Original Article

Effects of Insulin on Methionine and Homocysteine Kinetics in Type 2 Diabetes With Nephropathy

Paolo Tessari,[1] Anna Coracina,[1] Edward Kiwanuka,[1] Monica Vedovato,[1] Monica Vettore,[1] Anna Valerio,[1] Michela Zaramella,[1] and Giacomo Garibotto[2]

Although hyperhomocysteinemia, an independent cardiovascular risk factor, is common in type 2 diabetes with nephropathy, the mechanism(s) of this alteration is not known. In healthy humans, hyperinsulinemia increases methionine transmethylation, homocysteine transsulfuration, and clearance. No such data exist in type 2 diabetes either in the fasting state or in response to hyperinsulinemia. To this purpose, seven male type 2 diabetic patients with albuminuria (1.2 ± 0.4 g/day, three with mild to moderate renal insufficiency) and seven matched control subjects were infused for 6 h with L-[methyl-$^2H_3$, 1-^{13}C]methionine. Methionine flux, transmethylation, and disposal into proteins as well as homocysteine remethylation, transsulfuration, and clearance were determined before and after euglycemic hyperinsulinemia (~1,000 pmol/l). In type 2 diabetic subjects, homocysteine concentration was twofold greater ($P < 0.01$) and methionine transmethylation and homocysteine clearance lower (from ~15 to >50% and from ~40 to >100%, respectively; $P < 0.05$) than in control subjects. The insulin-induced increments of methionine transmethylation, homocysteine transsulfuration, and clearance were markedly reduced in type 2 diabetic subjects (by more than threefold, $P < 0.05$ or less vs. control subjects). In contrast, methionine methyl and carbon flux were not increased in the patients. In conclusion, pathways of homocysteine disposal are impaired in type 2 diabetes with nephropathy, both in postabsorptive and insulin-stimulated states, possibly accounting for the hyperhomocysteinemia of this condition. *Diabetes* 54:2968–2976, 2005

Methionine, a sulfur-containing amino acid abundant in meat proteins, is converted to homocysteine by transmethylation (1). Homocysteine, an independent vascular risk factor (2–4) and an associated feature of the insulin-resistant syndrome (5,6), is common in type 2 diabetes with proteinuria and/or kidney insufficiency, hypertension, and obesity (7–13). The mechanisms of homocysteine-induced vascular damage are multifaceted, probably all linked to posttranslational protein modifications (14).

The pathophysiology of hyperhomocysteinemia in type 2 diabetes with nephropathy is unknown. It may be caused by increased production, decreased removal, or both. Although homocysteine concentration is inversely related to renal function (7,15) and is associated with the development of microalbuminuria (16), the precise kinetic mechanism(s) leading to the hyperhomocysteinemia of diabetes with nephropathy has never been investigated. Also, a possible link(s) between insulin resistance and alterations of homocysteine metabolism is largely unknown (12,17). With respect to this, contrasting data exist regarding insulin resistance and amino acid/protein metabolism in type 2 diabetes (18–20).

Homocysteine production is linked to methionine flux and transmethylation, whereas its catabolism occurs through either (reversible) remethylation to methionine (catalyzed by the enzymes methylene-tetrahydrofolate-reductase [MTHFR] and methionine synthase) and by (irreversible) transsulfuration to cystathionine (catalyzed by cystathionine-β-synthase [CBS]) (1,21). These pathways can be investigated in vivo by means of stable isotope methodology (22–24). We have recently shown that insulin acutely increases homocysteine transmethylation, transsulfuration, and clearance in healthy humans (24), thus stimulating homocysteine removal from plasma. No such data exist in diabetes. Therefore, this study was designed to measure methionine and homocysteine kinetics in type 2 diabetic patients with nephropathy and in control subjects, under both basal and postabsorptive conditions and after a euglycemic-hyperinsulinemic clamp.

RESEARCH DESIGN AND METHODS

Seven male type 2 diabetic patients and seven male age- and weight-matched nondiabetic volunteers were recruited (Table 1). The type 2 diabetic patients had established diabetic nephropathy, either clinical (with macroalbuminuria, $n = 6$) or incipient (with microalbuminuria, $n = 1$), a long disease duration (from 7 to 30 years), and diabetic retinopathy. Urinary albumin excretion was measured by two 12-h urine collections. Three patients had mild to moderate renal insufficiency (creatinine concentrations 140–200 mmol/l), whereas it was normal in the remaining four. The glomerular filtration rate was estimated from plasma creatinine concentration (25) and, in the type 2 diabetic subjects only, it was also directly measured with ^{51}Cr-labeled EDTA (26). All subjects had been adapted for >1 month to a diet containing 50% calories as carbohydrates, 20% as proteins (80–100 g/per day, unrestricted), and 30% as lipids. Two patients were treated with oral hypoglycemic agents (OHAs), two with OHAs plus one or two doses of intermediate-acting insulin, and three with split insulin doses. All of the diabetic and three of the control subjects were hypertensive and treated with combinations of hypotensive drugs. Four diabetic subjects and one control subject were hyperlipidemic and treated with statins. All of these agents were suspended the night before the study.

From the [1]Department of Clinical and Experimental Medicine, Chair of Metabolism, University of Padova, Padova, Italy; and the [2]Division of Nephrology, Department of Internal Medicine, University of Genova, Genova, Italy.

Address correspondence and reprint requests to Prof. Paolo Tessari, Department of Clinical and Experimental Medicine, Chair of Metabolism, Policlinico Universitario, via Giustiniani 2, 35128 Padova, Italy. E-mail: paolo.tessari@unipd.it.

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CBS, cystathionine-β-synthase; MTHFR, methylene-tetrahydrofolate-reductase; OHA, oral hypoglycemic agent; TTR, tracer-to-tracee ratio.

TABLE 1
Clinical and biochemical characteristics of the type 2 diabetic and control subjects studied

	Type 2 diabetic subjects	Control subjects
Age (years)	60 ± 3	54 ± 5
BMI (kg/m^2)	27.2 ± 1.0	26.4 ± 0.9
Disease duration (years)	17 ± 3	—
A1C (%)	8.5 ± 0.5*	5.4 ± 0.2
Fasting glucose (mmol/l)	9.8 ± 0.8*	4.8 ± 0.1
Fasting insulin (pmol/l)	117 ± 18*	34 ± 8
Plasma creatinine (μmol/l)	130 ± 17†	92 ± 3
Albuminuria (g/day)	1.21 ± 0.4*	<0.02
Fibrinogen (mg/dl)	427 ± 40†	302 ± 19
Total cholesterol (mmol/l)	5.2 ± 0.3	4.8 ± 0.3
HDL cholesterol (mmol/l)	1.1 ± 0.1	1.3 ± 0.1
Triglycerides (mmol/l)	2.3 ± 0.3*	1.0 ± 0.1
Plasma folates (μg//l)	5.74 ± 0.48	5.73 ± 0.41
Plasma vitamin B_{12} (ng/l)	319 ± 30	395 ± 48

Data are means ± SE. *$P < 0.015$ and †$P < 0.05$ for type 2 diabetic vs. control subjects.

The control subjects had no history of either diabetes or impaired glucose tolerance, and their fasting plasma glucose, HbA_{1c} (A1C), urinary albumin excretion, and general blood chemistry were normal (Table 1). The aims of the study were explained in detail, and each subject signed an informed consent. The protocol was approved by the ethical committee of the medical faculty at the University of Padova and was performed according to the Helsinki Declaration (1983 revision).

Experimental design. At 7:00 A.M. after the overnight fast, 18-gauge polyethylene catheters were placed, one in an antecubital vein of the right arm for isotope, insulin, and glucose infusion and another in a contralateral wrist vein in a retrograde fashion, this hand being maintained at 55°C in a heated box for venous-arterialized blood sampling. The type 2 diabetic subjects were moderately hyperglycemic (Table 1). After baseline blood and expired air, a primed (1.27–1.35 μmol/kg body wt) continuous (1.7–1.8 μmol · kg body wt^{-1} · h^{-1}) infusion of L-[methyl-$^2H_3$, 1-^{13}C]methionine (isotope purity 99%; Isotech, Miamisburg, OH) and L-[5,5,5-$^2H_3$]-leucine (prime 9 μmol/kg, continuous infusion 9 μmol/kg × h) were started at ~7:30 A.M. (i.e., time −180′). Samples were collected half-hourly to assess the achievement of steady state (data not shown). Thereafter, between the 120th and the 180th min of infusion (i.e., at times −60′ and 0′), 3–4 samples of both blood and expired air were collected for measurements of basal plasma isotope enrichments, substrate and hormone concentrations, and $^{13}CO_2$ enrichment. Expired air was collected as previously described (24). Between 90′ and 120′, total CO_2 production was determined with a calorimeter (Deltatrac; Datex Italia, Milano, Italy).

Starting at $t = 0'$, an euglycemic-hyperinsulinemic clamp was performed (24). Insulin (Humulin R; Eli Lilly, Indianapolis, IN) was infused at the rate of 1.9 mU/kg × min^{-1} (doubled in the first 10′) for 180′ (24). Euglycemia (i.e., ~5 mmol/l) was maintained by 20% dextrose infusions derived from maize starch and was monitored every 10′. In the diabetic subjects, blood glucose decreased slowly, reaching euglycemia within 55 ± 8 min, and thereafter it was maintained at ~5 mmol/l for an additional 120′. Thus, total study duration was nearly the same (~180′) in both groups. Between 120′ and 180′, 3–4 samples of blood and expired air were taken for measurements at the new steady state. Total CO_2 production was again measured between 160′ and 180′.

Because the expired $^{13}CO_2$ enrichment during the clamp may be artificially increased by the oxidation of the infused maize-derived starch glucose (27), in repeated studies performed in four control subjects and in four additional male type 2 diabetic patients with proteinuria and mild renal insufficiency, $^{13}CO_2$ production was determined after a euglycemic insulin clamp without the methionine tracer infusion, otherwise repeated under identical conditions (see calculations below). The resulting mean correction factors were 6.24 ± 1.53 and 4.65 ± 0.77 μmol/kg × h^{-1} per gram of glucose infused in the type 2 diabetic and normal control subjects, respectively (P = NS between the groups). These figures were subtracted from total $^{13}CO_2$ production measured during the methionine tracer infusions to determine accurately the rate of L-[methyl-$^2H_3$, 1-^{13}C]methionine oxidation. A retention factor of 20% for the expired $^{13}CO_2$ was applied.

Analytical measurements. Plasma methionine, homocysteine, leucine, and α-ketoisocaproate enrichments were measured by gas chromatography mass spectrometry (5790; Agilent, Palo Alto, CA) as previously described (24,28). The expired $^{13}CO_2$ enrichment was determined by gas chromatography isotope ratio mass spectrometry (Delta Plus GC-C-IRMS; Thermoquest, Bremen, Germany) (24). Enrichments were expressed as tracer-to-tracee ratios (TTRs) (29). Plasma insulin (in the insulin-treated type 2 diabetic subjects after polyethyleneglycol treatment), homocysteine, and amino acid concentrations and other metabolite concentrations were measured as previously reported (24,30).

Calculations. All calculations were performed using the mean values of the two steady-state periods (i.e., between −60′ and 0′ of the basal state and between 120′ and 180′ of the insulin clamp period) (Fig. 2, Table 2). Leucine rate of appearance (R_a) was calculated using the reciprocal pool model (31). Methionine-homocysteine kinetics were expressed in μmol/kg × h^{-1} (except for clearance, in ml/kg × h^{-1}) and were calculated using three models. The first, a plasma model, was proposed by Storch et al. (22) based on plasma methionine enrichments. The second, an intracellular model, was based on a surrogate of intracellular methionine enrichment, calculated by multiplying plasma [1-^{13}C, methyl-$^2H_3$]methionine (M+4) TTR times the ratio between α-ketoisocaproate/leucine plasma TTR measured in each subject. A similar correction factor was proposed also by Storch et al. (22), who, however, assumed a fixed 20% lower α-ketoisocaproate than leucine TTR in plasma. This model was designed as an intracellular model, α-ketoisocaproate/leucine correction. Finally, the third model was based on an estimate of intracellular methionine TTR calculated by multiplying plasma [1-^{13}C, methyl-$^2H_3$]methionine (M+4) TTR times the ratio between [1-^{13}C]homocysteine and [1-^{13}C]methionine TTR in plasma, according to MacCoss et al. (23). This model was defined as the intracellular model, homocysteine/methionine correction.

TABLE 2
Steady-state plasma methionine and homocysteine concentrations (in μmol/l); isotope TTRs of [1-^{13}C, methyl-$^2H_3$]methionine (i.e. [M+4]-methionine), of [1-^{13}C]methionine (i.e. [M+1]-methionine), of [1-^{13}C]homocysteine, of L-[5,5,5-$^2H_3$]leucine, and of [5,5,5-$^2H_3$]-α-ketoisocaproate; total CO_2 production (VCO_2, in μmol/kg × h^{-1}); and $^{13}CO_2$ production ($V^{13}CO_2$, in μmol/kg × h), either uncorrected or corrected for the contribution of $^{13}CO_2$ from glucose oxidation during the clamp, in the basal postabsorptive state, and after the euglycemic-hyperinsulinemic clamp

	Type 2 diabetic subjects		Control subjects	
	Basal	Clamp	Basal	Clamp
Methionine concentration	20.7 ± 1.9	14.2 ± 1.8*	23.7 ± 0.9	16.6 ± 1.8*
Homocysteine concentration†	19.6 ± 3.5	19.5 ± 3.6	10.3 ± 0.6	10.2 ± 0.7
[1-^{13}C, methyl-$^2H_3$]-methionine (M+4)	9.08 ± 0.98	12.00 ± 1.15*	9.09 ± 0.39	11.14 ± 0.68*
[1-^{13}C]-methionine (M+1)	1.03 ± 0.14	1.71 ± 0.15*	1.61 ± 0.31	3.71 ± 0.90*
[1-^{13}C]-homocysteine†	0.74 ± 0.07	0.59 ± 0.08	0.62 ± 0.03	0.37 ± 0.04*
L-[5,5,5-$^2H_3$]-leucine	0.106 ± 0.006	0.143 ± 0.01*	0.100 ± 0.005	0.130 ± 0.007*
[5,5,5-$^2H_3$]-α-ketoisocaproate	0.081 ± 0.006	0.103 ± 0.007*	0.071 ± 0.003	0.090 ± 0.005*
VCO_2 (μmol/kg × h^{-1})	111 ± 3	124 ± 12	106 ± 4	123 ± 4
$V^{13}CO_2$ (μmol/kg × h^{-1})	22.15 ± 3.22	48.46 ± 3.53	20.52 ± 3.53	64.6 ± 4.36
$V^{13}CO_{2\ corrected}$	22.15 ± 3.22	15.96 ± 4.37‡	20.52 ± 3.53	32.19 ± 6.51

Data are means ± SE. *$P < 0.025$ vs. basal (by ANOVA, post hoc test); †$P < 0.025$ for type 2 diabetic patients vs. control subjects (by ANOVA, group effect); ‡$P < 0.05$ for type 2 diabetic vs. control subjects (by ANOVA, interaction effect).

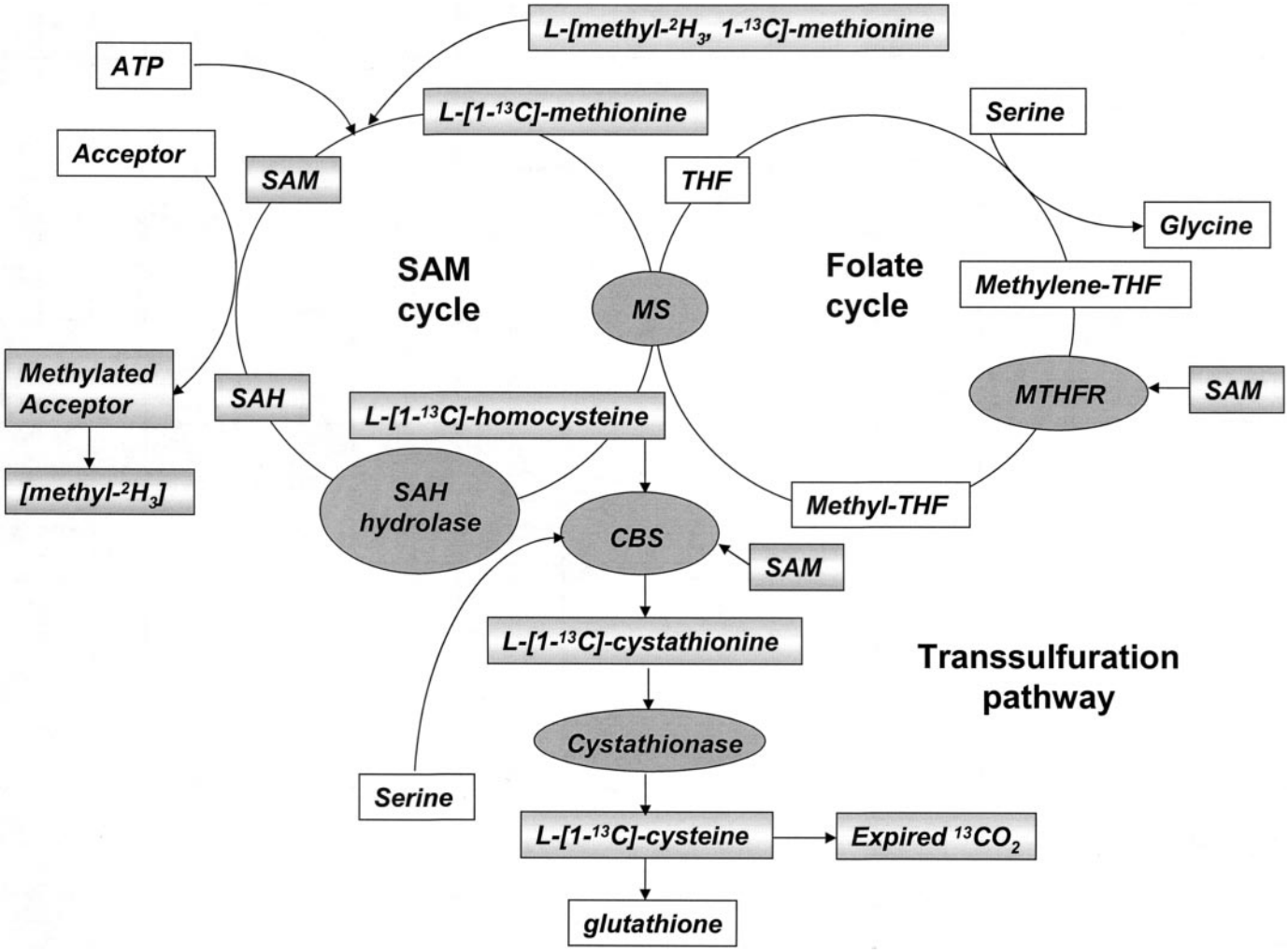

FIG. 1. Pathways of methionine and homocysteine metabolism, with depiction of routes of entry, transfer, and disposal into and out of the different pools, of the carboxyl (^{13}C) and the ($^{2}H_3$) methyl tracers. On the left side of the figure, the S-adenosylmethionine (SAM) cycle is depicted, and on the right the folate cycle is shown. Shaded boxes indicate labeled compounds, rounded boxes the enzymatic steps, and square white boxes unlabeled substrates. The bi-labeled [1-^{13}C, methyl-$^{2}H_3$]methionine enters the S-adenosylmethionine cycle, where it loses the [methyl-$^{2}H_3$] tracer to a methylated acceptor at the demethylation step. The resulting mono-labeled [1-^{13}C]homocysteine and, after remethylation, the [1-^{13}C]methionine are returned back to the S-adenosylmethionine cycle. The [^{13}C]homocysteine enters the transsulfuration pathway, where the $^{13}CO_2$ is lost at the site of cysteine catabolism. MS, methionine synthase; SAH, s-adenosylhomocysteine; THF, tetra-hydro-folate.

The corrections outlined in the second and third were applied only to plasma [1-^{13}C, methyl-$^{2}H_3$]methionine (M+4) TTR because the [1-^{13}C]methionine (M+1) TTR is already an "intracellular" TTR, derived from the intracellular demethylation of [1-^{13}C, methyl-$^{2}H_3$]methionine.

The rates of methionine appearance (R_a) in plasma were calculated as:

$$Q_m = I_{tr} \times E_{tr}/E_4 \quad (1)$$

$$Q_c = I_{tr} \times E_{tr}/(E_1 + E_4) \quad (2)$$

where Q_m and Q_c indicate methionine R_a using either the (M+4) or the (M+1) masses; I_{tr} is the tracer infusion rate (in μmol/kg $\times$ h^{-1}); E_{tr} is tracer enrichment (expressed as TTR); and E_4 and E_1 are [methyl-$^{2}H_3$, 1-^{13}C] (M+4) and [1-^{13}C] (M+1) tracer enrichments in plasma, respectively. The (M+1) mass is generated by the demethylation of the doubly labeled [methyl-$^{2}H_3$, 1-^{13}C] tracer due to the irreversible loss of the [methyl-$^{2}H_3$] group into the methyl pool(s) intracellularly (Fig. 1). Total [1-^{13}C] (M+1) tracer enrichment is the sum of the [1-^{13}C] (M+1) and [methyl-$^{2}H_3$, 1-^{13}C] (M+4) tracers because both contain a ^{13}C carbon (23).

Methionine remethylation (RM) was calculated as:

$$RM = Q_m - Q_c \quad (3)$$

The irreversible homocysteine loss through transsulfuration (TS) is conventionally assumed to be equivalent to oxidation (22). Oxidation was calculated by dividing the rate of $^{13}CO_2$ expiration (in μmol/kg $\times$ h^{-1}) (augmented by 20% because of fixation in the body bicarbonate pool) over plasma [^{13}C]methionine total TTR, i.e., the sum of the (M+4) and the (M+1) TTR:

$$TS = V^{13}CO_2 \times (1 \div [^{13}C]\text{methionine } E_{pl} - [1 \div {}^{13}C]\text{methionine } E_{tr}) \quad (4)$$

Where $V^{13}CO_2$ is the rate of $^{13}CO_2$ expiration (in mol/kg $\times$ h^{-1}), in turn calculated by multiplying the atom percent excess of the expired $^{13}CO_2$, converted into micromoles and corrected for bicarbonate fixation, times total CO_2 production (VCO_2, in ml/kg $\times$ h^{-1}). [^{13}C]methionine E_{pl} is total [^{13}C]methionine enrichment in plasma. Transsulfuration was also corrected for the contribution of the infused glucose during the clamp (see above).

Methionine transmethylation (TM) was calculated as:

$$TM = RM + TS \quad (5)$$

The nonoxidative methionine disposal, i.e., the index of whole-body protein synthesis (PS), was calculated by subtracting transsulfuration from Qc:

$$PS = Q_c - TS \quad (6)$$

Finally, homocysteine (Hcy) clearance (in ml/kg $\times$ h^{-1}) was calculated by dividing the sum of homocysteine removal through remethylation and transsulfuration, or transmethylation, over homocysteine concentration:

$$\text{Hcy clearance} = [RM + TS] \div [Hcy] \quad (7)$$

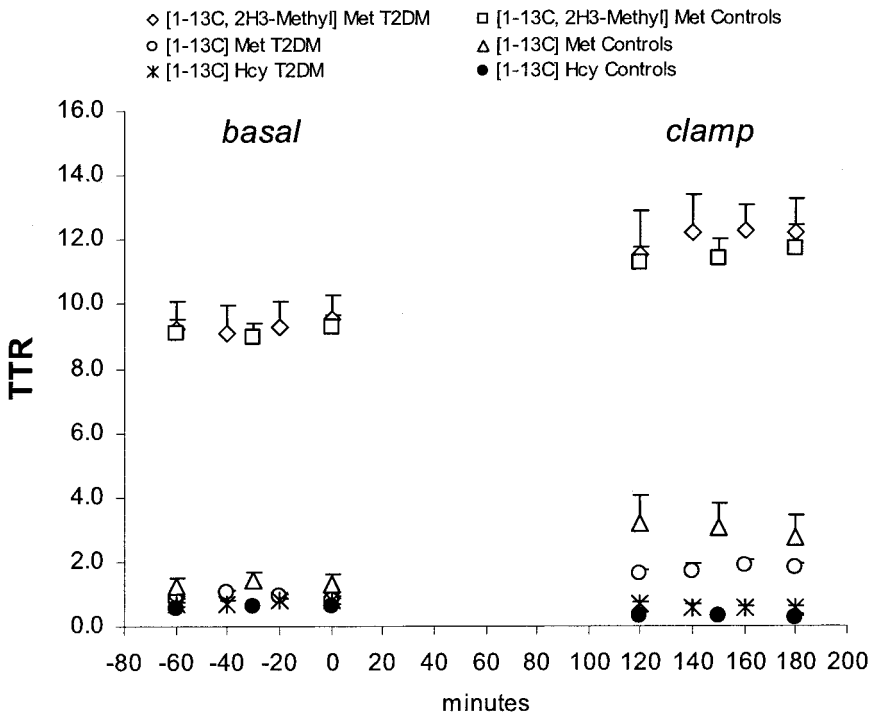

FIG. 2. Pattern of methionine and homocysteine isotope enrichments (expressed as TTRs in plasma) in the basal postabsorptive state and after the euglycemic-hyperinsulinemic clamp at steady state.

Where [Hcy] is plasma homocysteine concentration (in μmol/l). Partial clearance rates through either transsulfuration or remethylation were also calculated in an analogous fashion.

The rates described in Eqs. 1–7 were also recalculated, using the estimated intracellular [methyl-$^2H_3$, 1-^{13}C]methionine (M+4) TTR, i.e., that multiplied by either the ketoisocaproate/leucine TTR ratio, or the homocysteine/methionine TTR ratio, as indicated above.

Statistical analysis. All data are expressed as the means ± SE. Two-way ANOVA for repeated measurements and post hoc analyses were used to compare the data of the basal and the clamp periods between diabetic and control subjects. Two-tailed Student's *t* test for unpaired data was used to compare a single set of data between the two groups. Log transformations of data were performed when the distribution was not normal. Statistica software (version 6; StatSoft, Tulsa, OK) was used. *P* values <0.05 were considered statistically significant.

RESULTS

Substrate, hormones, insulin-mediated glucose disposal, isotope enrichments, carbon dioxide production, and MTHFR isoforms. The type 2 diabetic subjects had higher fasting glucose, A1C, insulin, fibrinogen, creatinine, and triglyceride plasma concentrations than the control subjects (Table 1). They also had a mean urinary albumin excretion rate of 1.2 g/day^{-1} and a lower glomerular filtration rate (calculated: 86 ± 11 ml/1.73 m^2 × min^{-1}; measured: 84 ± 13 ml/1.73 m^2 × min^{-1}; $P < 0.025$ and $P < 0.05$, respectively) than the control subjects (calculated: 122 ± 12 ml/1.73 m^2 × min^{-1}). During the clamp, plasma insulin was increased and maintained to similar levels in the diabetic (1,094 ± 140 pmol/l) and the control subjects (988 ± 111 pmol/l). Insulin-mediated glucose disposal was 4.9 ± 0.3 mg/kg × min^{-1} in the type 2 diabetic patients and 7.3 ± 0.7 in control subjects ($P < 0.01$ between the two groups).

Homocysteine concentration in the type 2 diabetic patients was twofold greater than in control subjects ($P < 0.03$) and did not change in either group after hyperinsulinemia (Table 2). Total amino acid concentrations were similar in both groups in both the basal (1,897 ± 81 and 1,806 ± 79 μmol/l in type 2 diabetic and control subjects, respectively) and the clamp period (1,328 ± 42 and 1,288 ± 52 μmol/l), being significantly decreased after the clamp (1,328 ± 42 and 1,288 ± 52 μmol/l, $P < 0.01$ vs. baseline).

Postabsorptive plasma methionine and homocysteine enrichments were not different between the groups (P = NS by ANOVA and post hoc test) (Table 2). After the clamp, plasma methionine enrichments decreased versus baseline to similar levels in both groups ($P < 0.001$) (Fig. 2, Table 2). Also, plasma homocysteine enrichment decreased in both groups ($P < 0.001$), but it remained greater in type 2 diabetes than in control subjects ($P < 0.025$). Consequently, the [1-^{13}C]homocysteine–to–[1-^{13}C]methionine enrichment ratio decreased during the clamp, but it remained greater in diabetic than in control subjects ($P < 0.05$).

Total CO_2 production increased during the clamp ($P < 0.05$ by ANOVA), without differences between groups (Table 2). The corrected rate of $^{13}CO_2$ expiration tended to decrease in the patients and to increase in the control subjects, albeit insignificantly.

The MTHFR alleles were substantially nondifferent between the two groups. One diabetic and two control subjects were homozygotic for the common phenotype, five diabetic and four control subjects were heterozygotic for the [677C→T] mutation, and one subject within each group was homozygotic for the [677C→T] mutation. Folate and vitamin B_{12} concentrations were normal and not different between the groups (Table 1).

Methionine and homocysteine kinetics (Table 3). Methionine carbon and methyl fluxes were similar in both groups in both the fasting and the hyperinsulinemic state. With hyperinsulinemia, methionine fluxes decreased versus basal using the plasma and the ketoisocaproate/leucine-corrected intracellular model, whereas it increased using the homocysteine/methionine-corrected intracellular model. Also, fasting leucine R_a was similar between type 2 diabetic and control subjects, and it was suppressed by 23% in both groups with hyperinsulinemia (data not shown).

TABLE 3
Steady-state methionine and homocysteine kinetics (expressed in μmol · kg^{-1} · h^{-1}, except for clearance, in ml · kg^{-1} · min^{-1}) in the basal state and after the euglycemic-hyperinsulinemic clamp

	Type 2 diabetic subjects		Control subjects	
	Basal	Clamp	Basal	Clamp
Plasma model				
Methyl R_a (Q_m)	17.00 ± 1.09	12.18 ± 0.66*	17.52 ± 0.96	13.69 ± 0.83*
Carbon R_a (Q_c)	15.47 ± 0.94	10.55 ± 0.55*	15.21 ± 0.68	11.06 ± 0.60*
Remethylation (RM)	1.53 ± 0.18	1.64 ± 0.20	2.31 ± 0.57	2.61 ± 0.62
Transsulfuration (TS)	2.18 ± 0.25	1.11 ± 0.28*†	2.00 ± 0.37	2.36 ± 0.50
Transmethylation‡ (TM)	3.71 ± 0.33	2.75 ± 0.43§	4.32 ± 0.68	4.97 ± 0.86
Transmethylation/transsulfuration	0.58 ± 0.04	0.34 ± 0.08	0.47 ± 0.09	0.48 ± 0.05
Protein synthesis (PS)	13.29 ± 0.94	7.07 ± 0.42*	13.20 ± 0.55	6.34 ± 0.27*
Homocysteine clearance‡	0.26 ± 0.08	0.35 ± 0.09*§	0.43 ± 0.07	0.75 ± 0.10*
Intracellular model, ketoisocaproate/leucine correction				
Methyl R_a (Q_m)	23.03 ± 1.86	17.6 ± 1.11*	25.47 ± 1.57	20.40 ± 1.01*
Carbon R_a (Q_c)	20.06 ± 1.35	14.40 ± 0.58*	20.92 ± 1.28	15.57 ± 0.93*
Remethylation‡ (RM)	2.98 ± 0.54	3.20 ± 0.58	4.56 ± 0.93	4.84 ± 0.82
Transsulfuration (TS)	2.81 ± 0.34	1.45 ± 0.36†‖	2.74 ± 0.54	3.20 ± 0.70
Transmethylation¶ (TM)	5.78 ± 0.75	4.65 ± 0.78†	7.30 ± 1.11	8.03 ± 1.20
Transmethylation/transsulfuration	0.49 ± 0.04	0.27 ± 0.07	0.38 ± 0.08	0.38 ± 0.04
Protein synthesis (PS)	17.25 ± 1.27	9.83 ± 0.46‖	18.18 ± 0.96	9.19 ± 0.47‖
Homocysteine clearance¶	0.40 ± 0.12	0.54 ± 0.15*§	0.73 ± 0.12	1.15 ± 0.14*
Intracellular model, homocysteine/methionine correction				
Methyl R_a‡ (Q_m)	22.62 ± 3.38	47.8 ± 13.09*	39.86 ± 8.54	107.69 ± 24.11*
Carbon R_a‡ (Q_s)	19.56 ± 2.61	28.68 ± 4.38*	26.75 ± 3.44	40.17 ± 4.13*
Remethylation‡ (RM)	2.85 ± 0.75	19.12 ± 8.89§	13.11 ± 5.49	67.52 ± 23.79*
Transsulfuration‡ (TS)	2.89 ± 0.62	3.00 ± 0.95§	3.35 ± 0.71	7.50 ± 1.52*
Transmethylation¶ (TM)	5.74 ± 1.33	22.12 ± 9.65*§	16.46 ± 5.85	75.02 ± 23.72*
Transmethylation/transsulfuration	0.53 ± 0.05	0.15 ± 0.04	0.35 ± 0.11	0.15 ± 0.03
Protein synthesis (PS)	16.89 ± 2.10	20.01 ± 3.11	23.40 ± 2.98	24.73 ± 2.62
Homocysteine clearance¶	0.44 ± 0.17	2.59 ± 1.52*†	1.6 ± 0.57	8.62 ± 2.76*

Data are means ± SE. The data are calculated with three kinetic models using: *1*) uncorrected plasma methionine TTR (defined as plasma model); *2*) plasma methionine TTR corrected for the ratio α-ketoisocaproate/leucine TTR in plasma (intracellular model, ketoisocaproate/leucine correction); and *3*) methionine TTR corrected for the ratio homocysteine/methionine TTR in plasma (intracellular model, homocysteine/methionine correction). *$P < 0.025$ vs. basal (by ANOVA and post hoc test); †$P < 0.05$ for type 2 diabetic vs. control subjects (by ANOVA, either interaction effect or post hoc analysis); ‡$P < 0.05$ for type 2 diabetic vs. control subjects (by ANOVA, group effect); §$P < 0.025$ for type 2 diabetic vs. control subjects (by ANOVA, either interaction effect or post hoc analysis); ‖$P < 0.05$ vs. basal (by ANOVA and post hoc test); and ¶$P < 0.025$ for type 2 diabetic vs. control subjects (by ANOVA, group effect).

In the type 2 diabetic subjects, methionine transmethylation was lower than in control subjects using all models, particularly after hyperinsulinemia. During the clamp, transmethylation in type 2 diabetes decreased versus baseline using the plasma and the ketoisocaproate/leucine-corrected intracellular models, whereas it did not change in the control subjects. Using the homocysteine/methionine-corrected intracellular model, transmethylation increased in both groups, but more in control subjects (Table 3). The resulting transmethylation relative increments after hyperinsulinemia were significantly lower in type 2 diabetic than in control subjects using all three models (Fig. 3).

Transsulfuration was not different between groups in the fasting state (Table 3). During the clamp, transsulfuration in type 2 diabetes decreased, whereas it did not change in the control subjects using either the plasma or the ketoisocaproate/leucine-corrected intracellular models. Using the homocysteine/methionine-corrected intracellular model, transsulfuration did not change in type 2 diabetes, whereas it was increased in control subjects (Table 3). The resulting relative increments of transsulfuration with hyperinsulinemia were lower in type 2 diabetic than in control subjects using all three models (Fig. 3).

Remethylation in type 2 diabetic subjects was lower than in control subjects when using both intracellular models, but not when using the plasma model. With hyperinsulinemia (Table 3), remethylation did not change in either group when using the plasma and the ketoisocaproate/leucine-corrected intracellular models, whereas it increased in both groups when using the homocysteine/methionine-corrected intracellular model, but to a greater extent in control subjects (Table 3).

Homocysteine clearance, i.e., (transmethylation + transsulfuration) ÷ homocysteine concentration, was >50% lower in the type 2 diabetic than in control subjects using all models, particularly in the hyperinsulinemic state (Table 3, Fig. 4). Homocysteine clearance increased significantly versus baseline in both groups, however, by a more than twofold greater extent in control than in type 2 diabetic subjects, using all models (Fig. 5). Both components of clearance (i.e., either transsulfuration or remethylation divided over plasma homocysteine concentrations) were significantly lower (by ANOVA) in type 2 diabetic than control subjects, using all models (data not shown).

Protein synthesis did not differ between the two groups, using all models. It decreased versus basal after hyperinsulinemia when using both the plasma and the ketoisocaproate/leucine-corrected intracellular models, whereas it did not change in both groups when using the homocysteine/methionine-corrected intracellular model (Table 3).

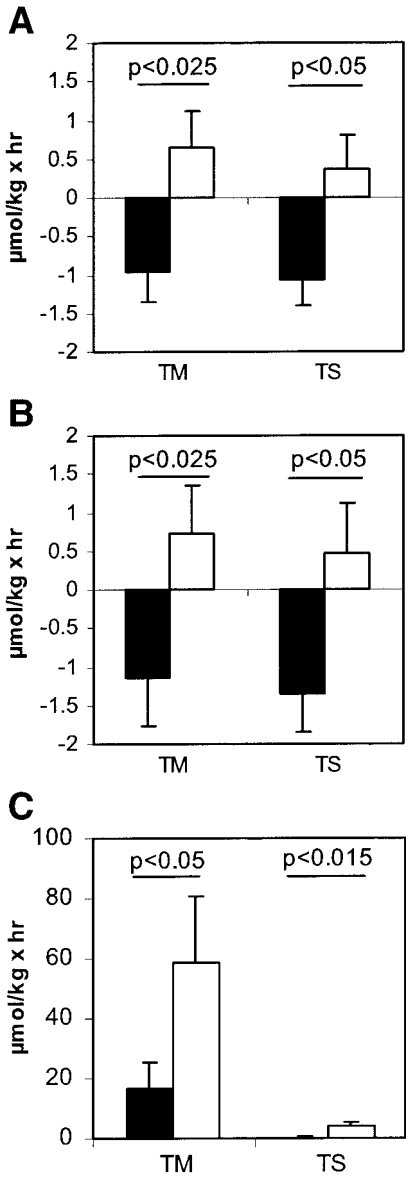

FIG. 3. Changes of transmethylation (TM) and of transsulfuration (TS; i.e., after the clamp versus the basal period [in µmol/kg × h^{-1}]) in the type 2 diabetic and control subjects, using the plasma (*A*); the intracellular model, ketoisocaproate/leucine correction (*B*); or the intracellular model, homocysteine/methionine correction (*C*). ■, type 2 diabetic subjects; □, control subjects.

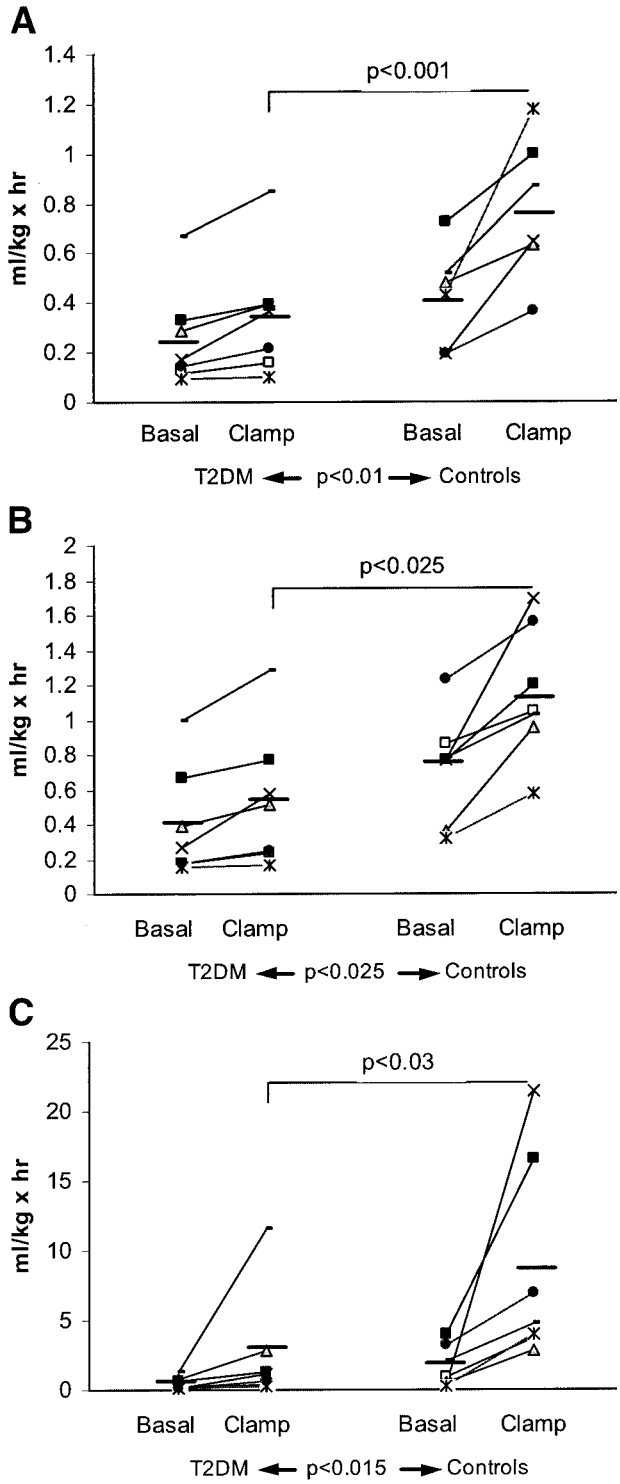

FIG. 4. Individual values of homocysteine clearance (in ml/kg × h^{-1}) in the basal postabsorptive state and after the euglycemic-hyperinsulinemic clamp at steady state in the type 2 diabetic subjects and in the control subjects, using the plasma (*A*); the intracellular model, ketoisocaproate/leucine correction (*B*); or the intracellular model, homocysteine/methionine correction (*C*). Horizontal short lines indicate the group means. There were significant differences between the two groups by ANOVA (group effect, indicated at the foot of each panel), as well as between the two groups after the clamp (by ANOVA and post hoc test). T2DM, type 2 diabetic subjects.

DISCUSSION

This study demonstrates that multiple metabolic abnormalities of methionine and homocysteine kinetics are present in type 2 diabetes with nephropathy. Homocysteine formation from methionine through transmethylation, and its metabolic clearance, were decreased in the type 2 diabetic subjects particularly after hyperinsulinemia. The insulin-induced changes in methionine transmethylation, homocysteine transsulfuration, and homocysteine clearance were also markedly reduced. In contrast, homocysteine production from methionine was not greater in type 2 diabetes. Taken together, these data demonstrate that the hyperhomocysteinemia of type 2 diabetes with nephropathy is caused by decreased homocysteine removal, and not by increased formation from methionine, particularly during hyperinsulinemia. This is particularly important in the view of the insulin resistance condition of type 2 diabetes (19,20). Also, fibrinogen kinetics was altered in type 2 diabetes after hyperinsulinemia in the direction of an exaggerated production (32). Thus, insulin

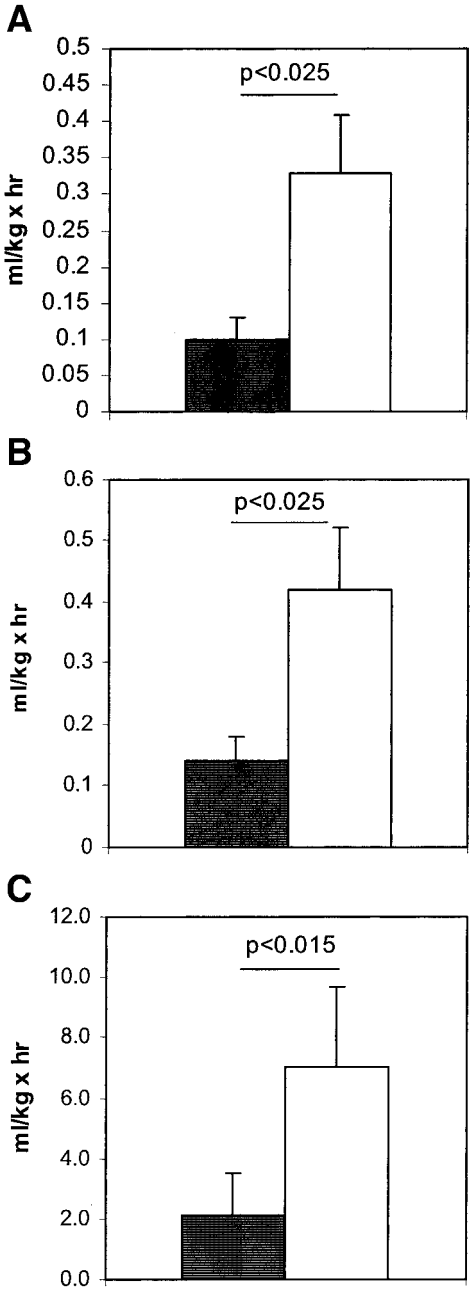

FIG. 5. Changes in homocysteine metabolic clearance (in ml/kg × h^{-1}) for clamp versus baseline euglycemic in type 2 diabetic and control subjects using the plasma (*A*); the intracellular model, ketoisocaproate/leucine correction (*B*); or the intracellular model, homocysteine/methionine correction (*C*). ▤, type 2 diabetic subjects; □, control subjects.

resistance in type 2 diabetes affects the kinetic regulation of two important vascular risk factors related to amino acid/protein metabolism.

The reduced transmethylation would lead to decreased S-adenosylmethionine concentration (Fig. 1). No data on this pathway in the whole body have been published in type 2 diabetes so far. However, in blood cells of type 2 diabetic patients, both S-adenosylmethionine concentration and MTHFR activity were reduced (33), in agreement with the mechanistic hypothesis of our study. A decreased S-adenosylmethionine concentration would in turn affect the rate-limiting step of homocysteine irreversible catabolism, i.e., transsulfuration, catalyzed by CBS (Fig. 1). CBS activity is mainly found in liver, kidney, small intestine, pancreas, adipose tissue, and brain in the rat; it is stimulated by phosphorylation/dephosphorylation, oxidizing conditions, methionine supplementation, and S-adenosylmethionine; but it is inhibited by S-adenosylhomocysteine, the direct homocysteine precursor (1,21,34–36). Thus, the impaired stimulation of both CBS and transsulfuration in type 2 diabetic subjects was likely associated with decreased intracellular S-adenosylmethionine concentration, resulting from decreased transmethylation (36). An inverse relationship between S-adenosylmethionine and the carotid intima-media thickness has been demonstrated (37), suggesting a link between reduced transmethylation, hyperhomocysteinemia, and vascular damage.

Insulin regulates CBS activity (35,38). In insulin-deficient diabetic rats, liver CBS activity was increased, and this increase was reversed by insulin treatment (35). In contrast, in diet-induced insulin-resistance models, an inverse relationship between insulin and CBS activity was observed (39). Thus, insulin-deficiency and insulin resistance differently affected CBS activity in experimental diabetes. Hyperinsulinemia, however, did not reduce, but actually increased, homocysteine transsulfuration in healthy individuals (24). The reasons for these discrepancies between the in vitro models of diabetes, as well as between in vitro and in vivo studies, are unknown. It is possible that some metabolic abnormalities associated with insulin deficiency directly affected CBS activity in animal models of diabetes. Notably, decreased homocysteine transsulfuration would diminish glutathione synthesis, a key antioxidant substance (1,21), which is often decreased in type 2 diabetes (40).

Transmethylation encompasses transsulfuration and remethylation, i.e., the two routes of homocysteine disposal (see calculations above). The differences between groups in both basal and insulin-stimulated transmethylation (i.e., transsulfuration + remethylation) became greater when it was divided over plasma homocysteine concentration to calculate homocysteine clearance (see Eq. 7). In theory, because amino acid clearance is inversely related to concentration (41), the lower homocysteine clearance in type 2 diabetes might theoretically have been caused just by the increased homocysteine concentrations, which, however, was not the case, as demonstrated above. These conclusions are further strengthened by the lower insulin-induced increments of clearance observed in the type 2 diabetic than in the control subjects, despite unchanged homocysteine concentrations in either group. Kidney and liver are possibly involved in the reduced transmethylation and transsulfuration also observed in the type 2 diabetic patients (1,21,34,36,38), although a role of peripheral tissues, such as muscle, cannot be excluded. A net homocysteine uptake was shown across the rat kidney (42). Only direct organ catheterization studies may elucidate this important point.

We intentionally selected type 2 diabetic patients with albuminuria because this condition is usually associated with hyperhomocysteinemia. Their renal function ranged from normality ($n = 4$) to a mild to moderate insufficiency (in the remaining 3). Because impaired renal function was associated with both hyperhomocysteinemia and abnormalities of methionine/homocysteine kinetics (7,43,44), it is possible that renal insufficiency played a role at least in three patients. Nevertheless, our type 2 diabetic subjects apparently exhibited a larger reduction of homocysteine clearance than that expected just from the degree of renal

insufficiency. Indeed, in the reported studies (43,44), the subjects with end-stage renal disease had a homocysteine concentration much greater (~4–6 times the control values) than that observed in the present study (twice the control values), whereas transmethylation and homocysteine clearance were ~25% and ~4 times lower, respectively, than control values, compared with the (proportionally) much lower transmethylation and homocysteine clearance of our study (Table 3). Additional studies in type 2 diabetic patients without nephropathy may add useful data to this issue. Interestingly, the S-adenosylhomocysteine concentration was increased in end-stage renal disease (43), well in agreement with the hypothesis discussed above. In the reported studies, however (43,44), the effects of insulin were not tested. Because the most dramatic differences between the two groups were observed after hyperinsulinemia (Table 3 and Figs. 3–5), the insulin clamp may thus be proposed as a specific test to elicit abnormalities of methionine/homocysteine kinetics in type 2 diabetes.

Both the methyl- and the carbon-methionine R_a were decreased by hyperinsulinemia in either group, using the plasma as well as the ketoisocaproate/leucine-corrected intracellular models, whereas they were apparently increased when using the homocysteine/methionine-corrected intracellular model. The former data are consistent with previous reports on the effects of insulin on endogenous amino acid appearance (18,30,45), whereas the latter are clearly unexpected. These discrepancies were essentially caused by opposite changes of plasma methionine and homocysteine enrichments after hyperinsulinemia (Table 2 and Fig. 2), which may at least in part reflect the complexity of homocysteine body pools (24,46), as well as a slow and incomplete equilibration between the various components of these pools, possibly different between diabetic and control subjects. Nevertheless, although plasma homocysteine enrichment might not have been entirely at steady state at the end of each experimental period in either group (23), the directions of changes of enrichments after hyperinsulinemia (an increase of methionine, a decrease of homocysteine) were so clear-cut that the resulting kinetic data should be considered, if anything, as conservative. Furthermore, the possible lack of homocysteine isotopic equilibrium in the type 2 diabetic subjects does not explain why homocysteine enrichment was not decreased in the type 2 diabetic subjects in the basal period. Finally, the main conclusions of our study on both transmethylation and clearance were consistent between the three models based on partially different assumptions and measurements.

Homocysteine concentrations were surprisingly unchanged in either group after hyperinsulinemia (Table 3), whereas other amino acids were decreased (24,30,45). The relationships between total plasma homocysteine concentrations, insulin sensitivity, and/or metabolic control are not clear yet, possibly conditioned by the complexity of homocysteine metabolic pools (12,17,47–49).

Our subjects had normal vitamin B_{12} and folate concentrations; therefore, no restriction by these cofactors of homocysteine remethylation (1,2) likely occurred. Conversely, no relationship between homocysteine concentration and vitamin B_{12} and folate concentrations was found in type 2 diabetes (12). The distribution of MTHFR was also not different between the two groups (see RESULTS); therefore, it did not likely account for the observed differences in methionine and homocysteine kinetics.

The patients' usual drug treatments (with ACE inhibitors, angiotensin receptor blockers, statins, and OHAs) might in theory have affected the results, possibly interfering with redox state and oxidative stress. Because drug combinations were, however, variable in each patient, a consistent effect would therefore be difficult to demonstrate. A possible "antioxidant" effect would theoretically be accompanied by an increased redox state, reduced transsulfuration, and decreased de novo formation of glutathione but an increase of its reduced form. Thus, a precise and univocal effect of the drugs on glutathione, the most important antioxidant substrate, would be difficult to demonstrate without a direct measurement.

All of the diabetic and three of the control subjects were hypertensive. The relationships between homocysteine and hypertension are unclear (50). Our hypertensive control subjects had an entirely normal homocysteine concentration (<10 μmol/l) and no other cardiovascular risk factors. Their kinetic values fell entirely within the mean ± 1 SD values of the control group (data not shown). Conversely, these otherwise metabolically normal, hypertensive subjects may serve also as control subjects for the hypertensive type 2 diabetic patients.

In conclusion, this study shows that methionine transmethylation and homocysteine disposal and clearance are impaired in type 2 diabetic patients with nephropathy, both in fasting and hyperinsulinemic conditions, thus suggesting a mechanism for the hyperhomocysteinemia of this condition.

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