clamp period. For cut tail–sampled mice, glucose and GIR values represent data obtained from n = 12 mice, and insulin, endoR_d, and R_g values represent data obtained from a subset of mice (n = 4). All values for artery-sampled mice represent data obtained from n = 7 mice. *P < 0.05 vs. cut tail.

**TABLE 2.** Effect of fasting duration on body composition and tissue glycogen content in C57BL/6J mice.

<table>
<thead>
<tr>
<th>Fasting Duration</th>
<th>Body Composition</th>
<th>Tissue Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-h fast</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>18-h fast</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Weight loss (g)</td>
<td>1.2 ± 0.1</td>
<td>3.5 ± 0.1*</td>
</tr>
<tr>
<td>Muscle mass loss (g)</td>
<td>0.8 ± 0.1</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>Fat mass loss (g)</td>
<td>0.2 ± 0.02</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Liver glycogen (mg glycogen units/g tissue)</td>
<td>31.8 ± 2.1</td>
<td>3.1 ± 1.2*</td>
</tr>
<tr>
<td>Gastrocnemius glycogen (mg glycogen units/g tissue)</td>
<td>5.3 ± 0.4</td>
<td>5.0 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. 5-h fast.
insulin sensitivity in these mice. The GIR required to maintain euglycemia was higher in 18-h– compared with 5-h–fasted mice at the end of the clamp (Fig. 4B and Table 4). GIR differences were not due to an effect on endoRₐ, because it was similarly suppressed in 18-h– and 5-h–fasted mice (Table 4). Instead, Rₐ was higher in 18-h–fasted mice (Table 4). Glycolytic rates were also higher in 18-h– compared with 5-h–fasted mice (Table 4). Given the lower Rₐ was not different between groups (51 ± 3 vs. 47 ± 5% Rₐ in 5-h– and 18-h–fasted mice, respectively).

Comparison of insulin delivery methods. Given the marked difference in insulin priming doses used in clamp studies, two general approaches for insulin priming were compared. Insulin clamps were performed on 5-h–fasted mice using a 16- or 300-mU/kg insulin prime. All samples were acquired from an arterial catheter. Clamp insulin levels were higher in mice receiving the 300-mU/kg prime compared with those receiving the 16-mU/kg prime (Table 4). Arterial glucose was clamped at similar levels in both groups (Fig. 5A). The GIR required to maintain euglycemia was stable throughout the clamp using the 16-mU/kg insulin prime (Fig. 5B). In contrast, clamps using the 300-mU/kg prime required a higher GIR to maintain euglycemia during the first 50 min of the clamp compared with clamps using the 16-mU/kg prime. The GIR decreased after the 300-mU/kg prime so that rates were not different in both groups (Fig. 5A and Table 4). However, when results were normalized for the difference in insulin primes used in clamp studies, clamp insulin levels were not different between groups by the end of the clamp (Fig. 5B and Table 4). Instead, clamp insulin levels were also higher in 18-h– compared with 5-h–fasted mice (Table 4). Instead, clamp insulin levels were not different between groups (51 ± 3 vs. 47 ± 5% Rₐ in 5-h– and 18-h–fasted mice, respectively).

Data are means ± SE and represent the average of five measurements during the last 40 min of the clamp period. *P < 0.05 vs. 5-h fast.

TABLE 3
Effect of fasting duration on basal glucose and insulin levels in C57BL/6J mice

<table>
<thead>
<tr>
<th></th>
<th>5-h fast</th>
<th>18-h fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>22.0 ± 0.7</td>
<td>19.4 ± 0.5*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>148 ± 7</td>
<td>111 ± 5*</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>17 ± 1</td>
<td>9 ± 1*</td>
</tr>
<tr>
<td>EndoRₐ (mg · kg⁻¹ · min⁻¹)</td>
<td>19 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>39 ± 1</td>
<td>39 ± 1</td>
</tr>
</tbody>
</table>

Data are means ± SE and represent the average of all studies using 5-h– and 18-h–fasted mice. *P < 0.05 vs. 5-h fast.

TABLE 4
Insulin clamp characteristics in C57BL/6J mice by time fasted and amount of insulin priming dose

<table>
<thead>
<tr>
<th></th>
<th>5-h fast</th>
<th>18-h fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>125 ± 1*</td>
<td>132 ± 3</td>
</tr>
<tr>
<td>GIR (mg · kg⁻¹ · min⁻¹)</td>
<td>41 ± 2†</td>
<td>36 ± 2‡</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>36 ± 3*</td>
<td>51 ± 3‡</td>
</tr>
<tr>
<td>EndoRₐ (mg · kg⁻¹ · min⁻¹)</td>
<td>4 ± 1*</td>
<td>9 ± 1‡</td>
</tr>
<tr>
<td>Rₐ (mg · kg⁻¹ · min⁻¹)</td>
<td>38 ± 2†</td>
<td>45 ± 2‡</td>
</tr>
<tr>
<td>Glycolytic rate (mg · kg⁻¹ · min⁻¹)</td>
<td>18 ± 1</td>
<td>16 ± 1‡</td>
</tr>
</tbody>
</table>

Data are means ± SE and represent the average of five measurements during the last 40 min of the clamp period. *P < 0.05 vs. 5-h fast, 300 mU/kg; †P < 0.05 vs. 18-h fast, 16 mU/kg; ‡P < 0.05 vs. 18-h fast, 300 mU/kg.
DISCUSSION

Despite increased use of the insulin clamp in assessing insulin action in mice, the lack of assessment of protocols used makes interpretation of results difficult and comparison between laboratories impossible. This is not a trivial issue, as many laboratories use insulin clamp protocols that differ from one another and from procedures used in human studies in both technical and conceptual aspects. Thus, in our studies, we systematically compared commonly used experimental variables in protocols for clamping conscious mice.

A key distinction between clamp protocols is the degree of mouse handling due to the manner of blood sample acquisition. Blood sampling from the cut tail in restrained mice requires manual squeezing of the tail. Previous studies have shown that handling of rodents markedly increases their circulating levels of catecholamines (37). In the studies presented here, cut tail–sampled mice exhibited elevated catecholamines compared with artery-sampled mice, indicating that cut tail sampling can be stressful (Fig. 1), although not as stressful as other forms of rodent handling (37). Catecholamine levels were not elevated in restrained mice with cut tails where blood for catecholamine analysis was obtained from an artery and only small volumes (~5 μl) were obtained from the cut tail (Fig. 1). Because blood from the cut tail does not often flow freely, the tail must be manually squeezed several times to obtain large blood samples (~100 μl), resulting in elevated catecholamines. Thus, the cut tail method is a viable technique if stress is not a concern of the study or if the sample volume needed is small (~5 μl). Basal glucose levels were higher in cut tail– compared with artery-sampled mice, resulting in lower initial GIR. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for seven mice/group. *P < 0.05 vs. 16 mU/kg.

![Insulin Clamp](image)

**Fig. 5.** Comparison of insulin delivery protocols in 5-h–fasted mice, showing arterial blood glucose (A) and glucose infusion rates (B) during a 120-min hyperinsulinemic-euglycemic clamp experiment in chronically catheterized, conscious mice. C57BL/6J mice were fasted during a 120-min hyperinsulinemic-euglycemic clamp experiment in 5-h–fasted mice were clamped using constant insulin infusion rates of 0.8, 2.5, 4, or 20 mU·kg⁻¹·min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for seven mice/group. *P < 0.05 vs. 16 mU/kg.

![Insulin Clamp](image)

**Fig. 6.** Comparison of insulin delivery protocols in 18-h–fasted mice, showing arterial blood glucose (A) and glucose infusion rates (B) during a 120-min hyperinsulinemic-euglycemic clamp experiment in chronically catheterized, conscious mice. C57BL/6J mice were fasted for 18 h before the experiment. Clamp experiments were performed with a 16-mU/kg (○) or 300-mU/kg (■) insulin priming dose. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for seven mice/group. *P < 0.05 vs. 16 mU/kg.
the experiment. A constant infusion of 0.8 (○) and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and compared with 5-h–fasted mice overexpressing hexokinase II. Many factors may play a role in the modulation of insulin sensitivity by fasting duration. Prolonged lowering of insulin, as with an overnight fast, has been shown to enhance insulin sensitivity in humans (41). In our studies, the higher hepatic glycogen content in 5-h– compared with 18-h–fasted mice might have contributed to the difference in clamp endoR, after the 300-mU/kg insulin prime (Table 4). Gastrocnemius glycogen content was not different between 5-h– and 18-h–fasted mice (Table 2). However, the possibility that glycogen content was reduced in other muscles in 18-h– compared with 5-h–fasted mice cannot be ruled out. A variety of behavioral and physiological adaptations in mice, including increased appetite and spontaneous light cycle activity, reduced metabolic rate, torpor, and decreased heart rate and blood pressure, can also occur after a single overnight fast (42). We observed that overnight fasting resulted in a significant decrease in body weight, attributed to a loss of lean body mass (Table 2). Thus, at clamp onset, 18-h–fasted mice are in a catabolic state compared with their 5-h–fasted counterparts. In summary, prolonged food deprivation results in a metabolic state that enhances insulin action during a clamp. As demonstrated by the GLUT4 (40) and hexokinase II (28) overexpression studies, phenotypes can be appreciably altered by fasting duration, although this effect may depend on the model used.

Given the large disparity in insulin-priming strategies used in clamp studies, we were interested in determining how the magnitude of the priming dose would affect glucoregulation throughout the clamp. A prime is typically used to quickly raise the concentration to target steady-state levels. Assuming insulin clearance and Vd are not affected by fasting duration, the 16-mU/kg prime was calculated to reach a theoretical peak insulin concentration of 80 μU/ml. The prime used in many clamps using a 2.5-mU · kg⁻¹ · min⁻¹ insulin infusion is 300 mU/kg (1–3), which is equal to the amount of insulin infused by the end of the clamp. This prime was calculated to raise the insulin concentration to a theoretical peak of ~1,600 μU/ml. Previous studies have shown that R, is maximal at plasma insulin of ~200–300 μU/ml in dogs and humans (43–45). This may explain the rapid stabilization of GIR in studies using the 300- compared with the 16-mU/kg prime in 18-h–fasted mice (Fig. 6B). As described below, other factors can modulate the glucoregulatory effects of different insulin primers.

In contrast to the results with 18-h–fasted mice, the GIR was quickly stabilized after the 16- compared with the 300-mU/kg insulin prime in 5-h–fasted mice (Fig. 5B). Furthermore, although the magnitude of the prime had no effect

![Diagram](Image)

**FIG. 7.** Comparison of insulin doses in 5-h–fasted mice, showing arterial blood glucose (A) and glucose infusion rates (B) during a 120-min hyperinsulinemic-euglycemic clamp experiment in chronically catheterized, conscious mice. C57BL/6J mice were fasted for 5 h before the experiment. A constant infusion of 0.8 (○), 2.5 (□), 4 (●), or 20 (○) mU · kg⁻¹ · min⁻¹ was used. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹. Arterial blood was obtained and measured for plasma [3-3H]glucose as described in RESEARCH DESIGN AND METHODS. Data are means ± SE for 7–9 mice/group. *P < 0.05 vs. 2.5, 4, and 20 mU · kg⁻¹ · min⁻¹; †P < 0.05 vs. 4 and 20 mU · kg⁻¹ · min⁻¹; §P < 0.05 vs. 0.8, 2.5, and 20 mU · kg⁻¹ · min⁻¹.
on the GIR at the end of the clamp, the GIR normalized for insulin concentration (i.e., whole-body insulin sensitivity) was higher in 5-h–fasted mice clamped after the 16- compared with the 300-mU/kg prime. EndoR_s was completely suppressed in 5-h–fasted mice after the 16- but not the 300-mU/kg prime, even though insulin at the end of the clamp was higher after the 300-mU/kg prime. These effects of the prime on insulin sensitivity and concentration were not observed in 18-h–fasted mice. It is possible that suprapharmacological insulin levels caused by the 300-mU/kg prime at clamp onset results in a high rate of 3H2O tracer equilibration but at the onset of the clamp (2,4–6,26,27,46–52). This is a reasonable strategy if one assumes that the insulin prime at clamp onset results in a marked expansion of the glucose pool (i.e., accumulation of intracellular glucose). Clamp endoR_s and R_d in those studies were similar to those presented here in equally fasted mice (18 h) using the same insulin prime (300 mU/kg). However, glycolytic rates were higher (69 ± 3% R_d) in those studies than in studies using the traditional tracer method (45 ± 2% R_d). It is likely that the large tracer prime at clamp onset results in a high rate of 3H₂O appearance and thus an elevated measurement of glycolytic rate.

The studies presented here compared several commonly used approaches to performing clamps in the mouse. By systematically evaluating individual clamp parameters, we have demonstrated that experimental variables affect results obtained with this technique. These effects are observed at different times throughout the clamp. Thus, the standard practice of reporting data as an experimental average does not always provide a thorough description of the events occurring throughout the entire clamp. A search of publications in which the insulin clamp was used in mice also shows a disturbing trend toward neither describing nor citing a reference that describes how experiments are performed. The results of the present studies show that this is a serious deficit, making comparisons of results from different laboratories impossible. It is also reasonable to suggest that genetic manipulations and strain differences may modulate the degree to which certain experimental parameters influence clamp results. Thus, the effects observed in wild-type mice may be masked or enhanced in transgenic mice (28,40) or mice of different strains. Because of these factors, it is necessary that time course changes in glucose and GIR be given as well as descriptions of how clamps are performed so that results obtained from this very powerful technique can be fully used.

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