Insulin in methionine and homocysteine kinetics in healthy humans: plasma vs. intracellular models

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METHIONINE, A SULFUR-CONTAINING AMINO ACID that is abundant in meat and other animal proteins, is converted intracellularly to homocysteine through transmethylation with a methyl acceptor (4). Homocysteine, i.e., the demethylated form of methionine, is not normally present in the amino acid sequences of proteins, but it can accumulate in blood as well as in intracellular fluids in a number of conditions, such as arteriosclerosis (21), renal failure (33), Alzheimer’s disease (2), neural tube defects (17), and others. Although not yet included in the cluster of factors associated with the insulin resistance syndromes (30, 36), homocysteine is usually increased in many of these conditions, such as type 2 diabetes, in particular with kidney insufficiency and/or proteinuria (35), obesity (13), and hypertension, particularly associated with diabetes (19). Thus a disturbance in the regulation by insulin of methionine/homocysteine metabolism can be suspected.

Physiological hyperinsulinemia inhibited whole body leucine and phenylalanine appearance from endogenous body proteins in a dose-response fashion (7, 27). Insulin also either decreased or did not affect leucine oxidation (7, 10, 28, 29), whereas it did not stimulate whole body leucine incorporation into proteins when no exogenous amino acids were supplemented (7, 10, 28, 29). No data exist so far on the effects of hyperinsulinemia on methionine and homocysteine kinetics in vivo.

In vitro studies have shown that insulin inhibits methionine/homocysteine catabolism at the step of transsulfuration, which is the first step of homocysteine irreversible degradation (1, 12). Thus insulin would exhibit a homocysteine-sparing effect in vitro. Therefore, under conditions of insulin resistance, homocysteine concentrations would be decreased. This concept, however, is not consistent with the increased homocysteine concentrations frequently observed in insulin resistance syndromes should insulin resistance be extended from glucose to amino acid (and methionine) metabolism. Furthermore, the effects of insulin on the regulation of other methionine/homocysteine metabolic steps, such as transmethylation and remethylation, are not known. Given these inconsistencies and/or insufficiency of data, the effects of hyperinsulinemia on homocysteine transsulfuration, as well as on other aspects of methionine and homocysteine kinetics in vivo, are worthy of study.

The key steps of methionine and homocysteine metabolism can be measured in vivo with isotope dilution methods by means of the labeled L-[methyl-2H3, 1-13C]methionine stable isotope (14, 26). A common problem in isotope dilution studies is the choice of the precursor pool on which kinetics are measured (16, 23). Because methionine is demethylated to homocysteine intracellularly, homocysteine enrichment has been proposed as a surrogate of intracellular methionine enrichment (15), in analogy with α-ketoisocaprate (KIC) for leucine (16, 23, 34).

Therefore, the aim of this study was to determine the effects of physiological hyperinsulinemia on methionine rate of appearance (Ra, both methyl- and carbon Ra) by 50% (P < 0.004 vs. basal) and methionine disposal into proteins by 50% (P < 0.0005), whereas it increased homocysteine clearance by ~70% (P < 0.025). With intracellular enrichments, insulin increased all kinetic rates, mainly because homocysteine enrichment decreased by ~40% (P < 0.001). In particular, transmethylation increased sixfold (P < 0.02), transsulfuration fourfold (P = 0.01), remethylation eightfold (P < 0.025), and clearance eightfold (P < 0.004). In summary, 1 physiological hyperinsulinemia stimulated homocysteine metabolic clearance irrespective of the model used; and 2) divergent changes in plasma methionine and homocysteine enrichments were observed after hyperinsulinemia, resulting in different changes in methionine and homocysteine kinetics. In conclusion, insulin increases homocysteine clearance in vivo and may thus prevent homocysteine accumulation in body fluids. Use of plasma homocysteine as a surrogate of intracellular methionine enrichment, after acute perturbations such as insulin infusion, needs to be critically reassessed.
volunteers. Both plasma and “intracellular” enrichments were used, and the kinetic data obtained with these models are compared and discussed.

MATERIALS AND METHODS

Subjects. Six middle-aged male volunteers (age 51 ± 5 yr, body mass index 26.7 ± 0.9 kg/m²) were recruited. No subject had any history of diabetes or impaired glucose tolerance, and their fasting plasma glucose concentrations, Hb A1c, and general blood chemistry were normal. They had been adjusted for ±4 wk to a diet containing ~50% calories as carbohydrates, 20% as proteins, and 30% as lipids. Protein intake ranged between 80 and 100 g/day. One patient was under mild hypotensive treatment (with a low-dose angiotensin-converting enzyme inhibitor), which was suspended 1 wk before the study. The aims of the study were explained in detail, and each subject signed his consent to the study. The protocol was approved by the Ethics Committee of the Medical Faculty at the University of Padova, Italy, and it was performed according to the Helsinki Declaration (as revised in 1983).

Experimental design. All subjects were admitted to the Metabolic Unit of the Department of Metabolic Diseases at 7 AM on the study day after the overnight fast. An 18-gauge polyethylene catheter was placed in an antecubital vein of the right arm for isotope, insulin, and glucose infusions. A contralateral wrist vein was cannulated in a retrograde fashion, and the hand was placed in a box heated at 55°C for venous arterialized blood.

The study was divided into two experimental periods, i.e., a basal and a clamp period. After baseline blood and expired air samples were taken for determination of natural isotopic enrichments, a primed (45 times the constant infusion rate per minute) continuous infusion of [1-13C]methyl[1-13C]methionine (Isotech, Miamisburg, OH; isotopic purity 99% for both methyl-13H3 and 13C labels) was started at 7:30 AM. The isotopic constant infusion rate was 1.82 ± 0.05 μmol·kg⁻¹·h⁻¹. Venous arterialized blood samples were drawn every 30 min for 120 min (data not shown) to assess the achievement of the steady state. Thereafter, between the 120th and the 180th min, samples of both blood and air were collected every 20 min for the measurements of basal isotopic enrichments, substrate and hormone concentrations in plasma, and 13CO2 enrichment in the expired air. The expired air was collected by means of a three-way valve into plastic bags containing evacuated, silicon-free glass tubes, which were rapidly capped before bag removal. Between 90 and 120 min, the rate of total CO2 production was determined by a calorimeter (Deltatrac; Datex Italia, Milano, Italy). Thereafter, a euglycemic hyperinsulinemic clamp was started. Regular insulin (Humulin R; Eli Lilly, Indianapolis, IN) was diluted in saline solution with the addition of 2 ml of each subject’s own blood and infused at the rate of 1.9 μU·kg⁻¹·min⁻¹ for 180 min. In the first 10 min, the rate of insulin infusion was doubled to rapidly prime the insulin pool. Euglycemia (i.e., between 85 and 90 mg/dl) was maintained by a variable exogenous 20% dextrose infusion. The source of the dextrose infused was maize starch. Plasma glucose concentration was monitored every 10 min by means of two reflectometers. Independent calibrations against an external reference (Beckman Glucose Analyzer 2; Beckman Instruments, Fullerton, CA) were frequently performed. After 120 min from the start of the insulin infusion, blood and expired air samples were again drawn every 20 min for an additional 60 min for the measurements at the new steady state. Between 160 and 180 min, the rate of total CO2 production was again determined by the calorimeter.

Because the maize-derived starch, from which the infused glucose is produced, may be naturally enriched in 13C above the basal enrichment of the subjects (22), the enrichment of the expired 13CO2 during the clamp may be artificially increased by the contribution of the oxidation of the infused glucose to CO2 production. As control for this potential bias, we determined in four subjects the rate of expiration of 13CO2 during an insulin clamp without the methionine tracer infusion, otherwise repeated under identical conditions (see Calculations).

Analytic measurements. Four to five milliliters of blood were collected into EDTA (6% wt/vol)-containing tubes and immediately kept on ice. The plasma was separated by centrifugation within ~1 h. Plasma enrichments of methionine, i.e., of the [methyl-13H1,1-13C1](M + 4) and of the [1-13C1](M + 1) isotopes, were measured by gas chromatography-mass spectrometer (GC-MS, model 5790; Agilent, Palo Alto, CA) as tert-butyldimethylsilyl derivatives and electron impact ionization, by monitoring the fragments [mass-to-charge ratio (m/z) 324/320 and m/z 321/320, respectively] (24). Plasma homocysteine enrichment was measured with a recently developed method (32). The intra- and interassay coefficients of variation were 1.5 and 1.8%, respectively. The enrichment in the expired 13CO2 was determined by means of a gas chromatography-isotope ratio mass spectrometer (Delta Plus GC-C-IRMS; Thermosquest, Bremen, Germany). Enrichments were expressed as tracer-to-tracer ratio (TTR) (34). Plasma insulin concentrations were measured by radioimmunoassay, as referenced elsewhere (29). Plasma homocysteine concentration was determined by fluorescence polarized immunooassay (FPIA; Abbott, Abbott Park, IL). Plasma amino acid concentrations were determined by cation exchange chromatography using a Beckman amino acid analyzer.

Calculations. The values of plasma methionine enrichments at the two steady-state periods, i.e., in the last 60 min of the basal, postabsorptive state, and in the last 60 min of the insulin clamp (i.e., between 120 and 180 min), were averaged. All calculations were performed using these mean values. Plasma methionine kinetics were calculated using the model of Storch et al. (26). Briefly, the model is based on the dilution of the infused doubly labeled methionine by the appearance in plasma of unlabeled methionine as well as by the differences in tracer enrichment attained in plasma of the [methyl-13H1,1-13C1](M + 4), and the [1-13C1](M + 1) species according to the following formulas. At variance with the suggestion of Storch et al., no correction of the data for estimated intracellular enrichments was performed.

\[
Q_m = I_s \times (E_{is}/E_{ii} - 1) \tag{1}
\]

and

\[
Q_c = I_s \times (E_{is}/E_{ii} + E_{ii} - 1) \tag{2}
\]

where \(Q_m\) and \(Q_c\) are the methionine rate of appearance (Ra) in plasma using either the \((M + 4)\) or the \((M + 1)\) masses; \(I_s\) is the rate of tracer infusion (in μmol·kg⁻¹·h⁻¹); \(E_{is}\) is tracer enrichment; and \(E_{ii}\) and \(E_{ii}\) are [methyl-13H1,1-13C1](M + 4), and [1-13C1](M + 1) tracer enrichments in plasma, respectively. The \((M + 1)\) mass is generated by the demethylation of the doubly labeled methyl-13H1,1-13C tracer due to the irreversible loss of the methyl-13H2 group into the methyl pool(s) intracellularly. Total [1-13C1](M + 1) tracer enrichment is the sum of the [1-13C1](M + 1) and of the [methyl-13H1,1-13C1](M + 4) tracer, since both contain a 13C carbon (26).

The rate of methionine remethylation (RM) was calculated as follows:

\[
RM = Q_m - Q_c \tag{3}
\]

The rate of the irreversible homocysteine loss through transsulfuration (TS) has been assumed to be equivalent to that of oxidation (15, 26). Therefore, methionine oxidation was calculated by dividing the rate of 13CO2 expiration (in μmol·kg⁻¹·h⁻¹, corrected for an 80% fixation in body bicarbonate pool), over plasma [13C]methionine total TTR, i.e., the sum of the \((M + 4)\) and the \((M + 1)\) TTR:

\[
TS = \frac{V^n}{13CO_2} \times (1/[13C]Met E_{pt} - [1/13C]Met E_{pt}) \tag{4}
\]

where \([13CO_2]\) is the rate of 13CO2 expiration (in mol·kg⁻¹·h⁻¹), in turn calculated by multiplying the atom percent excess of the expired 13CO2, converted into micromoles and corrected for bicarbonate fixation, times total CO2 production (VCO2, in ml·kg⁻¹·h⁻¹); and
Innovative Methodology

\[ [1^{13}C]\text{Met} E_{\text{basal}} \text{ is total } [1^{13}C]\text{methionine enrichment in plasma, i.e., the sum of the } (M + 4) \text{ and the } (M + 1) \text{ TTR. TS was also corrected for the contribution of the infused glucose during the clamp, by subtracting the rate of } [1^{13}CO_2] \text{ expiration measured in the four control studies, from the rate of total } [1^{13}CO_2] \text{ expiration in the corresponding clamp studies performed with the infusion of the methyl-}2H_3,1-^{13}C\text{ tracer. In the remaining two subjects in whom the repeated control study was not performed, we used the mean correction factor obtained in the four control studies (see below).}

The rate of methionine transmethylation (TM) was calculated as follows:

\[
TM = RM + TS
\]

\[ \text{The rate of nonoxidative methionine disposal, e.g., the index of whole body protein synthesis (PS), was calculated by subtracting TS from Qc.}

\[
PS = Qc - TS
\]

Finally, homocysteine clearance was calculated by dividing the sum of homocysteine removal through remethylation and transsulfuration over its concentration, as follows:

\[ [RM + TS]/[Hcy] \]

where [Hcy] is plasma homocysteine concentration (in $\mu$mol/l).

All rates described in Eqs. 1–7 were also recalculated using the plasma enrichments corrected for (i.e., multiplied times) the homocysteine/methionine $^{13}C$ TTR (15). This approach has been proposed to estimate intracellular methionine kinetics (15) in analogy with leucine and valine kinetics (3, 23).

Statistical analysis. All data are expressed as means ± SE. The comparison between the clamp and the basal values was performed with the two-tailed Student’s t-test for paired data. A two-way ANOVA for repeated measurements was employed to compare the plasma vs. intracellular kinetic data. A $P$ value of <0.05 was considered statistically significant.

RESULTS

Control studies. In the four subjects in whom the 180-min euglycemic clamp was repeated without tracer infusion, insulin was increased from 4.8 ± 1.6 to 168.8 ± 16.8 $\mu$U/ml. The rate of $^{13}CO_2$ expiration rose to 33.61 ± 4.8 $\mu$mol·kg$^{-1}$·h$^{-1}$ above basal. The insulin-mediated glucose disposal was 7.5 ± 0.9 mg·kg$^{-1}$·min$^{-1}$. The calculated increase above background in the rate of $^{13}CO_2$ expiration, normalized per gram of glucose infused, was 4.63 ± 0.78 $\mu$mol·kg$^{-1}$·h$^{-1}$ per gram of glucose. In these four subjects, the individually determined increase above baseline of the rate of $^{13}CO_2$ expiration in the control study was subtracted from the $^{13}CO_2$ expired during the tracer infusion to recalculate the “true” rate of $^{13}CO_2$ expiration from methionine oxidation. In the two remaining subjects in whom the repeated control study was not performed, the aforementioned mean factor was used to recalculate the true rate of $^{13}CO_2$ expiration during the tracer infusion clamp.

Isotope enrichments, total $V_{CO_2}$, and expired $^{13}CO_2$. Near-steady-state conditions were attained in both the basal and the clamp periods (Fig. 1). In the fasting state, the ratio between homocysteine and methionine $^{13}C$ enrichment was 0.652 ± 0.128, in good agreement with a published report (15). During the clamp, both the $[M + 4]$ and the $[M + 1]$ methionine TTR increased significantly vs. basal by 28–67%, respectively ($P < 0.001$), whereas that of homocysteine decreased by ~45% ($P < 0.001$) (Table 1). Consequently, the ratio between homocysteine and methionine $^{13}C$ enrichment decreased to 0.189 ± 0.027 ($P < 0.0001$). During the clamp, the rate of total $V_{CO_2}$ increased by ~16% ($P < 0.0002$). An increase in the rate of expiration of $^{13}CO_2$ was also observed, which was, however, not significant when this was corrected for the glucose infused (Table 1).

Methionine and homocysteine kinetics. By use of plasma enrichments, fasting methionine $R_a$, TS, TM, and RM were in good agreement with published reports in postabsorptive healthy volunteers (Table 2) (15, 26). After the hyperinsulinemic clamp, methionine $R_a$ was decreased by ~25% ($P < 0.003$ vs. basal) and PS by ~50% ($P < 0.0005$), whereas clearance was increased by ~70% ($P < 0.025$). No significant changes on TS, RM, and TM were observed.

With intracellular enrichments (i.e., by correcting for the homocysteine enrichment in plasma), fasting methionine $R_a$, TS, TM, and RM were again in good agreement with the data of MacCoss et al. (15) in postabsorptive healthy volunteers. All rates were significantly greater than those determined with plasma methionine enrichments ($P < 0.01$ or less by ANOVA). During the hyperinsulinemic clamp, however, in contrast with the plasma data, the methionine $R_a$ (both methyl- and carbon), was increased by ~170% ($P < 0.05$) and by ~40% ($P = $not significant) vs. basal, respectively. TS was increased more than twofold ($P < 0.012$), TM about sixfold ($P < 0.02$), RM about eightfold ($P = 0.025$), and clearance about eightfold ($P < 0.003$). Methionine disposal into PS was not significantly changed. The data of the single hypertensive subject did not deviate from the group’s means (data not shown).
Plasma insulin, insulin-mediated glucose disposal, vitamin status, and methylenetetrahydrofolatereductase isoforms. During the euglycemic hyperinsulinemic clamp, insulin was raised from 5.0 ± 1.1 to 147.8 ± 14.5 μU/mL. Plasma glucose was maintained at the fasting values of 85 ± 2 mg/dl by means of the variable 20% dextrose infusion. The insulin-mediated glucose disposal was 7.6 ± 1.2 mg·kg⁻¹·min⁻¹. Plasma folate concentration was 4.9 ± 1.3 μg/l (normal range: 1.8–14) and that of the vitamin B₁₂ was 339 ± 85 ng/l (normal range: 180–900). Two subjects were heterozygotic for the [677C → T] mutation of the methylenetetrahydrofolatereductase (MTHFR) alleles, and the other four were homozygotic for the common phenotype (i.e., no mutation). Plasma amino acid concentrations are reported in Table 3. Most amino acids decreased with hyperinsulinemia, but homocysteine concentrations did not change.

Correlations. Significant positive correlations were found between the insulin-mediated glucose disposal rate and the relative increase vs. basal of methionine transmethylation (r = 0.8, P < 0.03) and of homocysteine clearance (r = 0.81, P < 0.0025), using the plasma calculations.

**DISCUSSION**

Methionine is a sulfur-containing essential amino acid that is the precursor of homocysteine, a powerful and independent risk factor for cardiovascular disease and for other conditions (2, 8, 13, 17, 19). In insulin-resistant states, homocysteine concentration is usually increased (8). Therefore, knowledge of the physiological effects of insulin on methionine and homocysteine kinetics in vivo is necessary to test the effects of the hormone in insulin-resistant states with the purpose to investigate the potential mechanism(s) linking hyperhomocysteinaemia to insulin resistance.

In this study, we used the doubly labeled L-[methyl-²H₃,1-¹³C]methionine stable isotope tracer, which allowed us to measure a number of methionine and homocysteine metabolic steps (26). Because methionine is transmethylated to homocysteine intracellularly and then released into plasma (4), plasma homocysteine enrichment has been proposed as a surrogate of intracellular methionine enrichment to calculate precisely methionine and homocysteine kinetics in vivo (15) in analogy to the leucine-KIC (23) as well as to the valine-isovaleric acid (3) systems. Therefore, in this study, the kinetic data were presented using both plasma (i.e., based on plasma methionine enrichments) and intracellular (based on plasma homocysteine enrichments) calculations.

By use of plasma calculations, physiological hyperinsulinemia decreased methionine Rₘ from endogenous proteolysis, expressed both as the methyl and as the carbon R₉, by ~25% at the present insulin concentration of ~150 μU/ml. This finding is in agreement with the effects of insulin on the R₉ of several essential amino acids and is consistent with an inhibition of endogenous protein breakdown (7, 10, 11, 27–29). Insulin also decreased methionine disposal into proteins, also in agreement with data obtained with other amino acids tracers, confirming the failure of the hormone to stimulate whole body protein synthesis without amino acid supplementation (7, 10, 11, 27–29). In addition, insulin increased homocysteine metabolic clearance (29). Thus hyperinsulinemia on the one hand inhibited methionine appearance into the plasma space but on the other hand stimulated homocysteine removal from plasma, both effects possibly restraining homocysteine accumulation into plasma. The finding of a positive correlation between the insulin sensitivity index and the increment of both

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### Table 1. Steady-state isotope TTRs in plasma of [M + 4], [M + 1], and [¹³C]homocysteine; total V˙¹³CO₂ in basal, postabsorptive state and after euglycemic hyperinsulinemic clamp

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>[methyl-²H₃,1-¹³C]methionine TTR</td>
<td>9.26 ± 0.39</td>
<td>11.82 ± 0.55*</td>
</tr>
<tr>
<td>[¹³C]methionine TTR</td>
<td>1.13 ± 0.22</td>
<td>1.89 ± 0.12*</td>
</tr>
<tr>
<td>[¹³C]homocysteine TTR</td>
<td>0.62 ± 0.04</td>
<td>0.35 ± 0.05**</td>
</tr>
<tr>
<td>V˙¹³CO₂ uncorrected, µmol·kg⁻¹·h⁻¹</td>
<td>6527 ± 213</td>
<td>7538 ± 260*</td>
</tr>
<tr>
<td>V˙¹³CO₂ corrected, µmol·kg⁻¹·h⁻¹</td>
<td>0.215 ± 0.041</td>
<td>0.675 ± 0.039*</td>
</tr>
</tbody>
</table>

Values are means ± SE. TTR, tracer-to-tracee ratio; [M + 4], [methyl-²H₃,1-¹³C]methionine; [M + 1], [¹³C]methionine; V˙¹³CO₂ production; uncorrected and corrected, V˙¹³CO₂ uncorrected or corrected for the contribution of ¹³CO₂ from glucose oxidation during the clamp. *P < 0.05 or less vs. basal.

### Table 2. Methionine and homocysteine kinetics, calculated using plasma or intracellular enrichments, in the basal state and during euglycemic hyperinsulinemic clamp

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Clamp</td>
</tr>
<tr>
<td>Methyl Rₘ</td>
<td>17.60 ± 2.13</td>
<td>13.44 ± 0.95†</td>
</tr>
<tr>
<td>Carbon R₉</td>
<td>15.39 ± 0.68†</td>
<td>11.25 ± 0.61†</td>
</tr>
<tr>
<td>Remethylation</td>
<td>2.21 ± 0.59</td>
<td>2.19 ± 0.35</td>
</tr>
<tr>
<td>Transsulfuration</td>
<td>2.10 ± 0.42</td>
<td>2.50 ± 0.57</td>
</tr>
<tr>
<td>Transmethylation</td>
<td>4.31 ± 0.79</td>
<td>4.69 ± 0.78</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>13.29 ± 0.57</td>
<td>6.27 ± 0.31</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as µmol·kg⁻¹·h⁻¹. Rₘ, rate of appearance. *P < 0.05, †P < 0.003 vs. basal.

### Table 3. Plasma amino acid concentrations in the basal state and after euglycemic hyperinsulinemic clamp

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>34.6 ± 4.5</td>
<td>28.9 ± 2.5*</td>
</tr>
<tr>
<td>Asparagine</td>
<td>22.7 ± 3.3</td>
<td>17.0 ± 2.5*</td>
</tr>
<tr>
<td>Threonine</td>
<td>123.7 ± 8.3</td>
<td>80.6 ± 6.3*</td>
</tr>
<tr>
<td>Serine</td>
<td>115.0 ± 0.9</td>
<td>77.5 ± 6.4*</td>
</tr>
<tr>
<td>Glycine</td>
<td>179.7 ± 15.9</td>
<td>158.6 ± 15.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>314.2 ± 15.3</td>
<td>264.9 ± 29.3</td>
</tr>
<tr>
<td>Citrulline</td>
<td>25.8 ± 0.9</td>
<td>160.0 ± 0.9*</td>
</tr>
<tr>
<td>Valine</td>
<td>231.3 ± 19.7</td>
<td>136.7 ± 9.4*</td>
</tr>
<tr>
<td>Methionine</td>
<td>24.0 ± 0.8</td>
<td>16.7 ± 2*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>58.1 ± 6.4</td>
<td>17.7 ± 2*</td>
</tr>
<tr>
<td>Leucine</td>
<td>156.7 ± 9.7</td>
<td>85.1 ± 2.6*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>57.2 ± 5.1</td>
<td>32.4 ± 1.9*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>46.5 ± 2.8</td>
<td>30.8 ± 0.6*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>53.3 ± 3.9</td>
<td>42.1 ± 3.9*</td>
</tr>
<tr>
<td>Lysine</td>
<td>187.1 ± 8.5</td>
<td>134.9 ± 8.2*</td>
</tr>
<tr>
<td>Histidine</td>
<td>78.7 ± 6.2</td>
<td>64.4 ± 3.6*</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>4.6 ± 1.2</td>
<td>3.5 ± 0.6*</td>
</tr>
<tr>
<td>Arginine</td>
<td>105.8 ± 22.0</td>
<td>62.8 ± 5.6*</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>10.3 ± 1.9</td>
<td>10.1 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed in µmol/l. *P < 0.05 or less, clamp vs. basal.
transmethylation and homocysteine clearance is in agreement with this concept.

This study was performed in middle-aged male volunteers. Although the age of our subjects was greater than that of usual “young healthy volunteers,” the extent of the inhibition of endogenous methionine Ra was well within the range of previous studies in younger subjects with the leucine isotope method (7, 10, 27). Nevertheless, it will be interesting in future studies to assess the effects of age on methionine and homocysteine kinetics. Also, the response of male vs. female subjects should be investigated.

The use of intracellular calculations led to markedly greater kinetic rates, as was expected because of the ~45% lower plasma homocysteine than methionine enrichment (Table 1). In the postabsorptive state, the absolute rates of methionine/homocysteine kinetics were in fair agreement with what reported by MacCoss et al. (15). During hyperinsulinemia, however, there were sharply different responses from those observed with the plasma calculations. Hyperinsulinemia increased methionine Ra by ~40–170%, although this was significant only for the methyl Ra (Table 2). It also markedly increased transsulfuration, transmethylation, remethylation, and homocysteine clearance, whereas it did not significantly suppress methionine incorporation into proteins. Thus insulin apparently increased both methionine release into plasma from intracellular sources and its disposal through transmethylation and homocysteine irreversible loss (through transsulfuration/oxidation). Therefore, there was a net exit (by 10.57 μmol·kg⁻¹·h⁻¹, i.e., corresponding to the increase of TS), of homocysteine molecules from the intracellular space(s).

A key point to correctly interpret these data is the achievement of a full equilibration between plasma (as reflected by methionine) and intracellular (as reflected by homocysteine) enrichments. In our study, the steady state in both methionine and homocysteine plasma enrichments was apparently attained in the final 60 min of each 180-min experimental period (i.e., those defined as basal and clamp periods; Fig. 1). It has been previously reported that, after the intravenous infusion of [1-¹³C]methionine, achievement of the steady state in plasma methionine enrichment required ~1 h, whereas that of homocysteine required up to 8 h (15). Notably, in the referenced study (see Fig. 3 of Ref. 15), plasma homocysteine enrichment after ~3 h was only ~25% lower than that after 8 h. As a matter of fact, the shorter experimental time of the basal and of the clamp periods of our study may have resulted in both an underestimation of homocysteine enrichment in the basal state and an overestimation following the clamp, since a clear-cut decrease was observed with insulin (Fig. 1 and Table 1). Nevertheless, in our study, the basal ratio between plasma homocysteine and methionine enrichment (0.65) was in good agreement with that of MacCoss et al. (15) (0.58). Although it may be possible that subtle additional changes in plasma homocysteine enrichments occurred beyond the 3 h of each experimental period, in the direction of a greater enrichment in the basal state and of a lower enrichment after the insulin infusion, our data should be considered, if anything, as conservative.

That a full equilibration between plasma and intracellular methionine and homocysteine pools may have failed to occur is, however, suggested by the key observation that methionine enrichment increased whereas that of homocysteine decreased during hyperinsulinemia. The complexity of circulating homocysteine pools may have accounted for this apparent discrepancy. Free (i.e., reduced) homocysteine concentration accounts for less than 2% and oxidized homocysteine [as either mixed (homocysteine-cysteine) or homologous (homocysteine-homocysteine) disulfides] for ~20% of total homocysteine, whereas the largest amount is bound to proteins either with thiol or with covalent bonds (4, 21). Erythrocytes may also represent relevant sources of circulating homocysteine (31). Thus a slow equilibration between any of the (reversible) homocysteine pools may have occurred. Another possibility is that plasma homocysteine enrichment does not reflect true intracellular methionine enrichment in all conditions. Further studies are required to elucidate this important point.

The insulin-stimulated increase of homocysteine irreversible disposal, i.e., of transsulfuration (with the intracellular model), as well as the stimulation of homocysteine clearance (with both models), indicates a protective mechanism by insulin against an accumulation of homocysteine in body fluids. Thus, under conditions of normal insulin availability and/or action, the removal of homocysteine from the plasma space is likely increased by insulin, which may be useful particularly after ingestion of a mixed, protein-rich meal. Notably, the calculation of homocysteine clearance in this study is not biased by any change in plasma homocysteine concentrations, which remained constant. In this respect, the finding of unchanged homocysteine concentrations despite the insulin-induced decrease of many other amino acids is surprising. This might be explained by the complexity of the homocysteine pools, as discussed above, and by a possible slow equilibration among them following an acute stimulus such as acute hyperinsulinemia.

The key enzyme of homocysteine irreversible catabolism is cystathionine β-synthase (CBS), which is the rate-limiting step of the transsulfuration reaction (2). In rats, this enzyme is located in liver, kidney, small intestine, pancreas, adipose tissue, and brain (4). CBS activity is stimulated by oxidizing conditions, by phosphorylation/dephosphorylation, by methionine supplementation, and by adenosylmethionine (which is formed in the transmethylation reaction), whereas it is inhibited by adenosylhomocysteine, the direct precursor of homocysteine (4). Our
finding of the insulin stimulation of both transmethylation and transsulfuration with the intracellular model supports a role of the hormone in favoring homocysteine disposal through the two abovementioned biochemical steps. Interestingly, oxidizing conditions would result in increased homocysteine oxidation and glutathione synthesis, thus increasing the availability of a key antioxidant substrate in homeostatic regulation.

In vitro data on the effects of insulin on CBS activity appear, however, to be in contrast with the in vivo findings. Insulin has been reported to regulate CBS activity (25). Experimental, insulin-deficient diabetes in rats increased CBS activity in liver, and this increase was reversed by insulin treatment (12, 20). However, it cannot be excluded that some metabolic abnormality secondary to insulin deficiency directly affected CBS activity. In contrast, in diet-induced insulin resistance models, an inverse relationship between insulin levels and CBS activity was found (5). Thus insulin deficiency vs. insulin resistance may play a different role in the regulation of homocysteine catabolism and, possibly, of its concentrations. In healthy humans, no relation between plasma total homocysteine concentrations and insulin sensitivity was found (9). Euglycemic hyperinsulinemia reduced plasma homocysteine concentrations in normal but not in type 2 diabetic subjects without, however, any dose-response effect (6). Conversely, the improvement of insulin sensitivity and metabolic control did not influence plasma homocysteine concentrations in type 2 diabetes (18). As a matter of fact, the issue of the regulation by insulin and/or insulin resistance conditions on homocysteine concentrations is still unresolved. Therefore, kinetic studies like the present one may add new information on this important issue, particularly in insulin-resistant states characterized also by hyperhomocysteinemia, such as diabetes mellitus with nephropathy, proteinuric syndromes, renal insufficiency, etc.

The vitamin metabolic status of our subjects was normal, as shown by the vitamin B12 and folate concentrations (see RESULTS). Thus no restriction by these cofactors of homocysteine remethylation likely occurred.

In conclusion, this study shows that physiological hyperinsulinemia increased methionine homocysteine clearance (with both the plasma and the intracellular model), as well as transmethylation and transsulfuration (with the intracellular model) in humans. Insulin thus exhibited a possible protective effect against the accumulation in body fluids of homocysteine, a potent cardiovascular risk factor. These insulin effects need to be tested in clinical conditions of hyperhomocysteinemia and/or insulin resistance. The validity of either model (i.e., plasma vs. intracellular) in the evaluation of methionine/homocysteine kinetics should be further investigated.

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