Hyperinsulinemic euglycemic step clamping with tracer glucose infusion and labeled glucose infusate for assessment of acute insulin resistance in pigs

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Gjessing PF, Fuskevåg O, Hagve M, Revhaug A, Irtun Ø. Hyperinsulinemic euglycemic step clamping with tracer glucose infusion and labeled glucose infusate for assessment of acute insulin resistance in pigs. Am J Physiol Endocrinol Metab 298:E1305–E1312, 2010. First published April 6, 2010; doi:10.1152/ajpendo.00701.2009.—The present study aimed to establish hyperinsulinemic euglycemic step clamping with tracer glucose infusion and labeled glucose infusate (step hot-GINF HEC) for assessment of acute insulin resistance in anesthetized pigs and to arrange for combination with invasive investigative methods. Tracer enrichment was measured during D-[6,6-2H2]glucose infusion before and after surgical instrumentation (n = 8). Insulin dose-response characteristics were determined by two step hot-GINF HEC procedures, with accordingly labeled glucose infusates performed at a total of six insulin infusion rates ranging from 0.2 to 2.0 mU·kg⁻¹·min⁻¹ (n = 8). Finally, three-step hot-GINF HEC (0.4, 1.2, and 2.0 mU·kg⁻¹·min⁻¹) was performed subsequent to major surgical trauma (n = 8). Trajectory enrichment, basal glucose kinetics, and circulating levels of C-peptide, cortisol, glucagon, and catecholamines were not influenced by surgical instrumentation. Mean intra-individual coefficient of variance levels for glucose infusion rates and repeatedly measured insulin, glucose, and tracer enrichment indicated stable clamping conditions. Basal and maximal insulin-stimulated glucose utilization was twice as high as in humans at ~5.5 and 21 mg·kg⁻¹·min⁻¹. Surgical trauma elicited pronounced peripheral and moderate hepatic insulin unresponsiveness (45% lower whole body glucose disposal and 19% less suppressed endogenous glucose release) and apparently diminished metabolic insulin clearance. Step hot-GINF HEC seems suitable for assessment of acute insulin resistance in anesthetized pigs, and combination with invasive investigative methods requiring surgical instrumentation can be accomplished without the premises for utilization of the technique being altered, but attention must be paid to alterations in metabolic insulin clearance.

porcine insulin dose-response characteristics; insulin unresponsiveness and insensitivity; tracer dilution technique; surgical trauma

THE PHENOMENON OF INSULIN RESISTANCE is most commonly known as a chronic metabolic hallmark of type 2 diabetes mellitus. Acute insulin resistance, on the other hand, arises after any major trauma and has been shown to increase complication rates and even mortality among critically ill surgical intensive care patients (27). Despite increasing interest and research on acute insulin resistance, the underlying mechanisms are still elusive, and further research is needed.

Hyperinsulinemic euglycemic clamping (HEC) was first introduced in 1979 by DeFronzo et al (7) and is still regarded as the gold standard in measurement of insulin sensitivity in vivo. The technique is based on infusion of insulin at a constant rate, whereas simultaneous infusion of glucose is titrated to euglycemia. Aside from non-insulin-dependent glucose uptake and residual endogenous release of glucose, the steady-state (SS) glucose infusion rate (GIR) equals the amount of glucose utilized in peripheral insulin-sensitive tissues, and thus, HEC gives a picture of whole body insulin sensitivity.

The liver and peripheral tissues generally exhibit different degrees of insulin sensitivity (17), and insulin resistance can be caused by unresponsiveness or insensitivity to the hormone or a combination of both (19). Therefore, hot-GINF HEC performed at stepwise increasing insulin infusion rates, so-called step clamping, gives more detailed information about sites, degrees, and specific features of insulin resistance than a clamp performed at a single insulin infusion rate. Furthermore, when combined with other tracers and experimental procedures, including invasive techniques such as regional or organ-specific flowmetry and arteriovenous balance measurements, HEC also is a versatile tool for investigation of various non-glucose-related domains of the metabolic effects of insulin (6, 10).

Accordingly, the aim of this study was to establish step hot-GINF HEC as a tool for assessment of hepatic and peripheral insulin sensitivity in studies on acute insulin resistance in anesthetized pigs. Second, to arrange for combination of step hot-GINF HEC with invasive research methods, we wanted to investigate whether surgical instrumentation would alter basal glucose kinetics and thereby change the premises for correct labeling of the glucose infusates.
E1306 ASSESSMENT OF ACUTE INSULIN RESISTANCE IN PIGS

MATERIALS AND METHODS

Animals

The protocols were approved by the committee of the Norwegian Experimental Animal Board, and all experiments were conducted in compliance with institutional animal care guidelines and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services (NIH) Publication No. 85-23, revised 1985]. Male Yorkshire/Landrace hybrid pigs weighing 30 ± 5 kg were stalled and acclimatized in the animal research facilities for 1 wk before experiments. Animals were submitted to a 12:12-h light-dark cycle, a standardized diet, and ad libitum access to water. Experiments were commenced between 7 and 8 AM after an overnight fast.

Anesthesia and Monitoring

After sedation by intramuscular injection of 15 mg/kg ketamine (Narcanet; Véloquinol, Ittigen, Switzerland), 1 mg/kg midazolam (Midazolam B. Braun; B. Braun, Melsungen, Germany), and 1 mg of atropine (Atropin; Nycomed Pharma, Asker, Norway), the pigs were cleaned and weighed. Following mask inhalation of 4% isoflurane (Isofluran Baxter; Baxter, Irvine, CA) in 100% O2, the pigs were orotracheally intubated and gas anesthesia was continued throughout the experiments at a minimal alveolar concentration of 0.8–1.5% mixed with 40–60% oxygen. Deep anesthesia was induced through an iv bolus of 0.01 mg/kg fentanyl (50 µg/ml Fentanyl-Hameln; Hameln Pharmaceuticals, Hameln, Germany) and maintained with iv infusion of 0.02 mg·kg⁻¹·h⁻¹ fentanyl and 0.3 mg·kg⁻¹·h⁻¹ midazolam. Respiration and minimal alveolar concentration were monitored through a Datex Capnomac ultima instrument (Datex, Tewksbury, MA) and adjusted according to blood gas analysis (ABL 800 FLEX; Radiometer, Copenhagen, Denmark) and snout reflex tests. Arterial pressure was measured through a 20-gauge arterial catheter (BD Arterial Cannula with FloSwitch; Ohmeda, Swindon, UK) and placed in the superficial saphenous artery and was continuously monitored (BD Arterial Cannula with FloSwitch; Ohmeda, Swindon, UK) and adjusted according to blood gas analysis (ABL 800 FLEX; Radiometer, Copenhagen, Denmark) and snout reflex tests.

Infusions

The pigs were equipped with an 18-gauge iv catheter (Optiva 2 18 G; Medex Medical, Haslingden, UK) in an ear vein for infusion of 0.9% sodium chloride (9 mg/ml Natriumklorid; B. Braun), fentanyl, midazolam, insulin aspart (NovoRapid; Novo Nordisk, Copenhagen, Denmark), 20% glucose (200 mg/ml glucose; Fresenius Kabi, Upperville, MA) and adjusted according to blood gas analysis (ABL 800 FLEX; Radiometer, Copenhagen, Denmark) and snout reflex tests. Arterial pressure was measured through a 20-gauge arterial catheter (BD Arterial Cannula with FloSwitch; Ohmeda, Swindon, UK) placed in the superficial saphenous artery and was continuously monitored (BD Arterial Cannula with FloSwitch; Ohmeda, Swindon, UK) and adjusted according to blood gas analysis (ABL 800 FLEX; Radiometer, Copenhagen, Denmark) and snout reflex tests.

Sampling and Chemical Analysis

Serum insulin was measured 30 min prior to the end of the basal period and every 30th min during the last hour of the successive clamp periods. Anesthesia was discontinued, the pigs were extubated, and after a period of 4 days the procedure was repeated with three different insulin infusion rates. On the basis of unpublished preliminary data, insulin infusion rates at 0.2, 0.8, and 1.6 mU·kg⁻¹·min⁻¹ were chosen to cover a wide array of serum insulin levels in the pig.

Study Design

The study was divided into three separate substudies (Fig. 1). In substudies A and B, eight pigs were examined in two stages separated by a 4-day interval. In substudy C, eight pigs were examined once. Substudy A was designed to yield prerequisite information on basal SS tracer enrichment in solely anesthetized pigs (control) for correct labeling of the step hot-GINF HEC glucose infusates. Second, we investigated whether surgical instrumentation (instrumentation), necessary for combination of step hot-GINF HEC with invasive research methods, would require a change in labeling of the glucose infusates due to alterations in basal glucose kinetics by elevated counterregulatory hormones. Substudy B was designed to delineate porcine insulin dose-response characteristics and to determine optimal insulin infusion rates for assessment of changes in hepatic and peripheral insulin sensitivity. Substudy C was designed to test the suitability of the step hot-GINF HEC technique for assessment of acute insulin resistance after major surgical trauma.

Study design substudy A. The glucose tracer was given as a 150-min long primed (6 mg/kg), continuous (0.12 mg·kg⁻¹·min⁻¹) infusion. The priming dose and infusion rate were calculated from the exponential decline of tracer enrichment after bolus injection of D-[6,6-²H₂]glucose (pilot study; data not shown). Glucose kinetics were calculated based on tracer enrichment measured during the last 30 min of infusion. Circulating concentrations of insulin, cortisol, glucagon, epinephrine, norepinephrine, and C-peptide were measured 10 min before and 30 min prior to the end of tracer infusion. The pigs were reanesthetized after a period of 4 days, and the procedure was repeated after surgical instrumentation, consisting of a median laparotomy and groin incision followed by dissection of the portal vein, common hepatic artery, right renal artery and vein, superior mesenteric artery and vein, and right femoral artery and vein. Instrumentation as described and two-layer closure of the incisions added up to a total duration of ~1 h.

Study design substudy B. Primed, continuous tracer infusion (basal period) was commenced 150 min prior to and continued throughout three consecutive clamp periods. Three 120-min long clamps were performed at stepwise increasing insulin infusion rates while glucose (200 mg/ml) labeled with tracer at an APE according to the measured level of basal SS enrichment from substudy A was titrated to euglycemia at ~4.5 mmol/l. Tracer enrichment in arterial blood was measured every 10th min during the last 30 min of the basal and successive clamp periods. Serum insulin was measured 30 min prior to the end of the basal period and every 30th min during the last hour of the successive clamp periods. Circulating concentrations of cortisol, glucagon, epinephrine and norepinephrine, C-peptide, and free fatty acids (FFA) were measured 30 min prior to the end of the basal and clamp periods. Anesthesia was discontinued, the pigs were extubated, and after a period of 4 days the procedure was repeated with three different insulin infusion rates. On the basis of unpublished preliminary data, insulin infusion rates at 0.2, 0.8, and 1.6 mU·kg⁻¹·min⁻¹ were chosen to cover a wide array of serum insulin levels in the pig.

Study design substudy C. A three-step clamp with insulin infusion rates at 0.4, 1.2, and 2.0 mU·kg⁻¹·min⁻¹ was performed after major surgical trauma consisting of a right-sided thoracotomy, midline laparotomy, total colectomy, and closure of the incisions. Tracer enrichment in arterial blood was measured every 10th min during the last 30 min of the basal and successive clamp periods. C-peptide was measured 30 min prior to the end of the basal and clamp periods. Serum insulin was measured 30 min prior to the end of the basal period and every 30th min during the last hour of the successive clamp periods.

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Roche Modular E 170 analyzer (Roche Diagnostics, Basel, Switzerland). Plasma FFA were measured using a Wako HR series NEFA-HR(2) colorimetric assay kit (Wako Diagnostics, Richmond, VA) and a Wallac Victor2 1420 Multilabel Counter (EG & G Wallac, Turku, Finland). For catecholamine measurements, blood with heparin (4 IU/ml), reduced glutathione (4.5 mmol/l), and EGTA (5 mmol/l) were kept on ice for 30 min before centrifugation at 1,000 \( g \) for 20 min at 4°C. Preparation and analysis of plasma were performed with the Plasma Catecholamine Kit (order no. 5000; Chromsystems, Munich, Germany) with a Dionex P680 and ASI-100 HPLC equipment (Dionex, Germering, Germany) and electrochemical detector (ESA Coulochem III; ESA Laboratories, Chelmsford, MA).

**Determination of Tracer Enrichment**

**Chemicals.** HPLC grade acetonitrile was supplied by Merck (Darmstadt, Germany). Milli-Q grade water was produced by a Millipore system (Millipore, Bedford, MA). \( ^{13} \)C-glucose was obtained from Isotec (Miamisburg, OH). Stock solutions of glucose, \( ^{6,6-2H_2} \)glucose, and \( ^{13} \)C-glucose were prepared in water with concentrations of 33.69, 2.45, and 12.3 mM, respectively. A seven-point calibration curve ranging from 0.1 to 6.4 mmol/l for glucose and from 2 to 128 \( /H_9262 \)M for \( ^{6,6-2H_2} \)glucose was prepared by dilution of the stock solutions in water. Quality control samples were prepared in the same manner. Samples were stored at 70°C before analysis. The method showed good linearity for the calibration curve, with \( r^2 \geq 0.99 \).

**Sample preparation and liquid chromatography-tandem mass spectrometry.** Protein precipitation was performed with the addition of 300 \( \mu l \) of ice-cold acetonitrile and 20 \( \mu l \) internal standard (1 mM) to 50 \( \mu l \) of plasma samples, calibrators, and controls, followed by vortexing in 10 s and centrifugation for 5 min at 10,000 \( g \) at 4°C. Preparation and analysis of plasma were performed with the Plasma Catecholamine Kit (order no. 5000; Chromsystems, Munich, Germany) with a Dionex P680 and ASI-100 HPLC equipment (Dionex, Germering, Germany) and electrochemical detector (ESA Coulochem III; ESA Laboratories, Chelmsford, MA).

**Fig. 1. Study design.** Control, anesthesia only; instrumentation, surgical instrumentation; tracer infusion, primed, continuous infusion of \( ^{6,6-2H_2} \)glucose; APE, blood sampling for analysis of \( ^{6,6-2H_2} \)glucose atom % enrichment; insulin, blood sampling for analysis of insulin; CRH/C-P, blood sampling for analysis of counter-regulatory hormones and C-peptide; FFA, blood sampling for analysis of free fatty acids. **Substudy A:** 8 pigs were examined in 2 stages separated by a 4-day interval. Basal steady-state tracer enrichment, stress hormone levels, and glucose kinetics were assessed first during tracer infusion in anesthetized condition only (control) and second after surgical instrumentation (instrumentation). **Substudy B:** 8 pigs were examined in 2 stages separated by a 4-day interval. Two step hot-GINF HEC (hyperinsulinemic euglycemic clamping) experiments were performed at a total of 6 different insulin infusion rates to delineate porcine insulin dose-response characteristics. **Substudy C:** A 3-step clamp with insulin infusion rates selected on the basis of the results from **substudy B** was performed after major surgery in 8 pigs to test the suitability of the technique for assessment of acute insulin resistance.
**ASSESSMENT OF ACUTE INSULIN RESISTANCE IN PIGS**

Innovative Methodology

**RESULTS**

**Substudy A**

**Tracer enrichment and glucose kinetics.** The mean intradinidueel coefficient of variation (CV) of the four tracer enrichment and serum glucose measurements ranged from 4.7 ± 0.4 to 7.3 ± 1.4%, indicating SS conditions (Table 1). Mean tracer enrichment and serum glucose were not statistically different before (control) and after surgical instrumentation (instrumentation) of the pigs, and glucose kinetics remained unchanged with EGR of 5.62 ± 0.16 and 5.45 ± 0.30 mg·kg⁻¹·min⁻¹ and WGD of 5.65 ± 0.15 and 5.55 ± 0.30 mg·kg⁻¹·min⁻¹, respectively.

**Circulating hormone concentrations.** All measured hormone concentrations, including counterregulatory hormones, remained unchanged, indicating a lack of stress responses to the surgical instrumentation (Table 2).

**Substudy B**

**Insulin dose-response characteristics.** The timelines of GIR, tracer APE, and serum glucose concentrations during the step clamps are shown in Fig. 2. As shown in Table 3, mean glucose concentration was significantly higher during the basal period of the first step clamp compared with the basal period of the second step clamp, but both concentrations were well within the normal fasting range. The mean intradinidueel CV of serum glucose and tracer enrichment during the last 30 min of each step clamp ranged from 3.2 ± 0.5 to 5.3 ± 1.6% and indicated SS conditions. The corresponding values for GIR during the last 40 min of each clamp also indicated SS conditions, except for the first clamp in each step clamp. At insulin infusion rates of 0.2 and 0.4 mU·mg⁻¹·kg⁻¹, GIR slowly continued to rise, resulting in mean intradinidueel CV of 24.5 ± 4.0 and 20.9 ± 6.9%, respectively. Circulating insulin levels were linearly correlated to rates of insulin infusion, ranging from 8.7 ± 0.5 µU/ml during 0.2 mU·kg⁻¹·min⁻¹ to a maximum of 31.6 ± 1.1 µU/ml during 2.0 mU·kg⁻¹·min⁻¹. The corresponding curve for SS GIR during the last 40 min of each clamp was more sigmoid shaped and reached a near-maximal level of 18.18 ± 1.0 mg·kg⁻¹·min⁻¹ at an insulin infusion rate of 1.2 mU·kg⁻¹·min⁻¹, followed by a slow ascent toward 20.46 ± 0.75 mg·kg⁻¹·min⁻¹ at 2.0 mU·kg⁻¹·min⁻¹ (Table 3). All calculated values for EGR and WGD put together revealed a relative difference in insulin responsiveness of the liver and peripheral tissues with abruptly diminishing EGR at modest insulin infusion rates, whereas higher infusion rates were needed to elicit a strong increase in WGD (Fig. 3). Based on the determined insulin dose-response characteristics and the linear relationship of infused vs. circulating insulin, the calculated half-maximal effective insulin doses for EGR and WGD were ~9 and 15 µU/ml, respectively.

**Circulating hormone and FFA concentrations.** Significant fluctuations of circulating glucagon during the step clamp procedures were detected. Except for these variations, no significant differences in hormone concentrations were observed between or within each experiment. Mean plasma FFA decreased to approximately one-half of basal levels and remained stable during the step clamps, probably due to insulin inhibition of lipolysis (Table 4).

**Substudy C**

**Insulin and C-peptide.** Compared with the second step clamp in **substudy B**, serum insulin was significantly higher during all clamps despite identical insulin infusion rates. As in

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**Table 1. Substudy A: tracer enrichment and glucose kinetics**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer APE, %</td>
<td>2.11 ± 0.05</td>
<td>2.19 ± 0.09</td>
</tr>
<tr>
<td>CV Tracer APE, %</td>
<td>5.4 ± 0.4</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>WGD, mg·kg⁻¹·min⁻¹</td>
<td>5.65 ± 0.15</td>
<td>5.55 ± 0.30</td>
</tr>
<tr>
<td>EGR, mg·kg⁻¹·min⁻¹</td>
<td>5.62 ± 0.16</td>
<td>5.45 ± 0.30</td>
</tr>
<tr>
<td>S-glucose, mmol/l</td>
<td>4.66 ± 0.19</td>
<td>4.54 ± 0.25</td>
</tr>
<tr>
<td>CV S-glucose, %</td>
<td>4.7 ± 0.4</td>
<td>5.6 ± 1.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. APE, atom % enrichment; CV, coefficient of variation; WGD, whole body glucose disposal; EGR, endogenous glucose release; S-glucose, serum glucose. Control, anesthesia only; instrumentation, surgical instrumentation. No significant difference noted between control and instrumentation.

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**Table 2. Substudy A: circulating hormone concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Instrumentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, µU/ml</td>
<td>7.5 ± 0.7</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>C-peptide, ng/ml</td>
<td>0.13 ± 0.05</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Cortisol, nmol/l</td>
<td>57.5 ± 20.32</td>
<td>59.0 ± 12.7</td>
</tr>
<tr>
<td>Glucagon, pmol/l</td>
<td>16.4 ± 1.2</td>
<td>16.1 ± 1.7</td>
</tr>
<tr>
<td>Epinephrine, nmol/l</td>
<td>0.56 ± 0.13</td>
<td>0.47 ± 0.10</td>
</tr>
<tr>
<td>Norepinephrine, nmol/l</td>
<td>0.47 ± 0.08</td>
<td>0.33 ± 0.04</td>
</tr>
</tbody>
</table>

Data are means ± SE. No significant difference noted between control and instrumentation.
substudy B, endogenous insulin release was completely abolished by the exogenous insulin infusion, as indicated by undetectable levels of C-peptide (Table 3), and therefore, it can be concluded that the increase in circulating insulin was probably due to a reduction in metabolic insulin clearance.

Glucose kinetics and tracer enrichment. Although a greater intraindividual variation in tracer enrichment was seen during the postoperative step clamp than during clamps in substudy B, the mean intraindividual CV of serum glucose, tracer enrichment, and GIR together indicated adequately stable clamping conditions (Table 3 and Figs. 2–4). Postoperative mean basal glucose concentration was significantly higher than during the second step clamp \( (P < 0.05) \) but not compared with the first step clamp in substudy B. GIR, tracer APE, and serum glucose concentration timelines for all step clamps are shown in Fig. 2. Due to higher levels of circulating insulin during substudy C than during substudy B, not all glucose kinetics data were directly comparable (Fig. 3). Therefore, differences in metabolic effects of low and high insulin were calculated by comparison of data from postoperative clamps 1 and 2 to the data from clamps 2 and 6 in substudy B, since the respective levels of circulating insulin during these clamps were similar. Too high insulin levels rendered the third postoperative clamp not directly comparable with any clamp in substudy B. Postoperatively, GIR was 56 and 31% (both \( P < 0.001 \)) lower during low and high insulin, respectively. WGD was 45 and 28% lower (both \( P < 0.001 \)) during low and high insulin, respectively, and even during postoperative clamp 3, WGD was 13% \( (P < 0.05) \) lower than during clamp 6 in substudy B despite twice as high insulin levels. Calculated as percent change from basal, significantly less increase in WGD was measured postoperatively during both low and high insulin \( (170 \pm 25 \text{ vs. } 39 \pm 5\%, P < 0.001, \text{ and } 275 \pm 24 \text{ vs. } 156 \pm 15\%, P = 0.001) \), and EGR was significantly less suppressed by insulin after surgery during low \( (66 \pm 2 \text{ vs. } 85 \pm 5\%, P < 0.01) \) but not during high insulin \( (84 \pm 6 \text{ vs. } 91 \pm 5\%, P = 0.39) \).

**DISCUSSION**

This study reports for the first time implementation of step hot-GINF HEC for assessment of acute hepatic and peripheral insulin sensitivity in a porcine model and determination of insulin dose-response characteristics for endogenous glucose release and utilization in anesthetized pigs by this technique. Step hot-GINF HEC can be used in both operated and surgically untouched animals, making it suitable for studies on acute insulin resistance. It is characterized by high reproducibility, flexible and controllable glucose and insulin concentrations, and easy combination with complementary investigative techniques such as different tracers, indirect calorimetry, and regional artero-venous balance (8, 10). When performed during anesthesia, clamping can also be combined with other invasive procedures that are hardly feasible in conscious animals.

Various hyperinsulinemic clamps have previously been applied in porcine experiments to examine different metabolic effects of insulin. Insulin sensitivity and glucose metabolism in particular have been assessed with clamping alone or in combination with glucose tracers, and in a limited number of studies tracer enrichment was kept constant by either increased infusion rates during clamping or addition of tracer to the glucose infusion (1, 3, 21). Most often, single clamps were performed, and insulin infusion rates differed widely among studies. Also, the age and race of the pigs, anesthetic regime or conscious restraint, and hormonal and metabolic substrate manipulation varied with experimental design. Owing to the relatively small amount and limited comparability of existing data, we sought to establish and test the step hot-GINF HEC technique before implementation in porcine studies on acute insulin resistance.

Because considerable differences regarding basal glucose kinetics exist between species, specific tracer glucose infusion rates are necessary to achieve adequate tracer enrichment levels. Anesthesia in general alters glycemic homeostasis, and inhalation anesthetics in particular are known to diminish glucose-stimulated insulin secretion (24). If combined with invasive experimental techniques, these basal premises for utilization of step hot-GINF HEC might additionally be altered by elevated counterregulatory hormones due to surgical instrumentation. Therefore, in substudy A, basal glucose kinetics and levels of tracer enrichment and counterregulatory hormones were measured during tracer infusion in a uniform group of eight pigs submitted to a standardized anesthetic regime. After
### Table 3. Substudies B and C: insulin, C-peptide, glucose kinetics, and tracer enrichment during step clamps

<table>
<thead>
<tr>
<th>Substudy B</th>
<th>Step hot-GINF HEC 1</th>
<th>Step hot-GINF HEC 2</th>
<th>Substudy C</th>
<th>Step hot-GINF HEC after surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Clamp 1</td>
<td>Clamp 2</td>
<td>Clamp 3</td>
</tr>
<tr>
<td>IIR, mU·kg⁻¹·min⁻¹</td>
<td>6.3 ± 0.4</td>
<td>8.7 ± 0.5</td>
<td>16.0 ± 0.6</td>
<td>25.9 ± 1.0</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>12.7 ± 4.2</td>
<td>5.3 ± 2.2</td>
<td>7.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>CV insulin, %</td>
<td>0.22 ± 0.05</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td></td>
</tr>
<tr>
<td>C-peptide, mg/ml</td>
<td>6.00 ± 0.30</td>
<td>6.77 ± 0.42</td>
<td>14.97 ± 1.05</td>
<td>19.78 ± 0.88</td>
</tr>
<tr>
<td>WGD, mg·kg⁻¹·min⁻¹</td>
<td>6.01 ± 0.30</td>
<td>3.65 ± 0.46</td>
<td>0.97 ± 0.34</td>
<td>0.71 ± 0.39</td>
</tr>
<tr>
<td>EGR, mg/kg·min⁻¹</td>
<td>2.91 ± 0.55</td>
<td>13.98 ± 1.00</td>
<td>19.20 ± 0.74</td>
<td></td>
</tr>
<tr>
<td>CV GIR, %</td>
<td>24.5 ± 4.0</td>
<td>3.4 ± 0.5</td>
<td>4.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>S-glucose, mmol/l</td>
<td>4.98 ± 0.19</td>
<td>4.65 ± 0.14</td>
<td>4.47 ± 0.03</td>
<td>4.66 ± 0.05</td>
</tr>
<tr>
<td>CV S-glucose, %</td>
<td>4.9 ± 2.4</td>
<td>4.3 ± 0.6</td>
<td>4.1 ± 0.4</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Tracer APE, %</td>
<td>2.00 ± 0.10</td>
<td>2.65 ± 0.14</td>
<td>2.63 ± 0.07</td>
<td>2.47 ± 0.03</td>
</tr>
<tr>
<td>CV Tracer APE, %</td>
<td>4.7 ± 0.8</td>
<td>5.2 ± 1.2</td>
<td>3.2 ± 0.5</td>
<td>4.1 ± 0.9</td>
</tr>
</tbody>
</table>

Data are means ± SE. HEC, hyperinsulinemic euglycemic clamping; IIR, insulin infusion rate; GIR, glucose infusion rate. Significant differences by dependent and independent t-test: *P < 0.001 vs. corresponding value in step hot-GINF HEC 2; #P < 0.001 vs. clamp 2 step hot-GINF HEC 1; †P < 0.001 vs. clamp 6 step hot-GINF HEC 2; &P < 0.05 vs. clamp 6 step hot-GINF HEC 2; eP < 0.01 vs. clamp 2 step hot-GINF HEC 1; fP < 0.01 vs. basal step hot-GINF HEC 1; gP < 0.05 vs. basal step hot-GINF HEC 2.

In substudy B, it could be argued that an actual SS was never achieved during clamp periods 7 and 4, as glucose infusion rate (GIR) was not maintained at a constant level. However, this is consistent with a recent study (20), indicating that basal unaltered serum glucose levels were unaffected. In substudy C, the unaltered serum glucose level was reduced by 10.2 ± 0.33 mg/kg/min. Thus, the unaltered S-glucose level was reduced in all cases, and as glucose infusion rates were not maintained at a constant level, this resulted in a significant change in glucose concentrations in circulating concentrations of insulin, glucagon, and epinephrine. The interplay of these factors may explain why serum insulin, glucagon, and epinephrine concentrations were lower in the unaltered SS glucose infusion rate as compared to the glucose infusion rate in the SS glucose infusion rate. However, the unaltered serum glucose levels were not significantly different from the glucose infusion rate in the SS glucose infusion rate, and the interplay of these factors may explain why serum insulin, glucagon, and epinephrine concentrations were lower in the unaltered SS glucose infusion rate as compared to the glucose infusion rate in the SS glucose infusion rate. Therefore, the unaltered serum glucose level was reduced in all cases, and as glucose infusion rates were not maintained at a constant level, this resulted in a significant change in glucose concentrations in circulating concentrations of insulin, glucagon, and epinephrine. The interplay of these factors may explain why serum insulin, glucagon, and epinephrine concentrations were lower in the unaltered SS glucose infusion rate as compared to the glucose infusion rate in the SS glucose infusion rate.

**Fig. 3.** Substudy B & C: glucose infusion rate (GIR) and whole body glucose disposal (WGD) from both step hot-GINF HEC procedures in substudy B (anesthesia only; n = 18) and the step hot-GINF HEC performed after major surgery (n = 10). Data are means ± SE.
slowly continued to rise. However, clamping conditions were accepted as adequately stable considering the stable serum glucose and tracer enrichment levels and the reasonable results. Compared with other species, pigs exhibit a high rate of insulin clearance (20), and despite a relatively high maximal insulin infusion rate at 2.0 μU·kg⁻¹·min⁻¹, a mean serum insulin concentration of only 31.6 μU/ml was achieved. However, this was sufficient to elicit peak metabolic efficiency in the liver and peripheral tissues and totally diminish endogenous insulin secretion, demonstrated by undetectable levels of C-peptide. The experiments were performed using the modified insulin aspart, which in pilot studies from our own laboratory exhibited equal metabolic effects but higher clearance rates than native human and porcine insulin (data not shown).

Circulating levels of cortisol, glucagon, and catecholamines remained stable within physiological ranges normally seen in conscious pigs used in biomedical research (16). Infusion of insulin during the step clamp approximately halved plasma FFA from basal levels. A downward trend, but no statistically significant difference in circulating FFA, was detected between the three consecutive clamp procedures. Thus, influence by the classical counterregulatory hormones or the glucose-fatty acid cycle (14) on the measured insulin dose-response characteristics is unlikely. The finding that the liver is about two times more sensitive to insulin than peripheral tissues is not unique to pigs but in accord with human insulin dose-response characteristics (17). Also in accord with previous data (1, 21), the basal glucose turnover rate and maximal rate of glucose utilization were approximately two times higher than those found in similar studies in man (17).

Reduced insulin responsiveness is indicated by a shift of the insulin dose-response curve to the right and should therefore be measured with hot-GINF HEC at serum insulin corresponding to the end of the steepest part of the dose-response curves. Insulin insensitivity, on the other hand, is best detected at higher insulin levels, where EGR is almost completely suppressed and WGD is reaching its limits. This does not necessarily require tracer methodology, provided normal suppression of EGR. Apparently, diminished insulin clearance resulted in higher postoperative serum insulin levels, precluding a direct step clamp comparison between substudies B and C. Clamp data compared on the basis of matching serum insulin levels revealed pronounced peripheral and moderate hepatic insulin unresponsiveness after surgery. Furthermore, WGD persistently failed to reach the maximal rate measured in solely anesthetized pigs, even at twice the level of serum insulin, indicating a component of peripheral insulin insensitivity. Postoperatively, no significant change in suppression of EGR was measured during high serum insulin (Fig. 4). These results are in accord with human studies on acute insulin resistance after elective surgery (25).

Published data on insulin clearance after trauma have been contradictory. It has been reported to be increased in patients with multiple injuries (2), unchanged after severe scald injury in rats (15), and decreased during hypovolemic shock in primates (4), indicating the possibility for both species and trauma variations. The mechanism of diminished insulin degradation is not apparent from the present study. However, metabolic insulin clearance during physiological hyperinsulinemia is inseparably linked to insulin action, since binding to the insulin receptor constitutes the first step of degradation (9) and substantially decreased insulin clearance has been demonstrated in patients suffering from type 2 diabetes mellitus, obesity, and other conditions characterized by severe target cell insulin resistance (13, 26).

In conclusion, step hot-GINF HEC is a feasible technique for assessment of insulin sensitivity in anesthetized pigs and seems well suited for porcine research models on acute insulin resistance, but attention must be paid to alterations in metabolic insulin clearance. Combination with invasive research techniques requiring extensive surgical instrumentation can be accomplished without circulating levels of counterregulatory hormones, basal glucose kinetics, or tracer enrichment levels being altered. The insulin dose-response characteristics established in the present study suggest appropriate insulin infusion

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**Table 4. Substudy B: counterregulatory hormone and FFA concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Step Hot-GINF HEC 1</th>
<th></th>
<th>Step Hot-GINF HEC 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Clamp 1</td>
<td>Clamp 2</td>
<td>Clamp 3</td>
</tr>
<tr>
<td>IIR, mU·kg⁻¹·min⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol, nmol/l</td>
<td>27.0 ± 9.5</td>
<td>8.8 ± 3.5</td>
<td>31.5 ± 21.3</td>
<td>22.9 ± 13.5</td>
</tr>
<tr>
<td>Glucagon, pmol/l</td>
<td>13.9 ± 1.1</td>
<td>18.2 ± 1.4*</td>
<td>14.7 ± 1.7</td>
<td>13.3 ± 1.4</td>
</tr>
<tr>
<td>Epinephrine, nmol/l</td>
<td>0.47 ± 0.15</td>
<td>0.47 ± 0.16</td>
<td>0.56 ± 0.16</td>
<td>0.57 ± 0.20</td>
</tr>
<tr>
<td>Norepinephrine, nmol/l</td>
<td>1.30 ± 0.15</td>
<td>1.39 ± 0.32</td>
<td>1.55 ± 0.46</td>
<td>1.41 ± 0.43</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>117.3 ± 23.9</td>
<td>71.6 ± 15.8</td>
<td>69.8 ± 16.3</td>
<td>66.0 ± 18.7</td>
</tr>
</tbody>
</table>

Data are means ± SE. FFA, free fatty acids. Significant differences by repeated-measures analysis of variance. *P < 0.05 vs. basal.
rates for detection of hepatic and peripheral insulin unresponsiveness and insensitivity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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