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Repeatability and reproducibility of the hyperinsulinemic-euglycemic clamp and the tracer dilution technique in a controlled inpatient setting

Duc Son N.T. Le*, Thomas Brookshire, Jonathan Krakoff, Joy C. Bunt

Obesity and Diabetes Clinical Research Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, AZ 85016, USA

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Abstract

The objective of the study was to evaluate the reproducibility and repeatability of the combined use of the hyperinsulinemic-euglycemic (H-E) clamp and tracer dilution techniques. Ten nondiabetic men underwent a low-dose (40 mU/[m^2 min]) H-E clamp that was repeated within 3 to 4 days using porcine or human insulin in a double-blinded, randomized, crossover design. Coefficients of variation (CVs) for intraindividual differences and repeatability coefficient were calculated to evaluate reproducibility and repeatability. The Bland and Altman method was used to quantify repeatability. The CVs for intraindividual differences were 5.7% ± 3.5% for steady-state (SS) insulin; 6.7% ± 6.2% and 54.2 ± 38.3% for basal and SS endogenous glucose product (EGP), respectively; and 10.3% ± 8.5% for total insulin-stimulated glucose disposal (M) values. Basal EGP, SS EGP, and SS glucose and insulin concentrations were similar for the 2 clamps; but glucose infusion rate (P = .02) and M (borderline significant, P = .06) were higher in the first clamp than the second clamp. No significant correlations between mean of differences and average of basal and SS EGP, SS insulin concentration, and M between the 2 clamps were observed. We also found that the different values were less than the repeatability coefficients of these parameters and that the 95% limits of agreement and the interval of repeatability coefficient of these parameters were similar. There were no differences in metabolic responses between clamps when compared by the type of insulin (porcine vs human) infused. Our findings indicate that, although SS EGP has a high CV, the clamp, which measures insulin action (ie, SS insulin, M), and the tracer dilution technique for assessing basal EGP are repeatable and reproducible. Decreased glucose infusion rate and M over a short period in the second clamp may reflect an accumulative effect of continued physical inactivity.

1. Introduction

Insulin resistance and compensatory hyperinsulinemia are risk factors of type 2 diabetes mellitus [1]. The hyperinsulinemic-euglycemic (H-E) clamp procedure is the best method for measuring insulin action in vivo and involves infusion of insulin at a constant rate (determined by body size) while maintaining the glucose at a fixed concentration. It is also reported that dietary intake, changes in body weight, and physical activity level are confounders when assessing changes in insulin action over time.

To evaluate 2 procedures or 2 measurements on the same subjects, investigation of both reproducibility and repeatability is recommended [2]. The reproducibility, measured by the coefficient of variation (CV), indicates whether or not the means of repeated measurements are close. The CV indicates how strong the agreement is between the replicate measurements. Repeatability may be an even more appropriate evaluation in this case, as repeatability is a measure of the variation in measurements under the same conditions. Repeatability is investigated by comparing the mean and standard deviation (SD) of the difference between the measurements. When the mean of the differences in the measurement is not different from zero and when 95% of the differences are within 2 SDs, the repeatability can be considered adequate. In addition, repeatability can be examined by calculating a repeatability coefficient ("Subjects and methods") to determine if measurements fall within the range of this calculation. The reproducibility of the clamp procedure has been evaluated [3-6]. However, these studies report the intraindividual CVs for steady-state (SS) insulin

* Corresponding author. Tel.: +1 602 200 5345; fax: +1 602 200 5335.
E-mail address: leds@ttdinhduong.org (D.S.N.T. Le).

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[4,6], glucose infusion rate (GIR) [4,5], or total insulin-stimulated glucose disposal (M) value [6] separately. Furthermore, determining the repeatability of the clamp technique using the Bland and Altman method, a more appropriate statistical approach to evaluate repeatability [2,7], is limited. Only 1 study [5] thus far has used this statistical method, but the repeatability coefficient [8,9] was not estimated in this study. Finally, updated information is useful for calculating statistical power in intervention studies where insulin action is a main outcome variable.

To estimate endogenous glucose production (EGP) during the glucose clamp procedure, the "cold" method (using single 3-[^{3}H] glucose tracer and unlabeled D20 glucose) and "hot" method (using both tritiated glucose tracer and labeled D20 glucose to measure constant specific activity) have been compared. Several investigations indicate that the hot method is more accurate than the cold method for estimating EGP [10,11]. However, because of patient exposure to additional radiation, the cold method is still widely applied during the clamps. Yet, assessment of the reproducibility of this technique is lacking.

Therefore, the aims of this study were to evaluate the reproducibility and repeatability of the H-E clamp technique including measures of EGP with the cold single tracer (3-[^{3}H] glucose) dilution technique using the Bland and Altman approach in an inpatient setting with controls over both diet and physical activity levels.

2. Subjects and methods

2.1. Subjects

This study includes 10 men (2 whites and 8 full-blooded Native Americans) with a mean ± SD age of 31 ± 10 years. All participants were healthy according to physical examination and routine laboratory tests. None of the subjects took medication known to affect glucose or insulin metabolism for at least 1 month before the study. After giving written informed consent, subjects were admitted to the Clinical Research Unit where they were fed a weight-maintaining diet (50% of calories from carbohydrate, 30% from fat, and 20% from protein) and abstained from strenuous exercise. After at least 3 days on the diet, the percentage of body fat and the oral glucose tolerance status were assessed. Thereafter, insulin action and basal and SS EGP were measured during H-E clamps and tracer dilution techniques twice in each 3 to 4 days apart. The second clamp was run by different individuals who did not know the results of the previous clamp. In addition, human or porcine insulin was administered in a double-blinded, randomized, cross-over design. All insulin infusions were prepared by the same individual who was not involved in administering any of the clamp procedures. The study was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and by the Tribal Council of the Gila River Indian Community.

2.2. Anthropometric measurements

Body composition was assessed by total body dual-energy x-ray absorptiometry (DPX-L; Lunar Radiation, Madison, WI) to determine percentage of body fat. Calculations of fat mass and fat-free mass were derived from these measurements [12].

2.3. Oral glucose tolerance test

After a 12-hour overnight fast, glucose tolerance was determined by a 75-g oral glucose tolerance test with measurement of fasting, 30-minute, and 2-hour glucose and insulin concentrations and classified according to the 2003 American Diabetes Association diagnostic criteria [13].

2.4. H-E glucose clamp with tracer dilution technique

Insulin action, using either porcine or human insulin, was assessed during a standard H-E glucose clamp that has been used since our ongoing longitudinal study was initiated in 1982 [14]. Because of the discontinuation of porcine insulin, a within-study comparison of porcine vs human insulin was conducted to establish internal validity for our longitudinal database. The experimental procedures have been described previously [15,16]. Briefly, after an overnight fast, a primed (30 μCi) continuous [0.3 μCi/min] 3-[^{3}H] glucose infusion (prepared and determined to be free of contamination at the Clinical Center Department of Nuclear Medicine of the National Institutes of Health [NIH], Bethesda, MD) was started to determine basal and SS EGP. At least 2 hours after starting the isotope infusion, a primed continuous intravenous insulin infusion was administered for 100 minutes at a constant rate of 40 mU/[m^2 min]. Blood samples for measurement of 3-[^{3}H] glucose specific activity (Beckman LS6500 scintillation counter; Beckman Instruments, Fullerton, CA) were collected at the end of the basal period and every 10 minutes during the final 40 minutes of insulin infusion. The specific activity of all plasma samples were adjusted for background specific activity measured in samples collected before the bolus and infusion of 3-[^{3}H] glucose. Under basal conditions, basal EGP was calculated as the 3-[^{3}H] GIR divided by the SS plasma 3-[^{3}H] glucose specific activity. During the insulin clamp, SS EGP was the difference between the appearance rate of glucose in the plasma calculated from 3-[^{3}H] glucose measurements during insulin infusion using the Steele non-SS equation [17] and the GIR. Although negative numbers for SS EGP may occur if GIR is greater than the appearance rate of glucose, these are usually interpreted as total suppression of hepatic glucose production and given a value of 0. However, for this analysis, SS EGP was allowed to be negative to investigate the full range of CVs. The M value was calculated for the last 40 minutes of the insulin infusion and corrected for SS glucose and insulin plasma concentrations for which CVs were less than 3%. The total M value derived from the glucose clamp was normalized to estimated metabolic body size (EMBS, or fat-free mass +17.7 kg) [18].

2.5. Analytic procedures

Plasma glucose concentrations were determined by the glucose oxidase method (Beckman Instruments) and plasma insulin concentrations by an automated analyzer (ICN Radiochemicals, Costa Mesa, CA).

2.6. Statistical analyses

All data are presented as mean ± SD. Nonnormally distributed variables were log transformed before statistical analysis to approximate a normal distribution. Paired *t* tests were used to compare means and to test for significant differences in each metabolic parameter between the 2 clamps either by insulin type or clamp order. Pearson product moment and Spearman rank correlations of metabolites were also calculated for the 2 clamps by clamp order. To estimate the repeatability for the clamp measurements, the means of difference against average values were plotted for each metabolic parameter. This quantifies the repeatability of a method from a replicated measurement obtained by the same method [8,9], and the within-subject SD and repeatability coefficient (2.77 * within-subject SD) were calculated [8,9]. Finally, the intraindividual CVs were calculated as SD divided by mean for each variable to determine the degree of reproducibility for 2 consecutive clamp procedures. All statistical analyses were done using the programs of the SAS Institute (Cary, NC). A *P* value less than .05 was considered significant.

3. Results

All subjects were nondiabetic, but two had both impaired fasting glucose and impaired glucose tolerance (fasting plasma glucose range, 86-113 mg/dL; 2-hour glucose concentrations, 89-148 mg/dL). The study population was young (age, 18-46 years) and had a wide range of body size (body weight, 78-128 kg; percentage of body fat, 22%-36%).

3.1. Reproducibility of the H-E clamp and tracer dilution technique

The intraindividual CVs were 6.7% ± 6.2% for basal EGP, 54.3% ± 38.3% for SS EGP, 5.7% ± 3.5% for SS plasma insulin concentration, 16.0% ± 14.4% for GIR, and 10.3% ± 8.5% for total M values.

3.2. First vs second clamp and the repeatability of the H-E clamp and tracer dilution technique

There were no differences in basal EGP, SS EGP, and SS plasma glucose and insulin concentrations between the first and second clamps (Table 1). Both GIR ($P = .02$) and total M ($P = .06$) were higher in the first clamp than the second clamp, regardless of insulin type (Table 1). Correlations between the basal ($r = 0.47$, $P = .05$) and SS ($r = 0.58$, $P = .05$) EGP, SS plasma insulin concentration ($r = 0.75$, $P = .01$), and M value ($r = 0.79$, $P = .006$) for the 2 clamps are shown in Fig. 1. The mean differences for SS insulin, SS EGP, and M value between the 2 clamps are shown in Table 2 and are not significantly different from zero. The plots of the differences against average of SS plasma insulin concentration (SS insulin) and total M value that are visual checks for variability of differences between the 2 measurements are also shown (Fig. 2). No significant correlations between the differences and averages of these parameters were found ($r = -0.24$, $P = .5$ for basal EGP and $r = -0.21$, $P = .6$ for SS EGP; $r = -0.04$, $P = .9$ for SS insulin concentration; and $r = 0.56$, $P = .09$ for M value). Using the log-transformed value of M, the correlation between the differences and the average of M was still not significant ($r = 0.51$, $P = .13$). The 95% limits of agreement and the repeatability coefficients of these parameters are also shown in Table 2 and show that all measurements fell within the range of the repeatability coefficients.

Table 1
Metabolic parameters between the first and second clamps

Variable	Mean (SD)	Range	*P* value
Basal EGP (mg/[kg FFM min])			.32
1st clamp	2.53 (0.24)	2.06-2.78	
2nd clamp	2.43 (0.26)	1.89-2.79	
SS glucose concentration (mg/dL)			.33
1st clamp	101 (3)	97-106	
2nd clamp	101 (6)	94-110	
SS insulin concentration (μU/mL)			.7
1st clamp	58 (8)	44-68	
2nd clamp	57 (8)	48-74	
GIR (mg/[kg FFM min])			.02
1st clamp	5.63 (2.51)	2.51-10.64	
2nd clamp	4.48 (1.80)	2.23-8.13	
SS EGP (mg/[kg FFM min])			.15
1st clamp	−0.64 (0.71)	−1.75 to 0.63	
2nd clamp	−0.26 (0.84)	−1.83 to 0.87	
Total M (mg/[EMBS min])			.06
1st clamp	4.55 (1.58)	2.36-7.61	
2nd clamp	4.02 (1.11)	2.50-5.76	
Suppress HGO (%)			.12
1st clamp	87 (25)	41-131	
2nd clamp	76 (15)	54-101	

Total M value was normalized to EMBS, or FFM + 17.7 kg, and corrected for SS insulin and glucose concentrations. All statistical analyses were performed on log-transformed values. Analyses were done using paired *t* test. FFM indicates fat-free mass; HGO, hepatic glucose output; EGP, endogenous glucose product; EMBS, estimated metabolic body size; SS, steady-state.

3.3. Human vs porcine insulin

Randomization resulted in half of the subjects (n = 5) receiving porcine insulin for the first clamp. There were no significant differences in basal (2.55 ± 0.19 vs 2.43 ± 0.29, $P = .2$) and SS (−0.39 ± 0.80 vs −0.67 ± 1.11, $P = .4$) EGP, SS plasma glucose (102 ± 4 vs 101 ± 5, $P = .6$) and insulin (58 ± 8 vs 56 ± 8, $P = .3$) concentrations, GIR (4.86 ± 1.87 vs 5.23 ± 2.49, $P = .5$), and total M (4.14 ± 1.03 vs 4.29 ± 1.40,

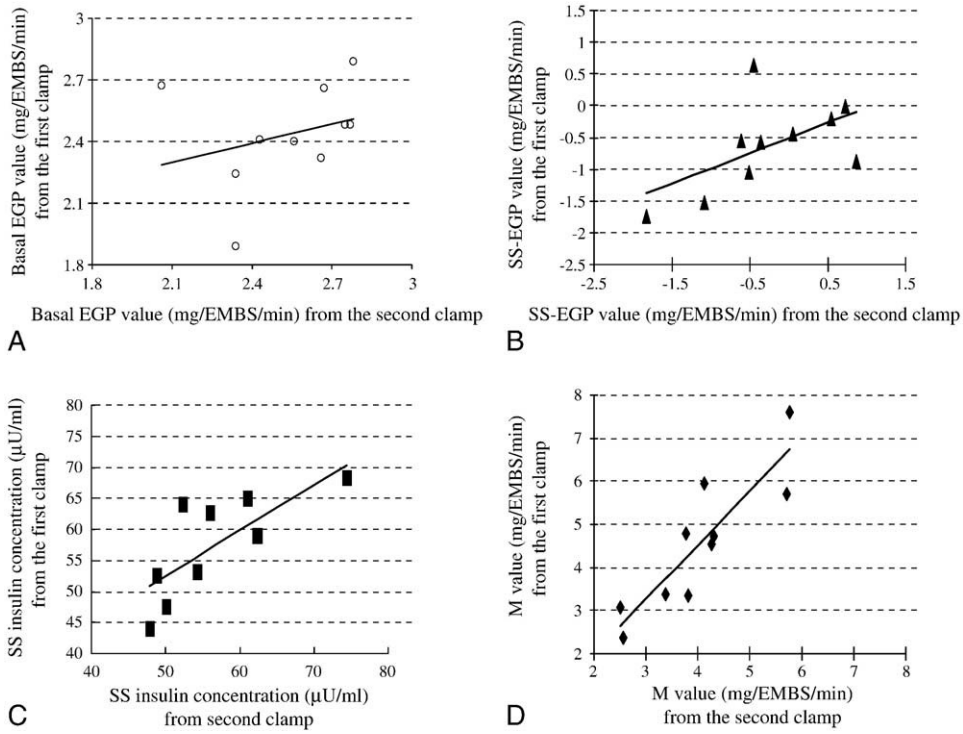

Fig. 1. Correlations of basal and SS EGP (A and B), SS plasma insulin concentration (C), and total M value (D) between 2 H-E clamps.

$P = .6$) values between the 2 clamps regardless of whether human or porcine insulin was administered in either the first or second clamp (Fig. 2).

4. Discussion

First, in agreement with earlier studies [19-26], we found that metabolic responses and insulin action in healthy people are similar during the H-E clamp when using either porcine or human insulin. Because of this interchangeability, we were able to evaluate the acute effect of an additional few days of physical inactivity on measurement of insulin action with the H-E glucose clamp. When the clamp procedure was repeated within 3 to 4 days after relative physical inactivity, GIR and total M value were consistently lower during the second clamp. Despite this trend, using a valuable approach, namely, the Bland and Altman plot, measures of basal EGP using the tracer dilution technique and insulin action using the H-E clamp are repeatable and reproducible.

We investigated the reproducibility of both the clamp and the tracer dilution techniques. The CVs for basal EGP (6.7%), SS plasma insulin concentration (5.3%), M (10%), and GIR (16%) were acceptable, indicating that the tracer dilution (in basal conditions) and the clamp techniques are reproducible. Our CVs were comparable with those obtained from previous investigations [3-6]. Compared with CVs of SS insulin concentration and M values obtained from DeFronzo et al [6] (3.4% and 2.6%, respectively), our CVs were higher. However, in the study of DeFronzo et al, the same type of insulin (porcine insulin) was used for both clamps and all participants were lean and normoglycemic, whereas in our study, we performed clamps on both lean and obese and both normal and impaired glucose-tolerant individuals using different types of insulin (human and porcine insulin). In other studies to investigate the variability and reproducibility of the H-E clamp in 32 middle-aged men [5] and in 7 middle-

Table 2
Metabolic values between the 2 clamps

Variables	Basal EGP	SS EGP	SS insulin	M
Mean difference between the 2 clamps[a] (1st − 2nd clamp)	0.10 (0.29)	−0.37 (0.73)	0.66 (5.69)	0.53 (0.80)
Average value of the 2 clamps	2.49 (0.20)	−0.45 (0.69)	57.25 (7.57)	4.28 (1.30)
95% Limits of agreement	−0.47 to 0.67	−1.80 to 1.06	−10.49 to 11.81	−1.04 to 2.10
Repeatability coefficient	±0.80	±2.02	±15.76	±2.22

[a] Not significantly different from zero.

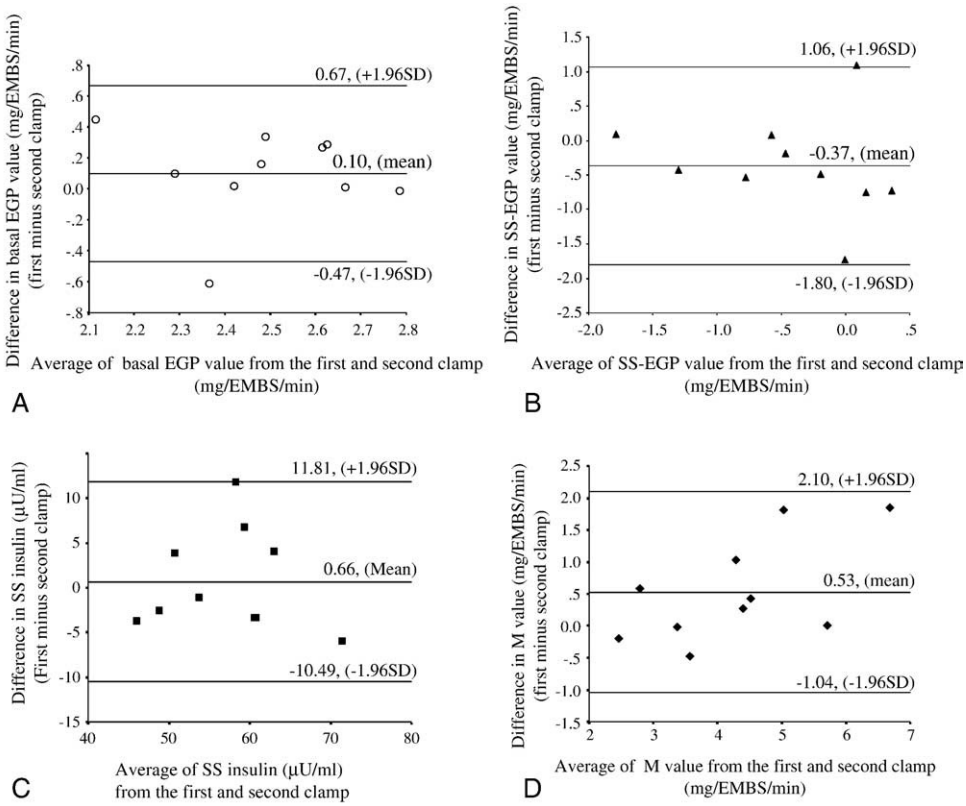

Fig. 2. The different values of basal and SS EGP (A and B), SS insulin concentration (C), and total M value (D) between 2 H-E clamps plotted against the average values of the 2 examinations.

aged healthy lean individuals [4] using human insulin, the CVs of GIRs were 15% and 5.8%, respectively. However, these studies did not report the CVs of other parameters of insulin action such as basal or SS EGP, SS insulin concentration, and M value [3-5]. In our study, we showed that the CVs were 6.7% for basal EGP and a much higher 54% for SS EGP. This higher variability in EGP during insulin infusion vs basal state may reflect a greater insulin sensitivity in the liver vs peripheral tissue. In fact, the human liver is exquisitely sensitive to small changes in insulin infusion [27]. Another explanation for the high CV for SS EGP is that SS EGP was calculated based on an exogenous GIR that varied during the clamp procedure. In addition, the range of SS EGP is narrow; thus, any small changes in absolute value may have a big effect on the calculated CV. Despite the high variability of EGP during insulin infusion (SS EGP), the total M (which includes both GIR and SS EGP) was not significantly different between clamps because SS EGP represents a relatively small proportion of total M (range, 0%-30%).

We also found that the clamp and the tracer dilution techniques were repeatable. In fact, to our knowledge, this is the first study to evaluate the repeatability of the clamp and tracer dilution techniques using the Bland-Altman method [8,9], which is a more appropriate and required approach to quantify the repeatability from replicated measurements obtained by the same method [7]. We did not find any significant correlations of the differences between the duplicate measurements and averages of these parameters derived from the 2 clamps (ie, basal and SS EGP, SS insulin concentration, and M value), indicating that any measurement error was constant. We also found that the different values were less than the repeatability coefficients of these parameters and that the 95% limits of agreement and the interval of repeatability coefficient of these parameters were similar. Taken together, this indicates that the clamp and the basal state tracer dilution techniques are repeatable.

In the comparison of human vs porcine insulin, our findings were consistent with previous studies performed in either diabetic or healthy individuals using either subcutaneous or venous injections or infusion during the clamp technique [19-26]. Despite the similarities in metabolic responses to porcine and human insulin, the GIR and total M values (borderline significant) were consistently higher in the first clamp compared with those in second clamp. Although factors such as dietary intake, changes in body weight, and physical activity level are potential confounders, dietary intake and body weight were controlled for throughout the study; and the level of physical activity (restricted to sedentary activities on our clinical research unit) was essentially unchanged across 3 to 4 days before the first through the second clamp. However, the accumulative effect of an additional 3- to 4-day period of inactivity may have affected the results of the second clamp. The association between physical activity and insulin sensitivity has been

well documented [28-30]. Recently, a longitudinal study using the clamp technique to measure insulin action indicated that physical activity was one of the important long-term predictors of insulin sensitivity [31]. Furthermore, the short-term effect of physical activity on lipids, especially for triglycerides and high-density lipoprotein 3, was reported [32]. Associations between increased triglyceride concentration and insulin resistance have been well documented [33]. Taken together, it is possible that an acute effect of sedentary activity was responsible for our observations. This has important implications for studies looking at the changes in insulin action over time. Specifically, differences in levels of physical activity between measurements can have a significant impact on results whether studies are in an inpatient or outpatient setting. In fact, failure to control for physical activity may explain the contrasting results of 2 investigations in which clamp procedures were performed at least 2 weeks apart in an outpatient setting and that showed higher SS GIRs in the second clamp than in the first clamp, indicating improved insulin action [5,6]. The other possibility is that a residual metabolic effect of the first clamp affected the second clamp because it was performed after a short interval. Consistent with our finding, Soop et al [4] reported that, with a 2-day interim, the GIR in the second clamp was lower than the first. The infusion of insulin may have a short-term effect on insulin action. In fact, comparison of subjects who did and did not receive insulin infusion 1 day before the clamp procedure demonstrated that those who received insulin infusion had higher fasting insulin and C-peptide concentrations. In addition, the total amount of glucose infused to achieve similar glucose concentration during the clamp was significantly lower in subjects who received insulin the prior day [34]. In addition, accumulation of insulin may result in an increased hepatic insulin resistance reflected by increased hepatic glucose production (as indicated by the higher EGP concentrations in our second clamp) [35].

Limitations of this study should be acknowledged. First, in this study, EGP was estimated using the single tracer dilution technique that has been reported to overestimate the suppression of glucose production and underestimate the stimulation of glucose [10,11]. However, this technique is still used globally to limit the radiation exposure for repeated-measures studies. Secondly, using a short period (ie, 100 minutes) may not be enough time to achieve an SS condition. However, 100 minutes is used as a standard period; and technically, it is unclear how long it takes before an SS can be achieved. Doberne et al [36] reported that, although clamp studies were carried out for 5 to 8 hours, the metabolic clearance rate continues to increase; thus, the authors suggested that comparisons of insulin sensitivity should be performed on different days, during the same period. The purpose of our study was to evaluate the repeatability, not the accuracy, of the H-E clamp using established standardized with identical conditions.

In conclusion, we confirm that the clamp procedure is reproducible and repeatable for measurements of whole-body insulin sensitivity. Although the tracer dilution technique is also reproducible and repeatable in the basal state, measurements of hepatic glucose output during insulin infusion is more variable. Therefore, if the main outcome of interest is to measure whole-body insulin action, a higher insulin infusion rate (eg, 80 mU/[m^2 min]) that can totally suppress EGP might be used without the tracer dilution technique during the clamp, with M calculated from the GIR. If hepatic insulin sensitivity is of specific interest, the hot tracer dilution technique would be preferable. Finally, our finding of lower whole-body insulin action in the second clamp may be due to the sedentary conditions of the inpatient setting. Therefore, the impact of variability in day-to-day or spontaneous physical activity should be accounted for in longitudinal or intervention studies conducted in either inpatient or outpatient settings.

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