Urinary urea nitrogen excretion during the hyperinsulinemic euglycemic clamp in Type 1 diabetes patients and healthy subjects

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Short Title: Urinary urea excretion during the clamp in Type 1 diabetes
Word count: 3970

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 HEC 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 hours divided into 3-hour periods and (b) before and during two-step HEC (1 and 10 mIU.kg^{-1}.min^{-1}; period 1 and period 2) combined with IC. The UE during HEC was corrected for changes in urea pool size (UEc). There were no significant differences in 24-hr UE between C and DM1; and no circadian variation in UE in either group. During 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 in DM1 ($p<0.001$). While UE did not change during HEC, UEc decreased significantly in both groups ($p<0.01$). UEc during HEC 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 hrs and UEc during clamp were statistically different both in C and DM1.

We conclude that urine collection performed during HEC with UE adjusted for changes in urea pool size is the most suitable technique for measuring substrate utilization during HEC both in DM1 and C. Urine collections during HEC cannot be replaced either by 24-hour sampling (periods I-VII) or by a single 24-hour 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.

INTRODUCTION

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

Calculations of substrate oxidation are based on measurement of urinary urea nitrogen excretion (UE). UE is influenced by accuracy of urine collection. Furthermore, during a hyperinsulinemic euglycemic clamp (HEC) 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, therefore UE adjustment for changes in (UEc) is an important factor in determining substrate utilization (Thorburn *at al*. 1991). In DM1, the effect of insulin on amino acid metabolism has been described (Tessari *at 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 HEC in healthy and DM1 subjects and to test the hypothesis that urinary collections during the clamp could be replaced by samples from 24-hour sampling of UE divided into 3-hour 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, and 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 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 hrs of sodium, 50-80 mmol/24 hrs of potassium, 80 g/24 hrs of proteins, 275-325 g/24 hrs of carbohydrates; total energy intake was 2,500-2,800 kcal/24 hrs.

Procedures

Hyperinsulinemic euglycemic clamp (HEC). A two-step HEC combined with IC was performed after an 8- to 10-hour overnight fast on day 3 after hospital admission. The HEC lasting 4 hours (period 1: 0-120 min and period 2: 120-240 min) was conducted as previously described (DeFronzo *at 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 raise 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 into 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 and 120 min and at 240 min.

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 and O_2 and CO_2 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 hours divided into 3-hour periods (periods I-VI from 06:00 to 24:00 hrs and one-night period VII from 24:00 to 06:00 hrs) 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 (HbA1c) 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 HEC was adjusted for changes in urea pool size (UEc) (Tappy *at al*. 1988). Insulin

action was estimated as the metabolic clearance rate of glucose (MCR) and glucose disposal (M) calculated at minutes 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 mean plasma glucose concentration (DeFronzo *at al*. 1979, DeFronzo *at al.* 1983)**.** Hepatic glucose production was not measured in this study, but it is known to fall by more than 90% at insulin levels > 50uU/ml in healthy men (Rizza *at al.* 1991). Thus, the total amount of glucose infused was a measure of the glucose metabolized by all cells of the body during clamp studies. Data were statistically analyzed by ANOVA with repeated measures. All data are expressed as means ± SD.

Results

Twenty-four-hour UE 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-hr UE between C and DM1. Evaluation of variation over time in UE output has been performed as coefficient of variation for the individual subjects, however, without statistical significances. During HEC, 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 HEC. 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 HEC, UEc decreased significantly in both groups ($p<0.01$). The insulin-induced decrease in UEc during HEC was attenuated in DM1: UEc during HEC was significantly higher in DM1 compared to C both in period 1 ($p<0.05$) and period 2

(p<0.01) indicating impaired insulin action on protein metabolism in DM1. 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.min), however, statistical significant insulin-stimulated decreases have been found during HEC 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 $ml.kg^{-1}.min^{-1}$; p<0.02), MCR_{glu} max (21.63 ± 6.47 vs 26.61± 4.45 $ml.kg^{-1}.min^{-1}$; p<0.05) and M_{glu}max (19.04 ± 5.6 vs 23.32 ± 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 hrs 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 hours and from 24-hours 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, (Thorburn *at al.* 1991, Tappy *at al.* 1988). 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 HEC between DM1 and C, the insufficient suppression of proteolysis could be responsible for this finding. This finding could imply that, in addition to IR in glucose metabolism, which has been documented by decreased M and MCR and was consistent with the results of other studies (DeFronzo *at al.* 1982,Yki-Järvinen *at al.* 1990, Wohl *at al.* 2004), DM1 patients are characterized by impaired insulin action in protein

metabolism as well. 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 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 protein load, resulting in an abnormally high postprandial urea production rate (Hoffer 1998). However, a significant correlation has not been demonstrated between UE and MCR suspiciously due to small groups of subjects included into our study. Although, not a completely new finding, this 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 under basal conditions equivalent. 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. However, also tracer methods could underestimate short-term variations in urea production in humans (Hamadeh at al. 1998). Effect of insulin on protein metabolism evaluated by tracers method is different in type 1 and type 2 diabetes, and dissociation of insulin effect on glucose and protein metabolism has been reported. Insulin action seems to be more effective in reducing catabolic pathways than anabolic processes in protein metabolism (Hoffer *at al.* 1998). In DM1, the increased leucine transamination associated with increased leucine oxidation has 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 *at al.* 2002). Moreover, there is an evidence that insulin may contribute to inhibition of protein oxidation and urea production by other mechanisms (Felig *at al.* 1971,Tessari *at al.* 1987,Gelfand *at al.* 1987). Fugawa *at al.* (1987) reported a reduction of

protein breakdown by insulin in healthy subjects. If this phenomenon occurs under the conditions of IR and DM1 still remains unclear.

During HEC, alterations in urine flow, urea pool size, and clearance, urinary nitrogen excretion and substrate oxidation rates occur as well and, under this condition, total urinary nitrogen increases (rather than decreases) by 47%, probably and largely due to increases in urea clearance (Thorburn *at al*. 1991). Increased urea clearance during the clamp is more likely to be due to an increase in urine output, since augmented urine flow is known to result in an increase in the fractional extraction rate of urea (Thorburn *at al*. 1991). This is so because urea reabsorbtion is a passive process completely dependent on the rate of water reabsorbtion, which establishes the diffusion gradient within the kidney tubules. Changes in the renal clearance of urea, the time 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 measurement of urea production based on urinary urea nitrogen excretion (Thorburn *at al* .1991, Mathews at al. 1984). In addition, increased insulin levels may play a role in altering sodium retention (Pelikánová *at 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 in diabetes and obesity as well (El Khoury 1996). In addition, daily obligatory urinary nitrogen excretion of diabetic patients was 18% higher than normal subjects and this is not influenced by strict normoglycemia and intensive insulin treatment. During this treatment there remain subtle impairments in amino acids recycling (Hoffer 1998). Mechanism of this fenomenon is still unknown. Relative insulin deficiency, different pathways of insulin application and insulin resistance dealing with disturbancies of protein metabolism in patients with Type 1 (DM1) and Type 2 (DM2) diabetes mellitus should be taken into account as well.

The circadian variations of UE were not significant in both groups in our study. Contrary to literary data, there were only non-significant trends (Thorburn *at al.* 1991). These 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. By contrast, the other authors concluded that there is little question that a diurnal rhythm of urea excretion exists in healthy subjects, but the physiological basis is still unclear (Steffe *at al.* 1981). However our results clearly indicate that UEc in urine collection during clamp cannot be replaced either by UE with 24-hour sampling (periods I-VII) or by single 24-hour urine collection. In addition, the study of Thornburn reported large day-to-day variability in basal urinary nitrogen, which might lead to substantial error in basal substrate oxidation rate (Thorburn *at al* .1991). UE is strongly related to protein feeding, 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 fed periods; however, in our study, dietary intake was standardized for all subjects (Steffe *at al.* 1981).

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, and neither basal UE nor clamp UE should be replaced by UE from 24 hour and 24-hour sampling. We conclude that UE collections performed during HEC with UE adjusted for changes in urea pool size are the most suitable for measuring substrate utilization during HEC both in DM1 and C. UEc in urine collection during HEC cannot be replaced by UE with 24-hour sampling (periods I-VII) or by a single 24-hour urine collection sample. Furthermore, we have not found 24-hour UE variability n both groups. Attenuated insulin induced decrease in UE implicates the impaired insulin effect on proteolysis, despite the protein oxidation is not altered in DM1. Future studies are needed for UE determination in DM1 and IR which could also furnish additional data to make measurement of substrate utilization more accurate.

Acknowledgements

Supported by grants from the Health Ministry of the Czech Republic (grant IGA NR 7880-3) The skilful technical assistance of Mrs. Dana Lapešová, Dagmar Šišáková and the help of Ms. Věra Lánská and Jelena Skibová with statistical evaluation of data are gratefully acknowledged.

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Tables

Table 1. Characteristics of study groups

	C (n = 11)	DM1 (n = 11)
Age (years)	28.72 ± 2.10	32.18 ± 5.72
BMI (kg/m^2)	24.09 ± 1.6	24.36 ± 2.15
Triglycerides (mmol·l^{-1})	1.11 ± 0.76	0.77 ± 0.40
Total cholesterol (mmol·l^{-1})	4.11 ± 0.88	4.23 ± 0.59
HDL-cholesterol (mmol·l^{-1})	1.27 ± 0.42	1.67 ± 0.29
HbA1c (DCCT) (%)	4. 82 ± 0.27	7.97 ± 1.34***
Daily insulin dose (IU/day)	----------	36 ± 9.8

Statistical significance: ***p<0.001

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

Hours (periods)	C (mmol/24 hrs)	DM1 (mmol/24 hrs)
24 hrs	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 mins (Period 1) and from 120 to 240 mins (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 hrs)	DM1 (mmol/ 24 hrs)
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.