

Urinary Urea Nitrogen Excretion during the Hyperinsulinemic Euglycemic Clamp in Type 1 Diabetic Patients and Healthy Subjects

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Summary

The hyperinsulinemic euglycemic clamp (HEC) combined with indirect calorimetry (IC) is used for estimation of insulin-stimulated substrate utilization. Calculations are based on urinary urea nitrogen excretion (UE), which is influenced by correct urine collection. The aims of our study were to improve the timing of urine collection during the clamp and to test the effect of insulin on UE in patients with type 1 diabetes (DM1; n=11) and healthy subjects (C; n=11). Urine samples were collected (a) over 24 h divided into 3-h periods and (b) before and during two-step clamp (1 and 10 mIU.kg⁻¹.min⁻¹; period 1 and period 2) combined with IC. The UE during the clamp was corrected for changes in urea pool size (UEc). There were no significant differences in 24-h UE between C and DM1 and no circadian variation in UE in either group. During the clamp, serum urea decreased significantly in both groups (p<0.01). Therefore, UEc was significantly lower as compared to UE not adjusted for changes in urea pool size both in C (p<0.001) and DM1 (p<0.001). While UE did not change during the clamp, UEc decreased significantly in both groups (p<0.01). UEc during the clamp was significantly higher in DM1 compared to C both in period 1 (p<0.05) and period 2 (p<0.01). The UE over 24 h and UEc during the clamp were statistically different in both C and DM1. We conclude that urine collection performed during the clamp with UE adjusted for changes in urea pool size is the most suitable technique for measuring substrate utilization during the clamp both in DM1 and C. Urine collections during the clamp cannot be replaced either by 24-h sampling (periods I-VII) or by a single 24-h urine collection. Attenuated insulin-induced decrease in UEc in DM1 implicates the impaired insulin effect on proteolysis.

Key words: Substrate utilization • Urinary urea nitrogen excretion • Insulin • Glycemic clamp • Indirect calorimetry

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Introduction

Indirect calorimetry (IC) has been used in combination with the glucose clamp technique to measure oxidative and non-oxidative components of glucose uptake, while simultaneously assessing fat oxidation and energy expenditure (DeFronzo *et al.* 1979, Jacot *et al.* 1982, Ferrannini 1988, Thorburn *et al.* 1991). IC is commonly performed before and during the clamp periods (Jacot *et al.* 1982, Ferrannini 1988, Thorburn *et al.* 1991).

Calculations of substrate oxidation are based on measurements of urinary urea nitrogen excretion (UE). UE is influenced by accuracy of urine collection. Furthermore, during a hyperinsulinemic euglycemic clamp insulin-induced changes in urea pool size have been reported in healthy subjects affecting the UE. Briefly, under clamp conditions, serum urea concentration will decrease, hence the UE adjustment for changes in (UEc) is an important factor in determining substrate utilization (Thorburn *et al.* 1991). In DM1, the effect of insulin on amino acid metabolism has been described (Tessari *et al.* 1986), but the effect of insulin on urea pool size and UE has not been evaluated. Thus, the aims of our study were a) to improve the timing of urine collection during the clamp in healthy and DM1 subjects and to test the hypothesis that urinary collections during the clamp could be replaced by samples from 24-h sampling of UE divided into 3-h periods; and b) to test the effect of insulin on UE in both groups.

Subjects and Methods

Subjects

The study groups consisted of 11 men with DM1 without specific diabetic vascular complications, and 11 male healthy control subjects (C) without a family history of diabetes mellitus, dyslipidemia, or other metabolic disease. All subjects gave their informed consent with the study protocol, which had been reviewed and approved by a local ethics committee. Patients were instructed to adhere to their ordinary lifestyle and avoid changes in food intake, alcohol consumption, and exercise one week before admission to the hospital. Characteristics of the study groups are shown in Table 1. All subjects were examined during 3-day hospitalization being on a standard dietary regimen. Dietary intakes in our study groups were: 150-170 mmol/24 h of sodium, 50-80 mmol/24 h of potassium, 80 g/24 h of proteins, 275-325 g/24 h of carbohydrates; total energy intake was 2500-2800 kcal/24 h.

Hyperinsulinemic euglycemic clamp (HEC)

A two-step HEC combined with IC was performed after an 8- to 10-h overnight fast on day 3 after hospital admission. The clamp lasting 4 h (period 1: 0-120 min and period 2: 120-240 min) was conducted as previously described (DeFronzo *et al.* 1979). Briefly, a Teflon cannula (Venflon; Viggo Helsingborg, Sweden) was inserted into the left antecubital vein for infusion of all test substances. A second cannula was inserted in the retrograde fashion into a wrist vein of the same hand for blood sampling, and the hand was placed into a heated (65 °C) box to achieve venous blood arterialization. A stepwise primed-continuous infusion (1 and 10 mU.kg⁻¹.min⁻¹ of Actrapid HM; Novonordisk, Copenhagen, Denmark) was administered to acutely rise and maintain the plasma concentration of insulin at 75 and 1400 µU/ml. Decreases in serum potassium concentrations during insulin infusion were prevented by co-infusion of potassium chloride with glucose (60 mmol/l KCl/l of 15 % glucose). Plasma glucose concentrations during the clamp were maintained at 5 mmol/l by continuous infusion of 15 % glucose. Arterialized blood plasma glucose concentrations were determined every 5-10 min. Before the clamp, only diabetics with fasting plasma glucose levels below 6 mmol/l were included in the study, and no glucose was infused until plasma glucose had declined to the desired level. Blood urea nitrogen was measured at times 0 min, 120 min and 240 min.

Table 1. Characteristics of study groups.

	C (n = 11)	DM1 (n = 11)
<i>Age (years)</i>	28.72 ± 2.10	32.18 ± 5.72
<i>BMI (kg/m²)</i>	24.09 ± 1.6	24.36 ± 2.15
<i>Triglycerides (mmol/l)</i>	1.11 ± 0.76	0.77 ± 0.40
<i>Total cholesterol (mmol/l)</i>	4.11 ± 0.88	4.23 ± 0.59
<i>HDL-cholesterol (mmol/l)</i>	1.27 ± 0.42	1.67 ± 0.29
<i>HbA1c (DCCT) (%)</i>	4.82 ± 0.27	7.97 ± 1.34***
<i>Daily insulin dose (IU/day)</i>	–	36 ± 9.8

Statistical significance: ***p<0.001

Indirect calorimetry (IC)

Substrate utilization and energy expenditure (EE) measurements were made in both groups by IC (Ferrannini 1988). Gas exchange measurements were taken during a 45-min basal period and during the final 45-min periods of the two insulin-infusion steps. A transparent plastic ventilated hood was placed over the subject's head and made airtight around the neck. A slight negative pressure was maintained in the hood to avoid loss of expired air. A constant fraction of air flowing out of the hood was automatically collected for analysis. Air flow as well as O₂ and CO₂ concentrations in expired and inspired air were measured by a continuous open-circuit system (metabolic monitor VMAX; Sensor Medics, Anaheim, CA, USA).

Urinary collections

Urine was collected on the second day after admission. UE during 24 h divided into 3-h periods (periods I-VI from 06:00 to 24:00 h and one-night period VII from 24:00 to 06:00 h) was measured. Urinary collection during the clamp was divided into the basal period (-120-0 min), period 1 (0-120 min), and period 2 (120-240 min).

Analytical methods

Plasma glucose concentrations were measured on a Beckman analyzer (Beckman Instruments, Fullerton, CA, USA) using the glucose oxidase method. Immunoreactive insulin (IRI) was determined by radioimmunoassay using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH a.s., Prague, Czech

Republic). Single measurement of glycosylated hemoglobin (HbA_{1c}) using the Bio-Rad hemoglobin A_{1c} column test (Bio-Rad laboratories, Munich, Germany) was performed before the testing. UE was measured by enzymatic urease reaction using a spectrophotometric UV method (Hitachi 912, Roche, Basel, Switzerland)

Data analysis

Calculations of substrate oxidation were made using standard equations (Ferrannini 1988). UE during the clamp was adjusted for changes in urea pool size (UEc) (Tappy *et al.* 1988). Insulin action was estimated as the metabolic clearance rate of glucose (MCR) and glucose disposal (M) calculated at 80 to 120 min (MCR_{glu} submax and M_{glu} submax) and between 200 and 240 min (MCR_{glu} max and M_{glu} max). MCR was calculated by dividing the amount of glucose infused, after adjustment for changes in glucose pool size, by means of plasma glucose concentration (DeFronzo *et al.* 1979, 1983). Data were statistically analyzed by ANOVA with repeated measures. All data are expressed as means ± S. D.

Results

Twenty-four-hour urinary urea excretion is shown in Table 2. We did not find a significant circadian variation in UE in C and DM1; likewise there were no significant differences in 24-h UE between C and DM1. Evaluation of variation over time in UE output was performed as a coefficient of variation for the individual subjects, however, without statistical significances. During the clamp, serum urea significantly decreased both in C (0 vs. 120 vs. 240 min: 4.67±0.73 vs. 4.29±0.61 vs. 3.8±0.68 mmol/l; p<0.01) and in DM1 (0 vs. 120 vs. 240 min: 6.34±1.73 vs. 5.22±1.7 vs. 4.73±1.21 mmol/l; p<0.01). Serum urea concentrations were significantly higher in DM1 compared to C during the basal (p<0.01) and clamp periods (p<0.05). Table 3 shows the UE and UEc during the clamp. The UE in the basal period was comparable in DM1 and C. During the clamp periods, UE was significantly higher as compared to UEc (p<0.001) in both groups. While UE did not change during the clamp, UEc decreased significantly in both groups (p<0.01). The insulin-induced decrease in UEc during HEC was attenuated in DM1 because UEc during the clamp was significantly higher in DM1 compared to C both in period I (p<0.05) and period II (p<0.01) indicating impaired insulin action on protein metabolism in DM1.

Table 2. Circadian urinary urea excretion (UE) in C (n=11) and DM1 (n=11).

Periods (hours)	C (mmol/24 h)	DM1 (mmol/24 h)
24 h	470 ± 140	452 ± 64
06 – 09 (I)	479 ± 134	431 ± 180
09 – 12 (II)	510 ± 213	423 ± 172
12 – 15 (III)	477 ± 229	428 ± 121
15 – 18 (IV)	474 ± 274	525 ± 102
18 – 21 (V)	576 ± 273	464 ± 135
21 – 24 (VI)	499 ± 103	503 ± 100
24 – 06 (VII)	436 ± 125	369 ± 108

Differences between DM1 and C are not statistically significant.

Table 3. Urinary urea excretion before (basal) and during the two-step hyperinsulinemic euglycemic clamp from 0 to 120 min (Period 1) and from 120 to 240 min (Period 2) before (UE) and after adjustment for changes in urea pool size (UEc) in C (n=11) and DM1 (n=11).

Period	C (mmol/24 h)	DM1 (mmol/24 h)
UE Basal	412 ± 125	412 ± 216
UE Period 1	462 ± 157	686 ± 228 ^b
UE Period 2	436 ± 138	522 ± 162 ^b
UEc Period 1	240 ± 170 ^d	374 ± 187 ^{a,d}
UEc Period 2	219 ± 122 ^{c,d}	274 ± 165 ^{a,c,d}

Statistical significance: a, p<0.05; DM1 vs. C, b, p<0.01; DM1 vs. C, c, p<0.01; UEc period 2 vs. UE basal, d, p<0.001; UEc Period 1 vs. UE Period 1, UEc Period 2 vs. UE Period 2.

C and DM1 did not differ in protein oxidation (basal: 0.70±0.19 vs. 0.97±0.68, period I: 0.42±0.27 vs. 0.69±0.39 and period II: 0.37±0.18 vs. 0.45±0.32 mg.kg⁻¹.min⁻¹). The protein oxidations dropped significantly during the clamp in both groups (basal period vs. period II; p<0.01).

Insulin action, as measured by MCR_{glu} submax (12.54±3.38 vs. 17.41±6.18 mg.kg⁻¹.min⁻¹; p<0.02), MCR_{glu} max (21.63±6.47 vs. 26.61±4.45 mg.kg⁻¹.min⁻¹; p<0.05) and M_{glu} max (19.0±5.6 vs. 23.3±3.0 mg.kg⁻¹.min⁻¹; p<0.01), was lower in DM1 compared with C, although the difference in M_{glu} submax did not reach significance (11.05±3.07 vs. 13.53±2.60 mg.kg⁻¹.min⁻¹). There were no significant relationships between UE and MCR_{glu}. UE over 24 h and UEc during

the clamp were statistically different both in C and DM1. Neither basal UE nor clamp UE could be replaced by UE from 24 h and from 24-h sampling.

Discussion

In this study, we have demonstrated significant insulin-induced changes in urea pool size in both DM1 and C subjects indicating that only UEc should be used for calculations of substrate utilization during the clamp studies. The results are in accordance with previous studies in healthy volunteers (Tappy *et al.* 1988, Thorburn *et al.* 1991). We have also found that hyperinsulinemia significantly decreases the UEc and protein oxidation in both groups.

The insulin-induced decrease in UEc was attenuated in DM1. Because there were no differences in protein oxidation during the clamp between DM1 and C, the insufficient suppression of proteolysis could be responsible for this. This finding could imply that, in addition to insulin resistance (IR) in glucose metabolism (DeFronzo *et al.* 1982, Yki-Järvinen *et al.* 1990, Wohl *et al.* 2004), DM1 patients are also characterized by impaired insulin action in protein metabolism. The increased plasma urea concentrations during clamp periods, as seen in DM1 subjects, could be explained partly by lower insulin sensitivity in DM1 and a lower volume load of glucose infusion during the clamp. Moreover, because an abnormal glucose tolerance test indicates impaired disposal of an oral glucose load, abnormal "protein tolerance" would be indicated by abnormal disposal of the protein load, resulting in an abnormally high postprandial urea production rate (Hoffer 1998). Although this is not a completely new finding, it is interesting in context of physiological and "mechanistic" studies. Thus, it seems that in our study hyperinsulinemia does not suppress proteolysis and urea production in subjects with DM1 as compared with non-diabetic subjects even though UE is equivalent under basal conditions. Our study supports the hypothesis that IR influences UE in DM1 without changes in protein oxidation. The importance of this phenomenon should be confirmed by studies using more precise methods such as tracer techniques which have not been used in our protocol.

Hepatic glucose production was not measured in this study, but it is known to fall by more than 90 % to insulin levels $> 50 \mu\text{U/ml}$ in healthy men (Rizza *et al.* 1991) and patients with DM1 (DeFronzo *et al.* 1982).

Thus, the total amount of glucose infused was assumed to be a measure of the glucose metabolized by all cells of the body during clamp studies. Nevertheless, we could not exclude the impaired suppression of hepatic glucose production by insulin and underestimation of MCR or M in DM1 patients.

However, tracer methods could also underestimate short-term variations in urea production in humans (Hamadeh and Hoffer 1998). Dissociation of insulin effect on glucose and protein metabolism has been reported by the tracer method in DM1 and type 2 diabetes. Insulin action seems to be more effective in reducing catabolic pathways than anabolic processes in protein metabolism (Hoffer 1998). In DM1, the increased leucine transamination associated with increased leucine oxidation has also been found, while in type 2 diabetic patients only changes in leucine transamination, but not in leucine oxidation or leucine kinetics have been shown (Halvatsiotsis *et al.* 2002). Moreover, there is evidence that insulin may contribute to the inhibition of protein oxidation and urea production by other mechanisms (Felig and Wahren 1971, Tessari *et al.* 1987, Gelfand and Barrett 1987). Fukagawa *et al.* (1987) reported a reduction of protein breakdown by insulin in healthy subjects. Whether this phenomenon occurs under the conditions of IR and DM1 still remains not clear.

During hyperinsulinemic euglycemic clamp, the alterations in urine flow, urea pool size, and clearance, urinary nitrogen excretion and substrate oxidation rates also occur. Under these conditions, total urinary nitrogen increases (rather than decreases) by 47 %, largely due to increased urea clearance (Thorburn *et al.* 1991). Increased urea clearance during the clamp is more likely due to an increase in urine output, since augmented urine flow is known to result in an increase of the fractional extraction rate of urea (Thorburn *et al.* 1991). This is due to the fact that urea reabsorption is a passive process completely dependent on the rate of water reabsorption, which establishes the diffusion gradient within the kidney tubules. Changes in the renal clearance of urea, the delay required for urea to pass through the kidney and to be collected in the bladder, and problems associated with subjects producing urine on demand. All this adds uncertainty to short-term measurements of urea production based on urinary urea nitrogen excretion (Thorburn *et al.* 1991, Mathews and Downey 1984). In addition, increased insulin concentrations may have a sodium retaining effect (Pelikánová *et al.* 1996) and, hence, changes in volume

and urea clearance. However, extrarenal metabolic pathways such as urea hydrolysis do not play an important role in whole-body urea homeostasis in healthy subjects, but there are no data about diabetes and obesity (El Khoury *et al.* 1996). In addition, daily obligatory urinary nitrogen excretion of diabetic patients was 18 % higher than in normal subjects and this is not influenced by strict normoglycemia and intensive insulin treatment. During this treatment there remain subtle changes in recycling of amino acids (Hoffer 1998). The mechanism of this phenomenon is still unknown. Relative insulin deficiency, different pathways of insulin application and insulin resistance dealing with disturbances of protein metabolism in patients with Type 1 (DM1) and Type 2 (DM2) diabetes mellitus should also be taken into account.

The circadian variations of UE were not significant in either group in our study. Such findings may be due to a small number of subjects as well as the fact that our measurements were not repeated over a period of several days. In contrast, Steffee *et al.* (1981) concluded that there is little doubt that a diurnal rhythm of urea excretion exists in healthy subjects, but the physiological basis is still not clear. In addition, Thorburn *et al.* (1991) reported large day-to-day variability in basal urinary nitrogen, which might lead to substantial error in the basal substrate oxidation rate. UE is strongly related to protein feeding (Steffee *et al.* 1981), differences in diet probably account for much variation in the reported urinary urea production rates, especially because several studies used collection periods that included both

postabsorptive and feeding periods; however, in our study, dietary intake was standardized for all subjects.

Based on our results, separate urine sampling seems to be the only option during a clamp for estimating substrate utilization both in DM1 and C. We conclude that UE collections performed during the clamp with UE adjusted for changes in urea pool size are the most suitable for measuring substrate utilization during the clamp both in DM1 and C. UEC in urine collection during the clamp cannot be replaced by UE with 24-h sampling (periods I-VII) or by a single 24-h urine collection sample. Furthermore, we have not found significant circadian variation in UE in both groups. Attenuated insulin-induced decrease in UE implicates an impaired insulin effect on proteolysis, despite not altered protein oxidation in DM1. Future studies are needed for UE determination in DM1 and IR which could also provide additional data to make measurement of substrate utilization more accurate.

Conflict of Interest

There is no conflict of interest.

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