Effects of Insulin on Glucose Turnover Rates In Vivo: Isotope Dilution Versus Constant Specific Activity Technique

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The conventional isotope dilution technique was compared with the more accurate constant specific activity (SA) method at six different insulin levels. Paired euglycemic clamp studies were performed in 30 normal subjects (4-hour insulin infusion: 5, 10, 20, 40, 80, and 160 mU · m^{-2} · min^{-1}) using primed-constant 3-^{3}H-glucose infusion and either conventional unlabeled glucose infusates (Cold-GINF) or labeled glucose infusates (Hot-GINF) to maintain constant SA. At all insulin levels, both glucose disappearance (R_d) and hepatic glucose production (HGP) were underestimated by the conventional technique, and errors during the first 2 hours correlated with glucose infusion rates (GIRs) (r = .93, P < .00001). During the second hour, mean underestimation of HGP varied from 20% ± 9% to 84% ± 16% of basal rates from low-dose to high-dose insulin infusion studies. During prolonged equilibration (3 to 4 hours), errors decreased but were still significant in the two low-dose insulin infusion protocols during the fourth hour. In conclusion, using the conventional isotope dilution technique, suppression of glucose production was overestimated and stimulation of glucose R_d was underestimated, and these errors were greater the higher the GIR. Thus, artifactually greater hepatic and smaller peripheral effects may have been assumed for factors or therapies that influence insulin sensitivity in previous studies using a conventional isotope dilution technique, and therefore, reevaluation of these issues may be relevant in future studies.

REGULATION of plasma glucose is of central importance in health and diabetes. Understanding this regulation requires sensitive and accurate methods that can measure the rates at which glucose is produced by the liver and used by the tissues.[1] Many issues of glucoregulation require assessment during non–steady-state.[2] Steele[3] in 1959 was the first to extend the primed-constant tracer infusion technique to the non–steady-state situation. Since then, numerous investigators have used the isotope dilution technique during non–steady-state in a variety of situations. Such experiments have led to the widely accepted belief that the liver is more sensitive to insulin than extrahepatic tissues.[4-9] Small increases in insulin have been reported to rapidly decrease hepatic glucose production (HGP) while having little apparent effect on glucose disappearance (R_d).[10,11]

However, in 1978 Allsop et al[12] compared tracer-determined rates with known glucose appearance rates (R_as) in hepatectomized, nephrectomized dogs in a variety of experimental situations and found that the conventional isotope dilution technique was inaccurate during all non–steady-state situations tested. In the same year, Radziuk et al[13] used high-dose glucose infusion based on the assumption that glucose production was thereby suppressed. They compared three different models for calculation of glucose R_a and found that a two-compartment model and a general-dispersion model performed no better than Steele's pool-fraction model. Absolute deviations of the calculated from the infused curves were 8.4%, 7.8%, and 9.5%, and it was concluded that the tracer infusion method can reliably measure R_a. It is important to realize that during such high glucose infusions, 10% errors for R_a translate into errors for endogenous glucose production of at least 50%, indicating a low degree of accuracy for glucose production. These two studies are the only attempts that has been made to validate the isotope dilution method, and in both cases the method appeared to be inaccurate. During the last decade, inappropriate negative rates for HGP have repeatedly been reported during high glucose infusion rates (GIRs) in euglycemic clamp studies.[9,14-20] Often, negative HGP rates have been considered a minor nuisance and positive rates assumed to be accurate. However, as suggested by Bergman et al,[21] negative HGP rates may represent only a symptom of a more general error in the conventional isotope dilution technique, and not only negative rates but also positive rates for HGP may be inaccurate. Based on theoretical analysis, Cobelli et al[22] and Norwich[23] suggested that if plasma specific activity (SA) is maintained constant during the non–steady-state experiment, then turnover rates can be accurately determined by the tracer technique. During the last few years, the constant SA approach has received renewed interest.[24-31] During euglycemic clamp studies, it is possible to maintain SA fairly constant by adding appropriate amounts of tracer to the glucose infusate (Hot-GINF).[27,29,32] Most recently, Bradley et al[33] have validated the constant SA approach by comparison to simultaneous measurements of hepatic arteriovenous balance during euglycemic clamp studies in dogs. Therefore, although the constant SA method has not yet received its final absolute validation, eg, comparison to known R_a in surgically prepared dogs as performed by Allsop et al,[12] it appears that more accurate turnover rates can be obtained with the constant SA method than have been achieved in previous studies with the conventional isotope dilution method.

Therefore, we found it appropriate to evaluate the magnitude and time course of errors in conventional isotope dilution studies by comparison to matched studies

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using the constant SA method. Paired euglycemic clamp studies were performed in 30 normal subjects over a wide range of physiological insulin concentrations using insulin infusions of 5, 10, 20, 40, 80, and 160 mU · m^{-2} · min^{-1}. For better illustration of differences in time courses, clamp studies were prolonged from the usual 2 hours' to 4 hours' duration. The study demonstrates that compared with the constant SA technique, glucose turnover rates were underestimated with the conventional technique at all insulin levels tested, and errors during the first 2 hours were larger the greater the GIR.

SUBJECTS AND METHODS

Subjects

Thirty healthy male subjects participated in the studies, with six subjects in each of the 5-, 10-, 20-, and 40-mU · m^{-2} · min^{-1} insulin infusion protocols. Six subjects participated in both the 80- and the 160-mU · m^{-2} · min^{-1} protocols in randomized order. In addition, eight subjects participated in a control study in which no glucose and insulin were infused, four from the 5-mU group, three from the 10-mU group, and one from the 40-mU group. Clinical characteristics of subjects in each protocol are listed in Table 1.

None of the subjects had a family history of diabetes, and all had normal results on screening blood tests of renal and hepatic function. The purpose and risks of the studies were carefully explained to all subjects before informed consent to participate was obtained. The protocol of the study was reviewed and approved by the regional ethics committee.

Oral Glucose Tolerance Test

Oral glucose tolerance tests were performed after a 10-hour overnight fast by giving each subject 75 g glucose dissolved in 300 mL water. Plasma was obtained at timed intervals from 20 minutes before until 180 minutes after the load for measurement of glucose and insulin levels.

Study Protocol

All subjects were studied twice, with at least a 4-week interval. On the first occasion, conventional unlabeled glucose infusates (Cold-GINF) were used for clamping, and on the second occasion, appropriate amounts of tracer were added to the glucose infusate (labeled glucose infusates [Hot-GINF]) to maintain plasma glucose SA at baseline level during the clamp. All studies began at 8 AM in the morning after a 10-hour overnight fast. Two catheters were inserted: one in an antecubital vein for infusion of insulin, glucose, and tracer, and another in a contralateral dorsal hand vein for collection of blood samples. During the studies, this hand was placed and maintained in a heated plexiglas box to obtain arterialized venous blood.[34] At 8 AM, after a 15-minute relaxation period, a primed-constant intravenous infusion of 3-^{3}H-glucose (DuPont-New England Nuclear, Boston, MA) was started and continued throughout the 6-hour study using a precision syringe pump (Harvard Apparatus, Natick, MA). The ratio of priming dose (10 mL) and constant infusion (0.1 mL · min^{-1}) was 100.[35] To achieve a common level of basal SA, the tracer infusion was adjusted for surface area by adjustment of infusate volume.[27] One vial of tracer was diluted in V mL 0.9% NaCl, where V was 120 mL divided by square meters of body surface area of the individual subject. After a 120-minute basal tracer equilibration period, insulin (Actrapid Human; NOVO, Bagsvaerd, Denmark) was infused at rates of 5, 10, 20, 40, 80, and 160 mU · m^{-2} · min^{-1} for 4 hours, and plasma glucose was maintained (clamped) at basal levels using a variable infusion of 18% glucose. In the first study, no tracer was added to the glucose infusates (Cold-GINF). In the second study, 3-^{3}H-glucose was added to the glucose infusates to maintain plasma SA constant at baseline levels during the clamp (Hot-GINF). Optimal labeling of the glucose infusate in the second study was calculated as previously described.[27] In this calculation, we used GIRs obtained in the preceding Cold-GINF studies, whereas glucose production rates of 40, 30, and 20 mg · m^{-2} · min^{-1} were assumed in 5-, 10-, and 20-mU · m^{-2} · min^{-1} insulin infusion studies. At the higher insulin infusion rates of 40, 80, and 160 mU · m^{-2} · min^{-1}, glucose production rates were assumed to equal zero.

During the studies, blood samples were collected in fluoride-treated tubes at timed intervals for determination of plasma glucose and plasma 3-^{3}H-glucose activity, and in heparin-trasylol–treated tubes for determination of plasma insulin. Blood samples were immediately centrifuged at 5°C, and plasma was stored at −20°C until assay.

Table 1. Clinical Characteristics of Subjects in Control Studies (no insulin or glucose infusion) and in Hyperinsulinemic-Euglycemic Clamp Studies Using Insulin Infusion Rates of 5, 10, 20, 40, 80, and 160 mU · m^{-2} · min^{-1}

Insulin Infusion Rate (mU · m^{-2} · min^{-1})	No. of Subjects	Age (yr)	Weight (kg)	Height (cm)	Body Mass Index (kg/m^2)
0	8	27 ± 2	79 ± 3	184 ± 4	23.4 ± 0.7
5	6	27 ± 2	81 ± 4	186 ± 4	23.3 ± 1.0
10	6	27 ± 2	79 ± 4	181 ± 4	24.0 ± 1.3
20	6	30 ± 4	82 ± 7	184 ± 4	24.1 ± 1.5
40	6	28 ± 2	80 ± 4	183 ± 2	23.7 ± 1.0
80	6	28 ± 4	77 ± 5	182 ± 4	23.1 ± 1.2
160	6	28 ± 4	77 ± 5	182 ± 4	23.1 ± 1.2

Assays

During the study, plasma glucose concentration was measured bedside using a glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). These values were used for adjustment of the variable glucose infusion during the clamp study. Calculations of glucose turnover rates were based on subsequent measurements of plasma glucose and 3-^{3}H-glucose activity on fluoride-treated samples, analyzed as previously described.[27] The purity of 3-^{3}H-glucose was evaluated using a high-performance liquid chromatography method.[36] This analysis revealed no labeled contaminants; all label was on glucose. Plasma insulin concentrations were measured using a double-antibody radioimmunoassay (Kabi Pharmacia Diagnostics, Uppsala, Sweden). Within-assay coefficient of variation was 5.6%, and total assay variation was 6.2%.

Calculations

Total glucose R_a and glucose R_d were calculated using Steele's non–steady-state equations[3,27]:

$$R_a = \frac{R_a^* - p \cdot V_D \cdot G(dSA/dt)}{SA} \quad (1)$$

and

$$R_d = R_a - p \cdot V_D \cdot \frac{dG}{dt}, \quad (2)$$

where R_a^* is the tracer infusion rate (counts per minute), SA is the specific activity of glucose in plasma (counts per minute per milligram), p is the pool fraction taken as 0.65.[37] V_D is the distribution volume of glucose taken as 200 mL · kg^{-1} body

weight,[38] and G is the plasma glucose concentration (milligrams per milliliter). In conventional Cold-GINF studies, R_a^* consists of the constant tracer infusion (I*) only, whereas in Hot-GINF studies, R_a^* is the sum of the constant tracer infusion rate (I*) and tracer infused with the Hot-GINF ($SA_{GINF} \cdot GINF$), where GINF is the glucose infusion rate (milligrams per minute) and SA_{GINF} is SA of the glucose infusate (counts per minute per milligram). Glucose production rates (HGP) were calculated as tracer-determined total glucose R_a minus exogenous GIR:

$$HGP = R_a - GIR. \quad (3)$$

From equation 3, it is clear that when HGP is underestimated, this error must be due to underestimation of tracer-determined R_a. Furthermore, from equation 2, it is seen that if R_a is underestimated in euglycemic studies, R_d is numerically equally underestimated. This is because at constant glycemia the derivative term in equation 2 reduces to zero. Thus, during constant glycemia, errors in HGP, R_a, and R_d will be numerically equal. Therefore, because more accurate rates are obtained when plasma SA is maintained constant,[33] errors in conventional isotope dilution studies were quantified as the difference between glucose production rates (HGP) in Hot-GINF and Cold-GINF studies. Relative errors for R_d were calculated as the difference in R_d (ΔR_d) between Hot-GINF and Cold-GINF studies corrected for differences in GIR, ie, $\Delta R_d\% = 100\,(Rd_{Hot} - Rd_{Cold} - [GIR_{Hot} - GIR_{Cold}])/Rd_{Hot}$.

In the past, several investigators have attempted to calculate the insulin concentrations that produce half-maximal (ED_{50}) stimulation of glucose disappearance and half-maximal suppression of HGP.[4-9,30] However, different approaches have been used for calculation of ED_{50} effects, and this may result in different ED_{50} values.[14] In the current studies, ED_{50} values were calculated after 2 hours for comparison to previous studies. ED_{50} for R_d was estimated as the insulin concentration that produced half-maximal stimulation of R_d above the baseline rate, assuming that R_d during the 160-mU study represents the maximum rate. ED_{50} for HGP was estimated as the insulin concentration that produced 50% suppression of basal HGP, assuming that HGP = 0 represents the maximal effect.

Statistical Analysis

All data are presented as the mean ± SEM. Statistical differences between Hot-GINF and Cold-GINF studies were evaluated using the Wilcoxon matched-pairs signed-rank test. Correlations were evaluated using the Spearman rank correlation test. Differences with $P < .05$ were considered significant.

RESULTS

Oral Glucose Tolerance Test

All subjects had normal fasting plasma glucose concentrations and normal glucose tolerance as evidenced by plasma glucose concentrations less than 6 mmol/L 2 hours after a 75-g oral glucose load. Fasting plasma insulin concentration averaged 6 mU/L and increased to a maximum of 57 ± 5 mU/L 30 minutes after the oral load, and thereafter was reduced. These peak insulin levels approximate the insulin levels of 60 mU/L obtained during the 40-mU $\cdot$ $m^{-2} \cdot min^{-1}$ insulin infusion.

Control Studies

Eight subjects participated in control studies in which no insulin or glucose was infused for 4 hours following the baseline period. During these 4 hours, glucose turnover decreased (HGP, 79 ± 2 to 72 ± 3 mg $\cdot$ $m^{-2} \cdot min^{-1}$; R_d, 80 ± 2 to 70 ± 3 mg $\cdot$ $m^{-2} \cdot min^{-1}$; both $P < .03$) and plasma glucose concentrations decreased slightly (5.0 ± 0.1 to 4.9 ± 0.1 mmol/L, $P < .04$), whereas plasma insulin did not change significantly (5.4 ± 0.5 to 4.6 ± 0.4 mU/L, $P = .07$).

Plasma Insulin and Glucose Concentrations and GIRs

Plasma insulin concentrations were at a mean level of 6 ± 1 mU/L during baseline. During the 4-hour insulin infusions, plasma insulin reached a stable plateau within 30 minutes and remained constant thereafter at 12 ± 1, 17 ± 1, 29 ± 2, 63 ± 3, 130 ± 5, and 310 ± 15 mU/L during insulin infusions of 5, 10, 20, 40, 80, and 160 mU $\cdot$ $m^{-2} \cdot min^{-1}$ (Fig 1). Mean plasma glucose was 5 mmol/L during baseline and was maintained unchanged during clamp studies. At each insulin level, plasma insulin and glucose concentrations were similar in paired Hot- and Cold-GINF studies both during the second and fourth hour.

Mean glucose infusions required to maintain euglycemia increased with increasing insulin dose (Fig 1). GIRs did not plateau during the first 2 hours, but continued to increase. Thus, at all insulin levels, GIRs during the fourth hour were larger than during the second hour (all $P < .03$; Table 2). GIRs in Hot- and Cold-GINF studies were not different (Table 2).

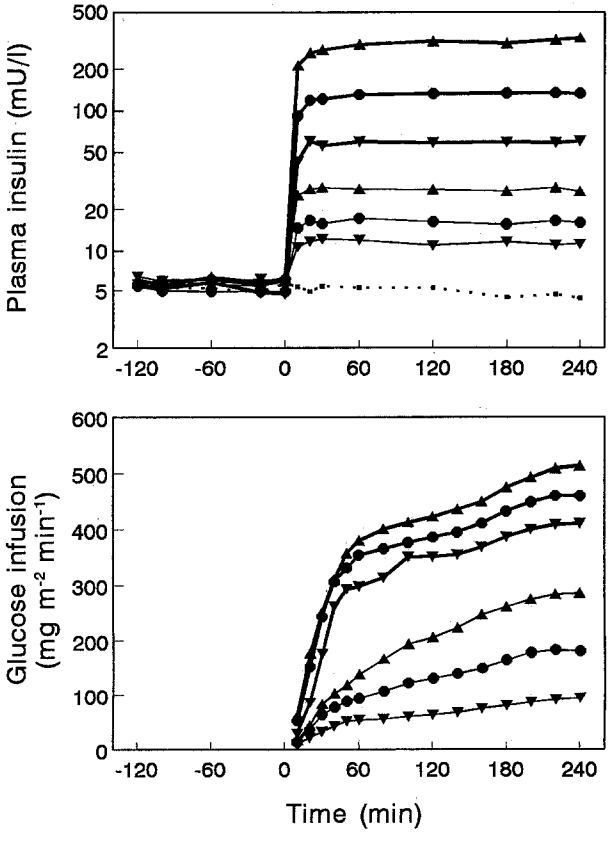

Fig 1. Mean plasma insulin concentrations and GIRs during euglycemic clamp studies using insulin infusions of 5 (▼), 10 (●), and 20 (▲) (thin lines) and 40 (▼), 80 (●), and 160 (▲) (thick lines) mU $\cdot$ $m^{-2} \cdot min^{-1}$. (·····) Concentrations in the control study. Error bars are omitted for clarity.

Table 2. GIR, Glucose R_a and R_d, and HGP (mg · m^{-2} · min^{-1}) during the Second and Fourth Hour of Euglycemic Clamp Studies Using Insulin Infusions of 0 (control study) or 5, 10, 20, 40, 80, and 160 mU · m^{-2} · min^{-1}

Insulin Infusion Rate (mU · m^{-2} · min^{-1})	Second Hour				Fourth Hour			
	GIR	R_a	R_d	HGP	GIR	R_a	R_d	HGP
0								
Control	0	75 ± 3	75 ± 3	75 ± 3	0	72 ± 3	70 ± 3	72 ± 3
5								
Hot-GINF	60 ± 4	109 ± 6*	111 ± 5*	50 ± 3*	91 ± 7	129 ± 5*	128 ± 6*	38 ± 3*
Cold-GINF	59 ± 5	92 ± 5	91 ± 5	33 ± 6	85 ± 6	108 ± 6	106 ± 5	23 ± 4
10								
Hot-GINF	120 ± 17	160 ± 22*	157 ± 20*	40 ± 6*	181 ± 20	204 ± 21*	199 ± 19*	23 ± 3*
Cold-GINF	92 ± 8	105 ± 8	107 ± 7	13 ± 3	154 ± 18	155 ± 16	151 ± 16	1 ± 4
20								
Hot-GINF	189 ± 34	221 ± 32*	216 ± 19*	32 ± 6*	281 ± 34	293 ± 32	292 ± 32	12 ± 4
Cold-GINF	173 ± 21	169 ± 17	169 ± 15	−4 ± 6	278 ± 35	275 ± 35	270 ± 32	−3 ± 8
40								
Hot-GINF	339 ± 49	357 ± 49*	354 ± 48*	18 ± 4*	407 ± 44	412 ± 43*	405 ± 44*	5 ± 3*
Cold-GINF	311 ± 25	275 ± 20	278 ± 19	−36 ± 6	389 ± 47	368 ± 20	372 ± 24	−21 ± 10
80								
Hot-GINF	376 ± 26	388 ± 24*	391 ± 24*	12 ± 3*	457 ± 25	463 ± 25	458 ± 25	6 ± 2
Cold-GINF	387 ± 26	340 ± 23	337 ± 21	−47 ± 9	449 ± 27	444 ± 30	444 ± 29	−4 ± 4
160								
Hot-GINF	412 ± 29	426 ± 28*	420 ± 26*	14 ± 7*	505 ± 37	509 ± 38	500 ± 35	4 ± 6
Cold-GINF	404 ± 20	355 ± 21	354 ± 17	−49 ± 7	452 ± 13	440 ± 9	437 ± 8	−12 ± 8

*$P < .05$: Hot-GINF *v* Cold-GINF studies.

Plasma Glucose SA

In control studies, plasma glucose SA increased from baseline to the second and fourth hour: 100% to 109% ± 1% and 115% ± 2% of baseline SA. In clamp studies using conventional unlabeled glucose infusates (Cold-GINF), plasma SA decreased in a dose-dependent manner and mean levels were 94% ± 3% and 80% ± 3%, 85% ± 4% and 59% ± 6%, 61% ± 7% and 36% ± 7%, 33% ± 3% and 23% ± 1%, 26% ± 2% and 19% ± 1%, and 23% ± 1% and 18% ± 1% of baseline SA during the second and fourth hour of the 5-, 10-, 20-, 40-, 80- and 160-mU clamp studies. In contrast, in labeled glucose infusates studies (Hot-GINF), plasma SA was maintained almost unchanged at 106% ± 4% and 105% ± 2%, 108% ± 7% and 104% ± 7%, 107% ± 4% and 105% ± 3%, 99% ± 4% and 99% ± 5%, 103% ± 2% and 101% ± 2%, and 100% ± 3% and 97% ± 2% of baseline SA during the second and fourth hour of the clamp in 5-, 10-, 20-, 40-, 80-, and 160-mU studies (Fig 2). This amelioration of changes in plasma SA was obtained using glucose infusates in which SA of the infusates was a mean of 46% ± 2%, 64% ± 2%, 76% ± 4%, 78% ± 4%, 78% ± 4%, 83% ± 2%, and 84% ± 3% of basal plasma SA, respectively.

Glucose Turnover Rates

The time course of HGP during six different insulin doses is illustrated in Fig 3. During high-dose insulin, markedly negative rates were calculated for HGP in Cold-GINF studies, whereas during low-dose insulin, negative rates were seldom encountered. Nevertheless, comparison to the paired Hot-GINF studies showed that HGP in Cold-GINF studies was underestimated at all insulin levels tested. However, the time course of errors differed between high- and low-dose studies. During high-dose insulin, initial errors were more marked but later reduced faster, whereas during low-dose insulin, initial errors were smaller but later reduced more slowly (Fig 3). Thus, during the second hour, errors were larger in high-dose (80 and 160 mU) than in medium-dose (20 and 40 mU) studies and smaller in low-dose (5 and 10 mU) studies (61 ± 4 *v* 45 ± 4 *v* 22 ± 5 mg · m^{-2} · min^{-1}, $P < .05$, high *v* medium and medium *v* low), and from the second to fourth hour, decreases in errors were larger in high-dose than in medium-dose and low-dose studies (47 ± 5 *v* 25 ± 5 *v* 3 ± 4 mg · m^{-2} · min^{-1}, $P < .05$, high *v* medium and medium *v* low).

During the first 2 hours of the clamp, the mean difference

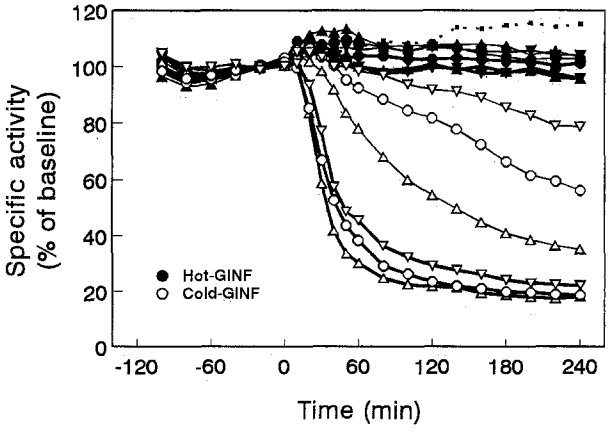

Fig 2. Plasma SA in percent of baseline levels during euglycemic clamp studies using Cold-GINF (open symbols) or Hot-GINF (closed symbols). Insulin infusions: 5 (▽), 10 (○), and 20 (△) (thin lines) and 40 (▽), 80 (○), and 160 (△) (thick lines) mU · m^{-2} · min^{-1}. (·····) SA in the control study. Error bars are omitted for clarity.

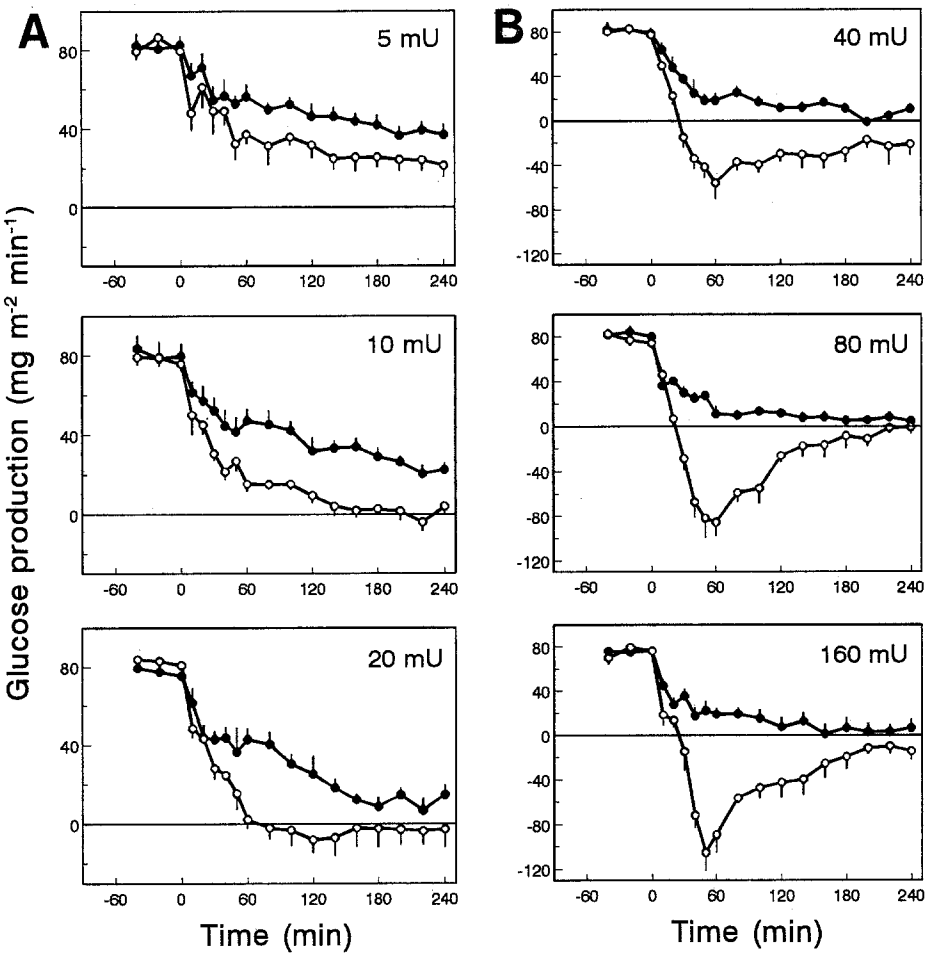

Fig 3. Glucose production rates in euglycemic clamp studies using Hot-GINF (●) and Cold-GINF (○) during insulin infusion rates of 5, 10, and 20 mU · m^{-2} · min^{-1} (A) and 40, 80, and 160 mU · m^{-2} · min^{-1} (B). Note that due to the larger errors in Cold-GINF studies during the 40-, 80-, and 160-mU studies, the scales on ordinates in A are smaller. Results are the mean ± SEM.

between HGP in Hot-GINF and Cold-GINF studies correlated with mean GIRs ($r = .93$, $P < .00001$; Fig 4). Also during the second hour, underestimation of HGP correlated with GIRs ($r = .75$, $P < .00001$), whereas no correlation was found during the fourth hour ($r = -.01$, $P = .95$).

Mean glucose turnover rates during the second and fourth hour are shown in Table 2. During the second hour, R_a, R_d, and HGP were lower in Cold-GINF than in Hot-GINF studies at all insulin levels. During the fourth hour, rates in Cold- and Hot-GINF studies were only significantly different in 5-, 10-, and 40-mU studies.

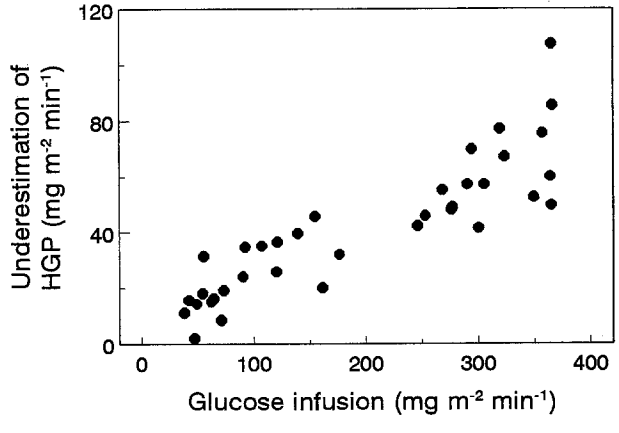

Fig 4. Relationship between mean GIRs and underestimation of HGP during the first 120 minutes of the clamp studies.

Assuming that rates determined in Hot-GINF studies were accurate, numerical underestimations of HGP in Cold-GINF studies were calculated as the difference between HGP in Hot-GINF and Cold-GINF studies (Table 3). Numerical underestimations of R_d in Cold-GINF studies were calculated as the difference between R_d in Hot-GINF and Cold-GINF studies corrected for differences in GIRs. Underestimations of R_d were of similar magnitude to underestimations of HGP at each insulin level. Relative errors for HGP determined during the second hour were greater the larger the insulin level, ranging from 20% ± 9% to 84% ± 16% of basal rates from low- to high-dose insulin. However, because R_d was greater the larger the insulin level, the relative underestimation of R_d was fairly constant at 15% to 18% across different insulin levels (Table 3).

Insulin Dose-Response Effect on R_d and HGP

In most previous studies, only 2 hours have been used for assessment of insulin action. Therefore, in dose-response curves in Fig 5, we have shown turnover rates determined during the second hour of clamp studies. Rates determined during the fourth hour are shown in Table 2. Figure 5A is

Table 3. Underestimation of Rates of HGP and Glucose R_d in Cold-GINF Studies During the Second and Fourth Hour

	Second Hour			Fourth Hour		
	Numerical Error	Relative Errors		Numerical Error	Relative Errors	
Insulin Infusion ($mU \cdot m^{-2} \cdot min^{-1}$)	ΔHGP ($mg \cdot m^{-2} \cdot min^{-1}$)	ΔHGP % of Basal HGP	ΔR_d (%)	ΔHGP ($mg \cdot m^{-2} \cdot min^{-1}$)	ΔHGP % of Basal HGP	ΔR_d (%)
5	17 ± 7	20 ± 9	15 ± 6	16 ± 4	18 ± 5	11 ± 3
10	26 ± 5	33 ± 6	16 ± 2	23 ± 6	27 ± 6	11 ± 3
20	36 ± 7	45 ± 8	18 ± 4	15 ± 9	19 ± 11	6 ± 3
40	54 ± 7	66 ± 7	16 ± 2	25 ± 12	30 ± 12	6 ± 2
80	58 ± 6	73 ± 8	15 ± 1	10 ± 5	13 ± 6	2 ± 1
160	63 ± 11	84 ± 16	15 ± 3	17 ± 9	22 ± 13	3 ± 2

Abbreviations: ΔHGP, underestimation of HGP in Cold-GINF *v* Hot-GINF studies; ΔR_d, underestimation of R_d in Cold-GINF *v* Hot-GINF studies.

the conventional plot using logarithmic insulin scale and different ordinate scales for R_d and HGP to illustrate relative changes. This representation is designed to estimate the half-maximal effective insulin dose (ED_{50}) for R_d and HGP. With Cold-GINF, the curve for suppression of HGP is shifted leftward and the curve for stimulation of R_d is shifted rightward compared with the curves from Hot-GINF studies. Based on the Cold-GINF curves, ED_{50} was estimated to be 10 mU/L for HGP and 43 mU/L for R_d, whereas based on the Hot-GINF curves, ED_{50} was 17 mU/L for HGP and 34 mU/L for R_d. Thus, with Cold-GINF the liver was four times and with Hot-GINF only two times more sensitive than peripheral tissues.

The same data are shown in Fig 5B, using a linear scale for insulin and similar ordinate scales for R_d and HGP to illustrate the quantitative aspects. This presentation illustrates that R_d and HGP were numerically equally underestimated in Cold-GINF studies. Furthermore, for the five higher insulin doses, stimulation of R_d in Hot-GINF studies was numerically much greater than suppression of HGP (all $P < .05$).

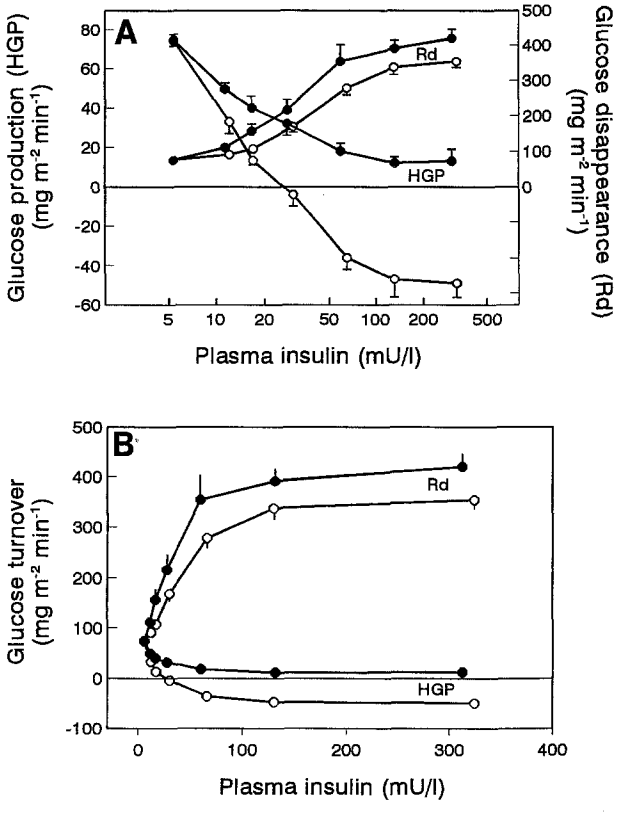

Fig 5. Insulin dose-response curves for glucose R_d and HGP determined after 2 hours in euglycemic clamp studies using Hot-GINF (●) or Cold-GINF (○). (A) Conventional presentation using log insulin scale and different ordinate scales for R_d and HGP to illustrate relative differences. (B) Linear insulin scale and common ordinate scale for R_d and HGP to illustrate absolute differences. Results are the mean ± SEM.

Insulin Action on R_d and HGP During Low-Dose Insulin Infusion

The time course of R_d and HGP during the low-dose insulin infusion study is shown in Fig 6. During insulin infusion of 5 $mU \cdot m^{-2} \cdot min^{-1}$, plasma insulin was elevated from basal levels of 6 mU/L to 12 mU/L during the clamp. Using a conventional tracer technique (Cold-GINF), this small insulin elevation apparently caused a marked suppression of HGP, whereas R_d remained almost unchanged. In contrast, constant SA studies demonstrate that changes in R_d and HGP were numerically similar (Fig 6). Thus, qualitatively different results may be obtained with the Cold-GINF and Hot-GINF technique, particularly at low physiologic insulin levels.

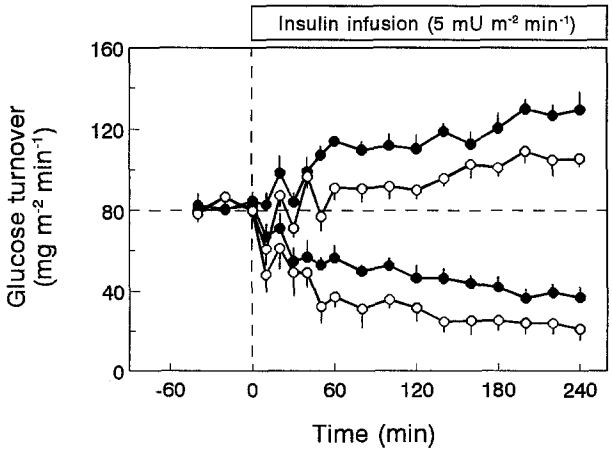

Fig 6. Time courses for stimulation of glucose R_d and suppression of HGP as determined using Hot-GINF (●) or Cold-GINF (○) during low-dose insulin infusion of 5 $mU \cdot m^{-2} \cdot min^{-1}$.

DISCUSSION

The current studies demonstrate that compared with the constant SA technique, glucose turnover rates were underestimated with the conventional isotope dilution technique at both high and low physiologic insulin levels. Thereby, stimulation of glucose disappearance was underestimated and suppression of glucose production was overestimated. It may be important to realize that if the conventional technique had not been compared directly with matched studies with constant SA, this underestimation would have gone unrecognized at the low insulin levels where HGP did not attain negative values. In addition, errors were shown to be both flux- and time-dependent. During the first 2 hours, errors with the conventional technique were greater the larger the GIR. Later, after 3 to 4 hours, errors decreased but were still significant in the low-dose insulin infusion studies.

Erroneous negative values for glucose production in conventional isotope dilution studies have been the subject of several previous studies. It has been suggested that these errors could be due to (1) presence of tracer contaminants in commercially available glucose tracers,[36,39] (2) an effect of tracer discrimination,[20,32] or (3) development of intercompartmental tracer gradients when plasma glucose SA is rapidly diluted during infusion of unlabeled glucose.[27] In the current studies, we used noncontaminated tracer. Isotope discrimination of ^{3}H-glucose moieties seems unlikely, since several studies have evaluated this question in vivo and have been unable to detect any significant effect of tracer discrimination.[26,40,41] The third suggestion is supported by several investigators, who suggested that errors in conventional isotope dilution studies were due to development of intercompartmental tracer gradients.[2,3,24,25,27] The problem seems to be that the glucose pool is not a single well-mixed pool, but rather consists of a small rapidly mixing plasma compartment and a much larger slowly mixing interstitial compartment.[42] During the basal tracer equilibration period, tracer and glucose are assumed to reach equilibrium, so that SA is similar in plasma and interstitial compartments. Then when unlabeled glucose is subsequently infused intravenously during the clamp, plasma SA is rapidly diluted, whereas interstitial SA may remain at a higher level for some time due to slow mixing in the interstitial compartment. Because the tracer will always move down its concentration gradient, there may be a net flux of tracer from the interstitial compartment back to the plasma, whereas during the clamp the net flux of glucose is from plasma to interstitial fluid and into cells. However, if the net flux of tracer differs from the net flux of glucose, then the tracer does not trace the net movement of glucose and obviously tracer-determined glucose turnover will be in error. Because only plasma concentrations are assessable, whereas interstitial fluid cannot be sampled, such gradients between interstitial compartments and plasma are concealed and can only be recognized by the errors they induce.

Previously, at a single insulin level, we have shown that in conventional isotope dilution studies the error patterns were as would be expected if errors were due to concealed gradients.[27] The current studies extend this observation to the whole range of physiological insulin concentrations. At low insulin levels, GIRs were relatively small and dilution of plasma SA was small and slow, whereas at higher insulin levels, GIRs were larger and dilution of plasma SA was more rapid and marked (Figs 1 and 2). During rapid dilution of plasma SA, larger initial gradients can be expected between interstitial compartments and plasma, resulting in greater initial errors than in studies in which dilution of plasma SA is smaller and less rapid. Furthermore, when glucose fluxes are large, later reduction of interstitial SA toward levels in plasma may proceed more rapidly and errors can be expected to reduce faster than when glucose fluxes are smaller. The current studies demonstrate that errors in conventional isotope dilution studies were both flux- and time-dependent, and the error patterns were as would be expected if errors were due to concealed gradients (Fig 3).

Several investigators have compared hepatic and peripheral insulin sensitivity in man by estimating the insulin concentrations that produce ED_{50} suppression of HGP and ED_{50} stimulation of glucose R_d (Table 4). Using different insulin infusions on separate days, three studies reported that HGP was four times more sensitive than R_d,[4-6] whereas two studies using sequential insulin infusions on a single day found HGP to be two times more sensitive than R_d.[7,8] These studies were performed using a conventional isotope dilution technique (Cold-GINF). Only one previous dose-

Table 4. Previous Studies Reporting ED_{50} Insulin Concentrations for Suppression of HGP and Stimulation of Glucose R_d in Normal Man

ED_{50} (mU/L)		Tracer	Clamp Duration	Study	Insulin Infusion Rates	
HGP	R_d	Method	(min)	Design	(mU · m^{-2} · min^{-1})	Reference
33	130	Cold-GINF	120	Single	15, 40, 120, 240, 1,200	4
30	120	Cold-GINF	120	Single	10, 20, 40, 200, 400*	5
20	90 ± 14	Cold-GINF	120	Single	15, 40, 120, 240, 1,200	6
29 ± 2	55 ± 7	Cold-GINF	120	Sequential	8, 20, 40, 80, 200*	7
26 ± 2	58 ± 5	Cold-GINF	120	Sequential	16, 40, 400*	8
14 ± 1	—	Cold-GINF	100	Sequential	4, 10, 20, 40, 100	9
16-19	29-33	Hot-GINF	180	Single	10, 24, 80*	30
11	43	Cold-GINF	120	Single	0, 5, 10, 20, 40, 80, 160	Current study
17	34	Hot-GINF	120	Single	0, 5, 10, 20, 40, 80, 160	Current study

*Insulin infusion rates originally reported in mU · kg^{-1} · min^{-1} have been converted to mU · m^{-2} · min^{-1} using a conversion factor of 40.

response study has used the constant SA technique. In this study, Katz et al[30] reported HGP to be two times more sensitive than R_d. In our own studies, HGP was four times more sensitive than R_d using the conventional technique, but only two times more sensitive than R_d using the constant SA method. However, although HGP was relatively less sensitive with Hot-GINF than with Cold-GINF, our ED_{50} values did not differ markedly from those previously reported. Thus, using ED_{50} analysis, the liver remained more sensitive than peripheral tissues. However, ED_{50} is only a qualitative measure, ie, the insulin concentration that produces a half-maximal effect, and does not include the fact that the maximal effect on R_d is quantitatively much greater (80 to 400 to 500 mg $\cdot$ m^{-2} $\cdot$ min^{-1}) than the maximal effect on HGP (80 to 0 mg $\cdot$ m^{-2} $\cdot$ min^{-1}). Therefore, the greater hepatic insulin sensitivity should not be taken to indicate that hepatic insulin action is more important than peripheral insulin action in glucose homeostasis. In the regulation of plasma glucose concentration, the balance between HGP and R_d, quantitative changes in HGP and R_d are important, not the qualitative sensitivity.

Quantitatively, insulin action on R_d and HGP differed markedly between Hot-GINF and Cold-GINF studies, in particular at low physiological insulin levels (Figs 5 and 6). Previous studies have suggested that low-dose insulin rapidly suppresses HGP while having little apparent effect on R_d.[10,11] Our studies confirm that such results are obtained with the conventional isotope dilution technique, but simultaneously demonstrate that such evidence may be incorrect due to underestimation of turnover rates with this technique (Fig 6). Paired studies with constant SA showed that stimulation of R_d and suppression of HGP were of similar magnitude during very-low-dose insulin infusion (Fig 6), and that stimulation of R_d was greater than suppression of HGP at all higher insulin doses (Fig 5).

In the current study design, GIRs determined in Cold-GINF studies were used in the calculation of appropriate labeling of glucose infusates in Hot-GINF studies. Therefore, the sequence was not randomized. Furthermore, a 4-week interval was allowed between each study. This study design obviously carries a risk of bias due to the interval between studies and the lack of randomization of the sequence. However, most recently, Katz et al[30] published double-tracer studies that may support our present data. One tracer, 6-^{14}C-glucose, was given both as a primed-constant infusion and as an addition to the glucose infusate in order to maintain constant SA (Hot-GINF), whereas the other tracer, 6-^{3}H-glucose, was given as a primed-constant infusion only (Cold-GINF). Because these tracer infusions were given simultaneously, day-to-day variation between Hot- and Cold-GINF studies was eliminated, but the design carries a risk of bias due to possible differences between tracers used. Katz et al[30] did not test for differences between rates calculated with the Hot- and Cold-GINF techniques. However, magnification of Figs 6 and 7 in Katz et al[30] reveals that there were marked differences between Hot- and Cold-GINF studies. In their Cold-GINF studies, underestimation of HGP was approximately 24%, 31%, and 64% of the basal rate during insulin infusions of 0.25, 0.6, and 2.0 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} (Fig 6, lower left panel, in Katz et al[30]), and similarly, underestimation of R_d can be estimated from Fig 7 in Katz et al[30] to 10%, 33%, and 67% of the basal turnover rate. These error levels in the Cold-GINF studies of Katz et al after 3 hours closely approximate the errors of 34%, 45%, and 74% observed in the current Cold-GINF studies after 2 hours during comparable insulin infusions (10, 20, and 80 mU $\cdot$ m^{-2} $\cdot$ min^{-1}). Therefore, although Katz et al did not analyze their data this way, their results strongly support our conclusions.

Much of our current knowledge about regulation of glucose turnover in vivo is based on conventional isotope dilution studies using unlabeled glucose infusates. Usually, only 2 hours have been allowed for assessment of insulin action. Therefore, the observation that during this period both R_d and HGP were underestimated is important. Since errors were greater the larger the GIR, these systematic errors may have biased assessment of the relative importance of peripheral and hepatic defects in insulin-resistant subjects in previous studies. Because sensitive control subjects will require larger glucose infusions than insulin-resistant patients during clamp studies, underestimation of both R_d and HGP will be greater in sensitive control subjects than in insulin-resistant patients. Thus, underestimation of R_d will be greater in control subjects and smaller in resistant patients, resulting in apparent smaller defects in peripheral insulin action in resistant subjects. Similarly, underestimation of glucose production will be greater in sensitive control subjects and smaller in resistant patients, resulting in apparent greater defects in suppression of glucose production in resistant patients. Thus, underestimation of glucose turnover in conventional isotope dilution studies may have resulted in the false impression of greater hepatic and smaller peripheral defects in insulin sensitivity in insulin-resistant patients. That this possibility may be more than theoretical is supported by data from recent studies. Based on a conventional technique, hepatic insulin resistance is a well-established feature in type II diabetes.[8,9] However, using the constant SA method, Katz et al[31] reported normal suppression of HGP and Pigon[43] and Stæhr et al[44] reported normal or near-normal hepatic insulin sensitivity in type II diabetic patients with marked peripheral insulin resistance. Therefore, reevaluation of peripheral and hepatic insulin sensitivity may be appropriate in obesity, type II diabetes, and other insulin-resistant states.

Similarly, evaluation of the main site of action (HGP or R_d) of pharmacological agents or different regimens that influence insulin sensitivity may have been biased in previous studies. If the regimen under study improves insulin action, greater GIRs will be required during clamp studies, resulting in apparent smaller improvements in peripheral insulin action and apparent greater improvements in hepatic insulin action. Thus, it is likely that therapies or regimens that improve insulin sensitivity may have greater effect on peripheral tissues and lesser effect on HGP than is currently assumed. Reevaluation of the mechanism of action of agents or regimens that influence insulin action therefore seems an appropriate topic for future studies.

This may comprise issues such as the action of the oral agents metformin, sulphonylureas, and glitazones, regimens such as exercise, dietary treatment, weight reduction, or intensive insulin therapy, and the effect of changes in concentrations of, for example, fatty acids or glucagon.

In summary, compared with the constant SA method, glucose turnover rates were underestimated with the conventional isotope dilution technique at all insulin levels tested. Thereby, stimulation of glucose R_d was underestimated and suppression of HGP was overestimated. During the first 2 hours, these errors were greater the larger the GIR. These systematic errors may have biased previous studies, resulting in apparent greater hepatic and apparent smaller peripheral defects in insulin-resistant states such as obesity and type II diabetes. Similarly, evaluation of different factors that influence insulin action may have been biased toward greater hepatic and smaller peripheral effects. Reevaluation of these issues therefore may be relevant in future studies.

Finally, the importance of appropriate validation of methods should be emphasized. Despite its widespread use, the conventional isotope dilution method has never passed adequate validation for assessment of glucose production. In contrast, the constant SA method is theoretically more appropriate, although also more difficult, and has been validated by comparison to the hepatic arteriovenous balance technique, suggesting that it can measure glucose production with reasonable accuracy. However, it is recognized that the constant SA method has not yet been compared with known R_as as can be done in the hepatectomized, nephrectomized dog model of Allsop et al.[12] Such studies are important and therefore should be encouraged.

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