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Original article

Impact on bulk ^{15}N natural isotopic abundance in hair of kidney function in type 2 diabetic nephropathy

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SUMMARY

Background and aims: Nephropathy is a serious long-term complication in type 2 diabetic patients. Few data are available about nitrogen metabolism in diabetic nephropathy. Bulk ^{15}N natural isotopic abundance (NIA) in hair has been shown modified in different pathologies with altered protein metabolism. We have compared bulk ^{15}N NIA in hair proteins in type 2 diabetic patients to look for differences between groups with or without nephropathy.

Methods: Case patients were type 2 diabetic patients with high urinary albumin concentrations ($\geq$20 mg/L or 30 mg/24 h on two of three urine collections) and/or an estimated glomerular filtration rate (eGFR) below 60 mL min^{-1} 1.73 m^{-2}, according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. Control subjects were type 2 diabetic patients of the same geographical origin with long-term diabetes, normo-albuminuria and eGFR above 60 mL min^{-1} 1.73 m^{-2}. The bulk ^{15}N NIA values for hair were measured by isotope ratio measurement mass spectrometry coupled with an elemental analyzer.

Results: Thirty-three patients in the nephropathy group and 31 in the control group were included. The mean age of the participants was 69 ± 8 years, and the mean duration of diabetes was 22 ± 8 years. Bulk hair ^{15}N NIA were 8.9 ± 0.7‰ in the nephropathy group and 9.0 ± 0.5‰ in the control group ($p = 0.41$). Positive correlations were found between bulk hair ^{15}N NIA with eGFR ($p < 0.01$) and with urinary urea concentration ($p < 0.01$), and a negative correlation with the urinary albumin-to-creatinine ratio ($p < 0.001$).

Conclusions: Bulk hair ^{15}N NIA might be an easily available, non-invasive biomarker of nitrogen metabolism in type 2 diabetic patients.

Abbreviations: ARB, angiotensin 2 receptor blockers; CKD, chronic kidney disease; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; $\delta^{13}C$ (‰), ^{13}C natural isotopic abundance; $\delta^{15}N$ (‰), ^{15}N natural isotopic abundance; eGFR, estimated glomerular filtration rate; irm-MS, isotope ratio measurement by mass spectrometry; NIA, natural isotopic abundance; RAS, Renin-angiotensin system; uACR, urinary albumin-to-creatinine ratio.

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1. Introduction

It is well established that protein metabolism is perturbed in conditions of nutritional imbalance and disease. The rate of protein synthesis is enhanced in obesity [1] and in type 1 and type 2 diabetes mellitus [2,3]. Accessing modifications to protein turnover is not facile and is classically carried out using short-term isotopic-tracer techniques [4]. Such an approach is restricted to short-term observations, however, and other methods are needed by which longer-term measurements can be obtained.

An attractive alternative approach might be to exploit the variation in bulk ^{15}N natural isotopic abundance (NIA) of nitrogenous compounds. NIA of whole body proteins partly depends on protein source, being higher in animal- than vegetable-derived proteins [5]. In addition, recent studies in rats exploiting both low-enriched ^{15}N-amino acid perfusion [4] and dietary-related studies [6] suggest that protein metabolism is a major determinant of bulk ^{15}N NIA values, as much as is protein intake. Amongst the factors contributing to the metabolic component determining bulk ^{15}N NIA is the transamination rate [7] and urea cycle flux rate [8]. Furthermore, it has been shown that $^{15}N/^{14}N$ ratios in nitrogenous compounds are influenced by nutritional imbalance and disease [5,8,9].

Within the nutritionally-related diseases, diabetes mellitus and associated diabetic nephropathy affects a growing proportion of the global population. Diabetic nephropathy, a multifactorial serious long-term complication in diabetes, is associated with an increased independent risk for all-cause mortality in both type 1 and type 2 diabetes mellitus [10]. Although the determinants of diabetic nephropathy remain poorly understood, high protein intake may be a key issue in nutritional care in chronic kidney disease (CKD), since it may be detrimental to renal function [11]. A randomized trial has shown the benefit of restriction of protein intake [12] in both diabetic and in non-diabetic populations [13]. Furthermore, a reduction of proteinuria was shown in type 2 diabetic proteinuric patients subjected to a low protein diet [14].

Clearly, then, nephropathy in diabetes mellitus is related to overall nitrogen metabolic status. To our knowledge, there is currently no simple and non-invasive method by which protein metabolism can be monitored in order to adjust protein intake in diabetic and non-diabetic populations. In view of the established relationship between protein metabolism and bulk ^{15}N NIA, we have tested the hypothesis that modified renal function in nephropathy in type 2 diabetes mellitus may alter bulk ^{15}N NIA values. To test this, we set up a case-control study involving 64 type 2 diabetic patients with (33 patients) or without (31 patients) diabetic CKD. To access bulk ^{15}N NIA values, we have exploited the easily-accessible protein of hair keratin, previously shown as a non-invasive means by which to observe potential modifications in protein metabolism [5,15,16].

2. Materials and methods

2.1. Population

Outpatients attending the diabetes mellitus department at the Poitiers University Hospital, France, were invited to participate in the Poitiers metabolic collection (INSERM CIC1402).

An independent committee, assessing key endpoints, reviewed the record of all participants for type 2 diabetes mellitus, diabetic retinopathy (according to ophthalmological reports including retinal examination, photographs and/or retinography), diabetic nephropathy and cardio-vascular events (including ECG data).

All patients were of European ethnicity. Patients were selected on the presence of type 2 diabetes mellitus, defined according to the ADA 2007 criteria. Exclusion criteria were definitive insulin requirement within less than 2 years after diagnosis, inability to understand study requirement, CKD stage 5 or total baldness. Patient's diet was not assessed but the whole population consumed a normal French diet.

Case patients were type 2 diabetic patients with high urinary albumin concentrations (uAC) of $\geq$20 mg/L or 30 mg/24 h on at least two of three sterile urine collections and/or a low estimated glomerular filtration rate (eGFR) of <60 mL min^{-1} 1.73 m^{-2}, according to the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation. Control subjects were type 2 diabetic patients of the same geographic origin with urinary albumin concentrations <20 mg/L or 30 mg/24 h on two of three urine collections and with an eGFR >60 mL min^{-1} 1.73 m^{-2}.

2.2. Laboratory assays

Urinary sample was a 24 h collection. Serum creatinine and uAC were measured by nephelometry on a Modular System P (Roche Diagnostics GmbH). Renal function was estimated by GFR using the CKD-EPI formula. Although renal function was not directly measured, calculation of eGFR is considered adequate for the assessment of renal function in patients with a stable condition [17]. Urinary creatinine concentration (uCC) was measured on a Hitachi 911 automatic analyzer (Roche Diagnostics, Meylan, France), allowing the determination of urinary albumin-to-creatinine ratio (uACR). Glycated hemoglobin (HbA_{1c}) was determined by using a high performance liquid chromatography method with an ADAMS A1C HA-8160 analyzer (normal values 4.0–6.0 %; Menarini, Florence, Italy).

2.3. Hair sample collection and preparation

A tuft of hair was cut close to the scalp, in the occipital area, the same day as laboratory assays, and stored at room temperature until processing. For isotopic analysis, the complete hair sample was transferred to a small glass bottle and washed in ethyl acetate (2 mL, 30 min) to remove adhesive tape residue and then in cyclohexane (2 × 2 mL, 30 min) to remove sebum (lipids) and residues (e.g. shampoo). Residual traces of solvent were removed by evaporation at 45 °C under a stream of pure nitrogen gas. The sample was cut into small sections (1 mm or less). An aliquot of each sample (~0.6 mg, giving ~0.08 mg N) was weighed with 10^{-6} g precision (ultra-microbalance XP6U, Mettler Toledo, Columbus, OH, USA) into tin capsules (solids "light" 5 × 9 mm, Thermo Fisher Scientific, Bremen, Germany).

2.4. Isotope analysis

The %N and %C and the bulk $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios were determined by flash combustion in an elemental analyzer (EA) and isotope ratio mass spectrometry (irm-MS), respectively, as previously described [18,19]. Briefly, capsules containing samples (approx. 80 μg N) were introduced into an Integra2 analyzer (Sercon, Crewe, Cheshire, UK), wherein they were flash combusted in an oxygen atmosphere in the EA and the resultant gases (CO_2 and N_2) were carried in a stream of helium to the irm-MS. Water was removed by a $Mg(ClO_4)_2$ water trap and N_2 was completely separated from CO_2 using an integrated gas chromatography column. Ion currents were measured for *m/z* 28, 29, 30 and *m/z* 44, 45, 46, for N_2 and CO_2, respectively, from which the bulk ^{15}N NIA (‰) and bulk ^{13}C NIA (‰) values could be calculated. Bulk NIA were expressed as the $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) ratios calculated as follows: