Insulin resistance of protein metabolism in type 2 diabetes mellitus

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ABSTRACT

Objective: We previously demonstrated that 1) obesity impairs and 2) gender influences insulin sensitivity of protein metabolism, while 3) poor glycemic control in type 2 diabetes (T2DM) accelerates protein turnover in daily fed-fasted states. We hypothesized that T2DM alters the insulin sensitivity of protein metabolism, and gender modulates it.

Research Design And Methods: Hyperinsulinemic, (~570 pmol/L), euglycemic (5.5 mmol/L), isoaminoacidemic (kept at postabsorptive concentrations) clamps were performed in 17 hyperglycemic T2DM and 23 subjects without diabetes matched for age and body composition, after 7d inpatient, protein-controlled, isoenergetic diets. Glucose and leucine kinetics were determined using tracers.

Results: In T2DM, postabsorptive (baseline) glycemia was 8-9 mmol/L, glucose production (Ra) and disposal (Rd) were elevated, and once clamped, endogenous glucose Ra remained greater and Rd was less (P <0.05) than in controls. Baseline leucine kinetics did not differ despite higher insulin levels. The latter was an independent predictor of leucine flux within each sex. With clamp, total flux increased less (P = 0.016) in T2DM men, though protein breakdown decreased equally (~20%) in male groups but less in female groups. Whereas protein synthesis increased in male controls and in both female groups, it did not in male T2DM. In men, HOMA-IR predicted 44%, whereas in women, waist-to-hip ratio predicted 40% of the change in synthesis.

Conclusions: During our clamp, men with T2DM have greater insulin resistance of protein metabolism than that conferred by excess adiposity itself, whereas women do not. These results may have implications for dietary protein requirements.

KEY WORDS: protein turnover, leucine kinetics, type 2 diabetes, insulin sensitivity, hyperinsulinemic clamps, obesity, human studies

ABBREVIATIONS:

α-KIC: α-ketoisocaproic acid
BCAA: branched-chain amino acids
FFA: free fatty acids
FFM: fat free mass
GCMS: gas chromatography-mass spectrometry
IAA: indispensable amino acids
IGT: impaired glucose tolerance
ISI: insulin sensitivity index (M/clamp insulin concentration)
HOMA-IR: Homeostasis model assessment of insulin resistance

HPLC: high performance liquid chromatography
M: glucose infusion rate (mg/min) per kg body weight
MUHC: McGill University Health Centre
OGTT: oral glucose tolerance test
Ra: rate of appearance
Rd: rate of disappearance
REE: resting energy expenditure
T2DM: type 2 diabetes mellitus
There is clear evidence for altered protein metabolism in type 1 (1-5), but in type 2 diabetes mellitus (T2DM), results have been inconsistent. That protein metabolism in T2DM has been reported to be both unaffected and altered may stem from differences in study design: tracer method, adiposity and gender of subjects, prevailing glycemia, normalization of data, and types of statistical analyses. We reported accelerated integrated fed-fasted kinetics of whole-body protein metabolism (using $[^{15}$N]-glycine) in obese T2DM subjects with hyperglycemia (6-9) compared with obese controls (6,7). Such studies required adjusting data for fat free mass (FFM), sex and age (6,8,9), and had precise control of protein and energy intake. When glycemic control was normalized with insulin (7), improved with oral antihyperglycemic agents (6) or normalized with oral agents and energy restriction (6), protein turnover was either improved or not different from that of obese controls.

Most reports showing no alterations in T2DM (10-14) assessed postabsorptive and post-insulin states, using amino acid tracers. However, one reported elevated postabsorptive catabolism in hyperglycemic T2DM, not corrected by insulin (15). Another showed elevated rates of leucine transamination that decreased with better glycemic control, without altering leucine oxidation (16). During the hyperinsulinemic, euglycemic clamp, suppression of breakdown was shown to be both blunted (15), and unaffected (12,13). Any decrease in catabolism decreases endogenous amino acid concentrations and availability, thereby producing “paradoxical” decreases in synthesis (17,18).

Therefore, defining the roles of insulin on synthesis and catabolism requires that plasma amino acids be maintained constant. We have used an hyperinsulinemic, euglycemic clamp with circulating amino acids clamped at fasting levels. This also avoids raising plasma amino acids, which themselves stimulate anabolism. Both the conventional clamp (19) and our approach are experimental models rather than simulation of the fed state after mixed meals with their hyperaminoacidemia and hyperglycemia. With this method, we found increased protein synthesis in lean men (20), a blunted response in obese women (21), and less net protein accretion in women without diabetes compared with men (22).

The hypotheses of the present study are that T2DM affects the protein anabolic responses to hyperinsulinemia and gender modifies these alterations. Hyperinsulinemic, euglycemic, isoaminoacidemic clamps with leucine and glucose tracers were performed in overweight and obese men and women with or without T2DM. Groups of the same sex were matched for body composition and age. Data from some of the control subjects have been published previously (20-23).

**METHODOLOGY**

**Subjects.** Seventeen T2DM (7 women, 10 men) and 23 control subjects (12 women, 11 men) were admitted to the Clinical Investigation Unit of the MUHC/Royal Victoria Hospital (Table 1). Consent was obtained according to the institutional Research Ethics Board. Subjects were screened by medical and dietary history, physical examination and laboratory evaluation to assure the absence of hepatic, hematologic, renal, pulmonary, thyroid and cardiovascular dysfunction. Inclusion criteria were non-smokers, stable weight for 6 months and protein intakes within the Dietary Reference Intakes (24). Control subjects took no medications that affect metabolism and underwent a 75g OGTT (25). The groups within each sex were matched for anthropometric variables. Diabetes medications were stopped for 1 week, lipid-lowering medications upon admission, but
anti hypertensive agents were continued in 4 subjects.

Subjects consumed an isoe energetic, protein-controlled liquid formula (Ensure®, Ross Laboratories, Montreal, Canada) for 7 days in control and 8 days in T2DM subjects, with or without additional energy as canola oil and a glucose polymer (20). Total energy intakes were 1.5 x resting energy expenditure (REE), by indirect calorimetry (Deltatrac®, SensorMedics, Yorba Linda, CA), with 60% from carbohydrate, 25% from fat, and 15% (1.7g/kg FFM/day) from protein.

Premenopausal women were studied during the follicular phase. 24-hour urine was collected daily for determination of nitrogen balance (9). Subjects were weighed daily. Physical activity was limited to walks in and around the hospital. Pre-meal capillary glucose determinations (Accu-Chek, Advantage®, Roche Diagnostics, Laval, Canada) were done in T2DM subjects. If hyperglycemia was >15 mmol/L, insulin (Humulin-R®, Eli Lilly Canada Inc, Toronto, Canada) was administered but not for 15 hours before the clamp. Energy in glycosuria was added daily (50% glucose polymer, 50% canola oil). Waist and hip circumferences were measured according to WHO (26). Body composition was assessed by bioelectrical impedance analysis (RJL-101A; RJL Systems, Detroit, MI) using equations validated for lean (27), overweight (28), obese (28), and older (29) subjects.

Hyperinsulinemic, euglycemic, isoaminoacidemic clamp protocol (Appendix 1) [available at http://diabetes.diabetesjournals.org]). On the last day, the clamp was performed, as detailed (20), with glycemia at 5.5 mmol/L and total branched-chain amino acids (BCAA) maintained at each individual subject’s postabsorptive concentrations. Briefly, catheters were inserted into an antecubital vein for infusions and a contralateral dorsal hand vein for sampling arterialized venous blood with the hand in a warming box at 65°C. At 8:00 AM, an oral bolus of 0.1 mg/kg of NaH13CO2 (MassTrace Inc., Woburn, MA) in water was ingested and a primed L-[1-13C] leucine (Isotec, Sigma-Aldrich, St. Louis, MO) (0.5 mg/kg), constant infusion at 0.008 mg/kg·min was started. D-[3-3H] glucose (PerkinElmer Inc., Life and Analytical Sciences, Boston, MA) was used for glucose kinetics, with prime of 22 µCi (814 kBq) in controls and 30 µCi (1110 kBq) in T2DM and continuous infusion of 0.22 µCi/min (8.14 kBq/min). A primed infusion of human insulin (Humulin-R®, Eli Lilly Canada Inc, Toronto, Canada) was started 180 min later then maintained at 40 mU/m2·min. At 184 min, the amino acid infusion (10% TrophAmine® without electrolytes; B Braun Medical Inc., Irvine, CA) was started and rates adjusted based on plasma BCAA determined every 5 min. Potato starch-derived glucose [20% (wt/vol) Avebe b.a., Foxhol, Netherlands] with added D-[3-3H]glucose [“hot ginf” (30)], was infused with rates adjusted every 5 min.

Postabsorptive and clamp physiological and isotopic steady-states were attained. Blood was collected for substrates, hormones, and glucose and leucine kinetic determinations at baseline, every 10 min for 40 min before insulin, then every 30 min until the last 40 min, then every 10 min. Expired air samples were collected then stored in Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Indirect calorimetry was performed for 20 min before and during the last 30 min of the clamp, and data used for calculation of leucine oxidation, nonprotein respiratory quotient and glucose, protein and fat oxidation.

Leucine kinetics were calculated according to (31), using plasma [13C] α-KIC enrichment (reciprocal model), providing leucine total Ra (flux), oxidation, endogenous rates of appearance (Ra) (protein breakdown), non-oxidative rates of disappearance (Rd) (protein synthesis), and net endogenous balance (synthesis minus breakdown). Recovery factors of 13C from the bicarbonate
pool for the calculation of postabsorptive and clamp leucine oxidation were 0.671 and 0.799 (20). Glucose turnover was calculated using OOPSEG (32). In subjects with T2DM, 20% glucose was begun when euglycemia was reached during insulin infusion. Postabsorptive and clamp isotopic steady-states for glucose specific activity and α-KIC enrichment were designated based on a slope not different from 0 for the last 30 min of each period. In the calculation of leucine oxidation rates, correction was made for the dilution effect of $^{13}$CO$_2$ due to the low $^{13}$C content of the glucose infusion, as previously described (20). The correction factor in additional clamp studies without $^{13}$C leucine in some T2DM and obese control subjects was 7% in both groups. Postabsorptive and clamp $^{13}$CO$_2$ recovery factors used also took this dilution effect into account.

**Assays.** The $^{13}$C enrichment of plasma α-KIC was analyzed by gas chromatography-mass spectrometry with electron-impact ionization (GCMS 5988A; Hewlett-Packard, Palo Alto, CA), after derivatization with N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (Regis Technologies Inc., Morton Grove, IL) (21,23). Expired air was analyzed for $^{13}$CO$_2$ enrichment by isotope ratio mass spectrometry (Micromass 903D, Vacuum Generators, Winsforce, United Kingdom) (20). Glucose was determined by the glucose oxidase (GM7 Micro-Stat, Analox Instruments, USA, Lunenberg, Mass) and BCAA by an enzymic, fluorometric assay (20). The radioimmunoassays for insulin and glucagon and analysis of glucose specific activity were previously documented (30,33). FFAs were measured using the NEFA C Kit (Wako Chemicals USA Inc, Richmond, VA). Reverse-phase High Performance Liquid Chromatography (HPLC) (Beckman Coulter Canada Inc, Mississauga, Canada) with automated pre-column β-phthalaldehyde derivatization was used to determine plasma amino acids.

**Statistical Analyses.** Results are presented as mean ± SEM. Subject characteristics of both sexes were compared separately by unpaired t-test, and steady-state baseline and clamp hormone and substrate concentrations were compared using analysis of variance (ANOVA). Kinetics were compared between control and T2DM groups separately by sex, by analysis of covariance (ANCOVA) with FFM as a covariate when found to have a predictive value from prior regression analysis (23,34). Responses to the clamp were analyzed using repeated measures ANOVA, (clamp as within-subject factor and T2DM as between-subject factor) for each sex. Unpaired t-tests were used to compare the percent change in leucine kinetics in subjects with and without T2DM. HOMA-IR and fasting insulin values were log-transformed to yield a normal distribution. Stepwise linear regression analysis was conducted to assess for independent predictors of flux and synthesis among those found by simple correlations. Significance was set at 0.05 and power at 80%. A minimum sample size of 7 per group was estimated with change in net leucine balance as endpoint and standard deviations from our previous studies. Analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL).

**RESULTS**

All subjects were overweight or obese (Table 1). Within each sex, age, body composition and body fat distribution did not differ between T2DM and control subjects. HOMA-IR was higher in T2DM subjects. Two-hour OGTT glycemia was 7.1 ± 0.3 mmol/L in control subjects. Diabetes duration was 6 ± 1 years. Substrates and hormones are presented in Table 2. Baseline hyperglycemia in T2DM subjects was by design, but total BCAA, IAA and FFA were not different. In men, dispensable amino acids being higher (data not shown), total amino acid levels were higher in T2DM. Results of individual amino acids are presented in Appendix 2. Insulin was
significantly higher in female T2DM. Baseline glucagon was not different. Sex differences included higher FFA and lower BCAA in women (P <0.05).

The clamp glycemic goal of 5.5 mmol/L was reached in all groups, but it took 76 ± 11 min in the T2DM subjects; BCAA were clamped at baseline levels with amino acid infusion rates that did not differ in T2DM but were lower in women (P <0.05), even when adjusted for FFM. Small, but significant changes in IAA and total amino acids were not different between control and T2DM subjects. FFA were suppressed equally, but to higher clamp levels in T2DM. Insulin reached the same typical postprandial concentrations among groups. Glucagon declined in controls but not in T2DM subjects.

Results of plasma $[^{13}C]$ α-KIC and expired $^{13}$CO$_2$ enrichments are shown in Appendix 3. Coefficients of variation of enrichment at each plateau were less than 3% in all groups. Leucine kinetics (Table 3) did not correlate with either clamp insulin or its change from baseline when controlled for FFM. Thus no adjustments for insulin were necessary. At baseline, within each sex, there was no T2DM effect in any kinetic variable, whether as absolute values or adjusted for FFM (data not shown), despite the presence of hyperinsulinemia and hyperglycemia. In contrast, the clamp increase in flux (total R$_a$) was smaller in male T2DM. Protein breakdown (endogenous R$_a$) decreased equally in T2DM and controls, but % change was less in women (P <0.001). Leucine infusion rates were not different in T2DM vs. controls. There was a significant increase in protein synthesis (nonoxidative R$_d$) in all groups except male T2DM. Leucine oxidation increased comparably in all groups, but % increase was greater in women (P = 0.039). These kinetic responses resulted in an increase in net endogenous balance that did not differ between T2DM and controls but was positive in control men and greater in men than women (P <0.001).

Endogenous glucose R$_d$ was higher at baseline in T2DM and decreased by a comparable amount to controls, to higher clamp rates (Table 4). Glucose infusion rates were much lower in T2DM. Total R$_d$ in T2DM was higher at baseline, and increased less to lower clamp levels. Oxidative R$_d$ did not differ at baseline, and increased to lower values in female T2DM. Nonoxidative R$_d$ in T2DM was higher at baseline, but decreased to lower clamp values, whereas it increased in controls. Fat oxidation decreased equally during the clamp in all groups, but was higher in female T2DM both at baseline and during the clamp, even when adjusted for FFM. There was no T2DM effect on REE at baseline or during the clamp. It increased during the clamp only in men. Glucose infusion rates and clamp nonoxidative R$_d$ adjusted for FFM were higher in male than in female controls (P <0.05).

Controlled for FFM, postabsorptive leucine flux and protein synthesis correlated positively with fasting plasma insulin in men ($r = 0.506, P = 0.023$ and $r = 0.528, P = 0.017$, respectively) and leucine flux correlated with fasting insulin ($r = 0.541, P = 0.020$) and waist circumference ($r = 0.579, P = 0.012$) in women. Stepwise regression analysis showed that FFM and log of fasting insulin were the two significant independent variables that predicted 74% of the variance in postabsorptive flux in men and 87% of that in women.

In men, the change in synthesis correlated with markers of insulin resistance of glucose: lnHOMA-IR (Figure 1A); fasting lninsulin ($r = -0.566, P = 0.008$) and fasting plasma glucose ($r = -0.576, P = 0.006$), and with markers of insulin sensitivity: M (glucose infusion rate/kg/min) ($r = 0.557, P = 0.009$), and insulin sensitivity index, ISI (M/clamp insulin, $r = 0.508, P = 0.019$). Lesser suppression of protein breakdown correlated with higher fasting lninsulin ($r = -0.450, P = 0.041$).
Unlike men, in women, the increase in net leucine balance did not correlate with the increase in protein synthesis, only with that in breakdown (r = −0.701, P = 0.001) and in oxidation (r = −0.497, P = 0.030). Changes in synthesis related to body fat distribution, with a significant correlation between waist-to-hip ratio and the change in synthesis (Figure 1B).

It is noteworthy that lnHOMA-IR correlated strongly with lnM and ISI (r = −0.816, P < 0.001 and r = −0.786, P < 0.001) in men and (r = −0.889, P < 0.001 and r = −0.813, P < 0.001) in women.

Data for percent changes in leucine kinetics during the clamp are compared with our previous results from lean subjects [9 men (BMI: 21.3±0.4, age: 26±1, FFM: 59±1) and 8 women (BMI: 20.8±0.3 kg/m², age: 24±1 y, FFM: 41±1 kg)] using the identical protocol, in Figure 2A for men and Figure 2B for women. This emphasizes that the abnormalities in T2DM, though relatively modest when compared with matched subjects without diabetes, are extremely large compared with lean subjects (20,21).

Stepwise regression analysis of the present study showed that in men, lnHOMA-IR predicted 37% of the variance in the increase in flux and 44% of that in synthesis and in women, waist-to-hip ratio and hip circumference predicted 66% of the variance in the increase in flux and waist-to-hip ratio predicted 40% of that in synthesis.

DISCUSSION
This study demonstrates that insulin-resistant overweight and obese persons with T2DM also have insulin resistance of protein metabolism that is expressed differently in men and women. While in the postabsorptive state, in both sexes, elevated plasma insulin, a marker of insulin resistance, is associated with elevated leucine flux, the response to the clamp is sex determined. The increase in total flux in male T2DM is blunted and this difference is driven by failure of stimulation of protein synthesis (Figure 2A). This abnormal response to hyperinsulinemia is predicted by multiple recognized markers of insulin resistance of glucose, indicating concurrent resistance of both. In women, T2DM does not have an additive effect on the already blunted responses in flux and synthesis of body composition-matched control women (Figure 2B).

Our postabsorptive leucine kinetic results are consistent with most studies comparing T2DM subjects with weight, BMI, percentage of ideal body weight, or percent body fat-matched controls (10-13,16). Elevated fasting rates of leucine oxidation in T2DM in one study is likely because only the T2DM subjects were on a hypoenergetic diet (35). The study of T2DM with highest A1C showing augmented postabsorptive protein breakdown (or flux) suggests that poor diabetes control is an important factor (15). That study and ours found positive correlations of breakdown with fasting insulin. Furthermore, we found a negative correlation between fasting insulin and net balance (all subjects included, P < 0.02). Therefore, given their greater hyperinsulinemia, our results support the presence of insulin resistance of postabsorptive protein metabolism in T2DM, as previously implied (12,13).

By clamping amino acids at fasting levels and preventing the hypoaminoacidemia of conventional clamps, we observe smaller increases in total leucine flux and no response of protein synthesis in T2DM men. This is due primarily to resistance during hyperinsulinemia at clinically relevant peak concentrations reached by similar subjects, after a mixed meal (data not shown). Our protocol maintained euglycemia, constant baseline BCAA, and total indispensable and total amino acids within ranges of interindividual variability and reference postabsorptive concentrations (36). The small changes in individual amino acids observed are unlikely to be of physiological significance, compared with
hypoaminoacidemia systematically observed in conventional clamp studies that do not report a T2DM effect (12,13,15).

A sustained glycemia of 5.5 mmol/L was a prerequisite for comparison of leucine and glucose kinetics to those of control subjects. Though this glycemic normalization was brief, it may have attenuated even greater abnormalities in T2DM. Despite this, there is a significant negative correlation between fasting glycemia and change in synthesis in the men. Their smaller insulin-induced increase in REE (Table 4) is consistent with the lack of stimulation of protein synthesis, an energy-requiring process (37).

Our fed-fasted 15N-glycine studies showed that protein flux, breakdown, and synthesis are elevated in hyperglycemic T2DM, while net balance is diminished (6-8). We now show that higher fasting insulin correlates with higher postabsorptive flux and more negative net balance. There is evidence that the accelerated fed-fasted whole-body protein turnover rates in T2DM likely lie in amino acid and insulin effects captured during the postprandial states. First, amino acid-induced protein anabolism could be impaired in T2DM, as has been found in cirrhosis, another insulin-resistant disorder (38). Second is the lower postprandial insulin response in T2DM (39). Third, improving glucose control with insulin or antihyperglycemic agents improves protein metabolism (6,7) and the rates of protein breakdown are proportional to glycemia (40). Hence, lesser postprandial insulin response, greater postprandial hyperglycemia, and impaired amino acid action could elevate proteolysis, thereby increasing the availability of substrates for protein synthesis. Increased amino acid availability also downregulates the insulin signaling pathway (41), thereby creating a cycle of insulin resistance of glucose and protein metabolism that would worsen as metabolic control deteriorates in T2DM.

The overweight-obese control groups have insulin resistance when compared with lean, normal subjects, as previously shown for obesity (21). Male T2DM subjects have a markedly smaller increment of flux and synthesis than lean and smaller than control subjects (Figure 2A). Clamp net balance is less than in lean men. Female T2DM and nondiabetic controls both have impaired stimulation of flux and synthesis compared to lean subjects, and clamp net balance is less (Figure 2B). The % increment in oxidation is not different among groups within each sex, but is more in women than men (not shown). That breakdown inhibition is not different among groups, within each sex, indicates that the level of hyperinsulinemia (that was the same in lean subjects) was sufficient for optimal suppression, and therefore, the processes involved may have different insulin dose-response relationships than for synthesis.

Sex differences in leucine kinetics are only present during the clamp. The women have significantly less suppression of protein breakdown than men (16 vs. 22%) (Table 3, Figure 2), as previously published (22). This is reflected in lower amino acid infusion rates, also consistent with less insulin sensitivity of protein metabolism. The increase in net balance in men results from the magnitudes in the decrease in breakdown, increase in synthesis and lower rates of oxidation. In women, the increase in net balance is due to the magnitude of suppression of protein breakdown concurrent with lesser increases in oxidation. Unlike in men, rates of synthesis did not relate to net balance. The fates of amino acids from infusion and from protein breakdown differ between male and female controls: percent increases in rates of synthesis are comparable, but those of oxidation are higher in women (40 vs. 18%, P = 0.039). This could be interpreted as “glucose-sparing”, suggested by their lower rates of glucose infusion and insulin-mediated nonoxidative Rd.
Of note is that the sex differences in clamp glucose $R_d$ in controls are not observed in T2DM subjects. In the latter, clamp FFA concentrations are higher, fat oxidation is higher (significantly so in women) and endogenous glucose production is less suppressed, such that glucose infusion rates and disposal are substantially less in both male and female T2DM vs. controls. This coexistence of insulin resistance of glucose and protein metabolism is reinforced by HOMA-IR as a predictor of the changes in flux and synthesis and the negative correlation between the change in synthesis and HOMA-IR in men (Figure 1A). The negative correlation between the change in protein synthesis and waist-to-hip ratio in women (Figure 1B) suggests that body fat distribution has a greater impact on protein metabolism than in men. This is consistent with a study showing that larger hip circumferences have a “protective” effect on insulin sensitivity of glucose (42).

The relative importance of insulin vs. amino acids in stimulating protein synthesis and anabolism in humans depends on their concentrations and availability. In skeletal muscle, where most protein turnover occurs in the postprandial state, both insulin and amino acids stimulate synthesis, but their complementary effects vary according to their concentrations (18). It has been suggested that insulin does not have additional effects on protein synthesis during hyperaminoacidemia (17). However, increasing amino acid concentrations with insulin at 500-600 pM, increased whole body protein synthesis stepwise (17). Since we maintained isoaminoacidemia at comparable amino acid infusion rates, it is probable that the differences are due to insulin resistance.

Our results suggest a change is required in the prevailing view that protein metabolism is “normal” in T2DM. Prior studies included 1) study groups having both sexes (15) or only women (10,11); 2) differing adjustments for body composition (10-16,35); 3) no prior diet control (12,35); 4) presence of comorbidities (11,35); 5) insulin therapy overlapping the kinetic study (11,12), attenuating possible differences; 6) pharmacologic hyperinsulinemia (15); 7) lean T2DM subjects (13,35); 8) and use of conventional hyperinsulinemic, euglycemic clamps (12,13,15). To our knowledge, no prior clamp study in T2DM was isoaminoacidemic.

Thus, using a clamp that maintains postabsorptive amino acids and glycemia and postprandial-level hyperinsulinemia helps resolve prior controversies regarding insulin resistance of protein metabolism in overweight and obese persons with T2DM. The magnitude of this defect is considerable when compared to lean, normal persons, but much of it is due to adiposity itself. (21). In addition, the sex influence on the protein anabolic action of insulin (22) contributes to differences in responses in T2DM. These findings have implications for dietary protein requirements, which are likely to be influenced by diabetes control, concurrent obesity or overweight, energy restriction and sex.

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### TABLE 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Male controls (n=11)</th>
<th>Male T2DM (n=10)</th>
<th>Female controls (n=12)</th>
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<td>Age (y)</td>
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<td>FFM (kg)</td>
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<td>48.8 ± 2.1 †</td>
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<tr>
<td>Body fat (%)</td>
<td>31.0 ± 1.7</td>
<td>28.0 ± 1.5</td>
<td>44.6 ± 1.5 †</td>
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<td>Waist circumference (cm)</td>
<td>103.4 ± 3.0</td>
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<td>Hip circumference (cm)</td>
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<td>0.99 ± 0.01</td>
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<td>HOMA-IR</td>
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<td>3.1 ± 0.3</td>
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<td>Triglycerides (mmol/L)</td>
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<td>2.3 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>3.3 ± 0.7</td>
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Mean ± SEM. T2DM vs. controls were analyzed for each sex separately by unpaired t-test; * P < 0.01 vs. controls. Men vs. women were analyzed for T2DM and controls separately by unpaired t-test. HOMA-IR: Homeostasis model assessment of insulin resistance \([\text{insulin (µU/mL) \times glucose (mmol/L)}]/22.5\). † P<0.01 vs. corresponding male subjects.
TABLE 2. Substrate and hormone concentrations at baseline and during clamp

<table>
<thead>
<tr>
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<th>Male controls</th>
<th>Male T2DM</th>
<th>T2DM effect on clamp response (P)</th>
<th>Female controls</th>
<th>Female T2DM</th>
<th>T2DM effect on clamp response (P)</th>
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<tbody>
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<td>**Plasma glucose (mmol/L) * **</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.09 ± 0.08</td>
<td>9.11 ± 0.55 †</td>
<td>4.97 ± 0.08</td>
<td>8.29 ± 0.77 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>5.50 ± 0.02</td>
<td>5.33 ± 0.03</td>
<td>&lt;0.001</td>
<td>5.53 ± 0.02</td>
<td>5.52 ± 0.02</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>BCAA (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>438 ± 24</td>
<td>461 ± 17</td>
<td>390 ± 11</td>
<td>395 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>436 ± 23</td>
<td>455 ± 17</td>
<td>---</td>
<td>397 ± 8</td>
<td>400 ± 20</td>
<td>---</td>
</tr>
<tr>
<td><strong>AA infusion rate (mg/min)</strong></td>
<td>43 ± 2</td>
<td>40 ± 2</td>
<td>33 ± 1</td>
<td>33 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IAA (µmol/L) †</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>933 ± 36</td>
<td>1024 ± 27</td>
<td>868 ± 24</td>
<td>876 ± 53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>956 ± 36</td>
<td>1031 ± 31</td>
<td>---</td>
<td>928 ± 21</td>
<td>913 ± 57</td>
<td>---</td>
</tr>
<tr>
<td><strong>% change</strong></td>
<td>2.7 ± 1.5</td>
<td>0.7 ± 1.6</td>
<td>7.3 ± 1.6</td>
<td>4.3 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total AA (µmol/L) §</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2399 ± 59</td>
<td>2667 ± 57 †</td>
<td>2375 ± 31</td>
<td>2367 ± 99</td>
<td></td>
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</tr>
<tr>
<td>Clamp</td>
<td>2344 ± 61</td>
<td>2556 ± 48 †</td>
<td>---</td>
<td>2405 ± 29</td>
<td>2324 ± 109</td>
<td>---</td>
</tr>
<tr>
<td><strong>% change</strong></td>
<td>-2.2 ± 1.5</td>
<td>-3.9 ± 1.5</td>
<td>1.3 ± 1.1</td>
<td>-1.9 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>**FFA (µmol/L) * **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>536 ± 61</td>
<td>610 ± 42</td>
<td>777 ± 34</td>
<td>854 ± 63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>103 ± 4</td>
<td>166 ± 21 †</td>
<td>---</td>
<td>118 ± 17</td>
<td>184 ± 18 †</td>
<td>---</td>
</tr>
<tr>
<td>**Insulin (pmol/L) * **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>71 ± 10</td>
<td>98 ± 13</td>
<td>83 ± 9</td>
<td>121 ± 16 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>605 ± 59</td>
<td>525 ± 19</td>
<td>---</td>
<td>583 ± 33</td>
<td>555 ± 28</td>
<td>---</td>
</tr>
<tr>
<td>**Glucagon (pmol/L) * **</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>27 ± 3</td>
<td>27 ± 4</td>
<td>22 ± 1</td>
<td>22 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>19 ± 2</td>
<td>28 ± 4 †</td>
<td>&lt;0.001</td>
<td>16 ± 1</td>
<td>21 ± 1 †</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Mean ± SEM. Diabetes effect was sought at baseline and clamp steady states by ANOVA. The response to the clamp was analyzed by repeated measures ANOVA; P values are stated where significant interaction is found, i.e. response to clamp is different between control and T2DM groups.


* P < 0.05, Clamp effect in all groups.
† P < 0.05, T2DM vs. controls of the same sex.
‡ P < 0.05, Clamp effect in female subjects.
§ P < 0.05, Clamp effect in male subjects.
### TABLE 3: Postabsorptive and clamp whole-body leucine kinetics

<table>
<thead>
<tr>
<th>Leucine kinetics</th>
<th>Male Controls</th>
<th>Male T2DM</th>
<th>T2DM effect on clamp response (P)</th>
<th>Female controls</th>
<th>Female T2DM</th>
<th>T2DM effect on clamp response (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Rₐ</strong> *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>164 ± 6</td>
<td>169 ± 10</td>
<td></td>
<td>126 ± 8</td>
<td>140 ± 10</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>177 ± 6</td>
<td>173 ± 9</td>
<td>0.016</td>
<td>141 ± 9</td>
<td>152 ± 11</td>
<td>---</td>
</tr>
<tr>
<td><strong>Endogenous Rₐ</strong> *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>164 ± 6</td>
<td>169 ± 10</td>
<td></td>
<td>126 ± 8</td>
<td>140 ± 10</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>131 ± 6</td>
<td>131 ± 8</td>
<td>---</td>
<td>106 ± 8</td>
<td>118 ± 10</td>
<td>---</td>
</tr>
<tr>
<td><strong>Leucine infusion rate</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>46 ± 2</td>
<td>43 ± 2</td>
<td></td>
<td>35 ± 1</td>
<td>35 ± 2</td>
<td></td>
</tr>
<tr>
<td><strong>Nonoxidative Rₐ †</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>129 ± 4</td>
<td>134 ± 8</td>
<td></td>
<td>102 ± 6</td>
<td>112 ± 7</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>136 ± 4</td>
<td>130 ± 7</td>
<td>0.001</td>
<td>105 ± 6</td>
<td>116 ± 8</td>
<td>---</td>
</tr>
<tr>
<td><strong>Oxidation</strong> *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>35 ± 3</td>
<td>36 ± 3</td>
<td></td>
<td>24 ± 2</td>
<td>28 ± 3</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>41 ± 2</td>
<td>43 ± 2</td>
<td>---</td>
<td>36 ± 3</td>
<td>36 ± 3</td>
<td>---</td>
</tr>
<tr>
<td><strong>Net endogenous balance</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-35 ± 3</td>
<td>-36 ± 3</td>
<td></td>
<td>-24 ± 2</td>
<td>-28 ± 3</td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>5 ± 2</td>
<td>0 ± 2</td>
<td>---</td>
<td>0 ± 3</td>
<td>-2 ± 2</td>
<td>---</td>
</tr>
</tbody>
</table>

Mean ± SEM. Each gender is analyzed separately. Diabetes effect was sought at baseline and clamp steady states by one-way ANCOVA with FFM as covariate. The response to clamp was analyzed by repeated measures ANOVA; P values are stated where significant interaction is found between control and T2DM groups; Total Rₐ: total leucine flux, including exogenous leucine infusion during the clamp period; endogenous Rₐ: index of protein breakdown; non-oxidative Rₐ: index of protein synthesis; net balance: protein synthesis minus breakdown.

* P < 0.05, clamp effect in all groups.

† P < 0.05, clamp effect in male T2DM subjects.
<table>
<thead>
<tr>
<th></th>
<th>Male controls</th>
<th>Male T2DM</th>
<th>T2DM effect on clamp response (P)</th>
<th>Female controls</th>
<th>Female T2DM</th>
<th>T2DM effect on clamp response (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endogenous Ra (mg/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>152 ± 7</td>
<td>188 ± 12 †</td>
<td>121 ± 3</td>
<td>168 ± 13 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>12 ± 10</td>
<td>45 ± 12 †</td>
<td>---</td>
<td>3 ± 6</td>
<td>35 ± 7 †</td>
<td>---</td>
</tr>
<tr>
<td><strong>Infusion rate (mg/min)</strong></td>
<td>418 ± 31</td>
<td>185 ± 25 †</td>
<td>320 ± 22</td>
<td>164 ± 17 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total R_d (mg/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>152 ± 7</td>
<td>202 ± 11 †</td>
<td>121 ± 5</td>
<td>175 ± 16 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>430 ± 31</td>
<td>232 ± 19 †</td>
<td>&lt; 0.001</td>
<td>326 ± 21</td>
<td>202 ± 14 †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Oxidative R_d (mg/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>84 ± 16</td>
<td>60 ± 19</td>
<td>47 ± 7</td>
<td>34 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>154 ± 15</td>
<td>132 ± 15</td>
<td>---</td>
<td>135 ± 8</td>
<td>89 ± 9 †</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>Non oxidative R_d (mg/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>68 ± 15</td>
<td>143 ± 20 †</td>
<td>74 ± 8</td>
<td>141 ± 14 †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>276 ± 30</td>
<td>100 ± 15 †</td>
<td>&lt; 0.001</td>
<td>191 ± 22</td>
<td>112 ± 12 †</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Fat oxidation (mg/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>61 ± 6</td>
<td>75 ± 7</td>
<td>66 ± 5</td>
<td>86 ± 6 †</td>
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<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>36 ± 6</td>
<td>46 ± 9</td>
<td>---</td>
<td>29 ± 5</td>
<td>59 ± 7 †</td>
<td>---</td>
</tr>
<tr>
<td><strong>REE (kcal/d)</strong> §</td>
<td>1782 ± 60</td>
<td>1836 ± 74</td>
<td>1516 ± 78</td>
<td>1741 ± 102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>1856 ± 47</td>
<td>1878 ± 83</td>
<td>1539 ± 83</td>
<td>1707 ± 97</td>
<td></td>
<td>---</td>
</tr>
</tbody>
</table>

Mean ± SEM. Each gender is analyzed separately. One-way ANCOVA, with T2DM as main effect and FFM as covariate, was used at baseline and clamp steady states. The response to clamp was analyzed by repeated measures one-way ANOVA, with T2DM as main effect; P values are stated where significant interaction was found. R_d: rate of disposal; EGP: endogenous glucose production; REE: resting energy expenditure.

* P < 0.05, clamp effect in all groups.
† P < 0.05, T2DM vs. controls of the same sex adjusted for FFM.
§ P < 0.05, clamp effect in male subjects.
Figure legends

**Figure 1.** Simple linear correlations between change in synthesis in μmol/min and lnHOMA-IR in men (r = −0.665, P = 0.001): white circles = male controls; black circles = male T2DM (Panel A) and waist-to-hip ratio in women (r = −0.629, P = 0.004): white squares = female controls; black squares = female T2DM (Panel B).

**Figure 2.** Percent change in protein flux, breakdown and synthesis from baseline to clamp, and clamp net leucine balance: black bars = lean subjects; white bars = control subjects; hatched bars = T2DM. * = P < 0.05 T2DM vs. lean, † = P < 0.05 controls vs. lean, ‡ P < 0.05 T2DM vs. controls. Unpaired t-tests were used for these comparisons. Panel A: male subjects, Panel B: female subjects.
Figure 1

A

```
controls
T2DM
```

B

```
controls
T2DM
```

```
0.0  0.5  1.0  1.5  2.0  2.5  3.0
0.0  0.5  1.0  1.5  2.0  2.5  3.0
```

```
0.70 0.80 0.90 1.00 1.10
0.70 0.80 0.90 1.00 1.10
```

```
Change in synthesis (umol/min)
Change in synthesis (umol/min)
```

```
Waist-to-hip ratio
```

```
Insulin resistance of protein metabolism in T2DM
```
Figure 2

A

B

% Change

Clamp leucine net balance (μmol/min)

-30

-20

-10

0

10

20

30

40

50

Lean men

Male controls

Male T2DM

Lean women

Female controls

Female T2DM

Flux

Breakdown

Synthesis

Net Balance