Irisin levels are associated with urotensin II levels in diabetic patients

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ABSTRACT

Aims/Introduction: Irisin is a newly identified myokine that can promote energy expenditure. Previous studies showed that circulating urotensin II (UII) levels were increased in diabetes, and UII could inhibit the glucose transport in skeletal muscle in diabetic mice and aggravated insulin resistance. We presumed that irisin levels are associated with UII in diabetic patients.

Materials and Methods: A total of 71 patients with type 2 diabetes and 40 healthy subjects were recruited. Blood and urinary irisin concentrations were measured by using enzyme-linked immunosorbent assay, and UII concentrations were measured by bioelectrical impedance analysis. Every participant’s body composition was analyzed by bioelectrical impedance.

Results: The serum irisin levels were significantly lower in diabetic patients than that of controls, whereas serum UII levels were significantly higher in diabetic patients than that in controls. Serum irisin levels were negatively associated with circulating UII, hemoglobin A1c and the natural logarithm transformation of urinary albumin excretion, whereas serum irisin was positively associated with estimated glomerular filtration rate, and low-density lipoprotein cholesterol and urinary irisin were positively associated with urinary UII. Furthermore, circulating irisin is positively associated with muscle mass, whereas circulating UII is negatively associated with muscle mass in diabetic patients. Hemoglobin A1c and circulating UII are independent determinants of circulating irisin by multiple regression analysis.

Conclusions: The present results provide the clinical evidence of an association between irisin and UII in diabetic patients. Hemoglobin A1c and circulating UII are independent determinants of circulating irisin. Our results hint that UII and high glucose might inhibit the release of irisin from skeletal muscle in diabetic patients.

INTRODUCTION

Muscle atrophy is a frequent complication of diabetes, and is associated with increased mortality of diabetic patients. Insulin resistance is an important cause of muscle atrophy1,2.

Irisin was first discovered as a novel myokine released into the circulation by cleavage and shedding of the membrane fraction of fibronectin type III domain-containing 5 (FNDC5)3. It was reported that circulating irisin was significantly lower in type 2 diabetes4,5; furthermore, irisin could alleviate insulin resistance in an animal model6.

Urotensin II (UII) was originally isolated from the urophysis of teleost fish. It is a somatostatin-like cyclic undecapeptide, identified as the most potent mammalian vasoconstrictor7,8. Previous studies showed that UII levels increased in diabetic patients8,9. Furthermore, it was reported that UII could inhibit the glucose transport in skeletal muscle in diabetic mice and aggravated insulin resistance10. According to previous studies, we speculate that UII can induce insulin resistance and then causes skeletal muscle atrophy in diabetic patients, and reduces the release of circulating irisin from skeletal muscle. In the
current study, we explored the relationship between irisin level and UII in diabetic patients.

METHODS
Study Population
From 30 December 2012 to 30 March 2013, a total of 71 patients with type 2 diabetes were recruited, 40 healthy subjects (all from medical staff at Peking University Third Hospital, Beijing, China) were selected as control subjects. Consent forms were signed by all the participants. Peking University Third Hospital ethical committee approved this study.

The diabetic patients were classified into two subgroups in the present study: (i) diabetes with normal albuminuria; and (ii) diabetic nephropathy. The diagnosis of diabetic nephropathy was confirmed either by the pathological examination of a renal biopsy carried out (10 patients) within 1 year before study enrolment or by the presence of clinical manifestations according to the Kidney Disease Outcomes Quality Initiative guidelines; that is: at least 5–10 years from the diagnosis of type 2 diabetes, the presence of diabetic retinopathy, presence of urinary microalbuminuria (urinary albumin excretion rate 20–200 μg/min) or overt proteinuria, (urinary albumin excretion rate >200 μg/min) and the absence of clinical or laboratory evidence of other kidney disease.

Measurement of Biochemical Parameters
Blood samples were obtained from participants after a 12-h fast. Aliquots of serum and plasma were stored at −80°C and were not thawed until analyzed. Serum irisin concentrations were measured in duplicate by using the enzyme-linked immunosorbent assay (ELISA) kits (Phoenix Pharmaceuticals, Burlingame, CA, USA) in accordance with the manufacturer’s instructions. The sensitivity of the assay was 0.1 ng/mL, and the linear range of the standard was 0.1–1,000 ng/mL. The intra- and interassay coefficients of variation (CV) were 4.5 and 8%, respectively. Serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride and high-density lipoprotein cholesterol (HDL-C) were measured enzymatically. Serum creatinine and urinary creatinine were measured by a picrate method. Blood glucose levels included fasting blood glucose (FBG) and hemoglobin A1c (HbA1c). HbA1c was measured by high-performance chromatography. The estimated glomerular filtration rate (eGFR) was assessed by a simplified MDRD equation: eGFR = 186 × (serum creatinine)−1.154 × (age)−0.203 × (0.742 if female).

For measurement of the albumin excretion rate, 8-h urine samples were collected (from 22.00 to 06.00 h) and assayed by chemiluminescent immunoassay (DPC, Inc., Los Angeles, CA, USA); this was carried out for each patient at least twice. The first fast morning urine samples were collected for measurement of UII, irisin, urinary neutrophil gelatinase-associated lipocalin (NGAL) and retinol binding protein 4 (RBP4) when the blood samples were collected on same day, and were also frozen at −80°C until the time of assay. NGAL and RBP4 were measured using an ELISA kit (RD, Inc., Minneapolis, MN, USA), and were calibrated by urine creatinine and expressed as the ratio of urinary concentrations of NGAL or RBP4 to grams of urinary creatinine (mg/g).

Radioimmunoassay of UII
According to our previous publications and other literature, both the blood and urine samples avoided repeated freeze/thaw cycles, and were redepolymerized with 0.75 mL 2 mol/L hydrochloric acid. After centrifugation for 20 min at 6,000 g, the supernatant was loaded onto cartridges that had been activated with 3 mL 100% methanol and 3 mL double-distilled deionized water. The cartridges were then washed twice with 3 mL 0.1% trifluoroacetic acid (TFA) and eluted with 3 mL 60% acetonitrile in 0.1% TFA. The eluants were freeze-dried overnight and resuspended in 250 μL of radioimmunoassay buffer. Then, 100 μL of standard UII or assay sample was incubated overnight at 4°C with 100 μL rabbit antisera. A total of 100 μL of labeled 125I-UII (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) were added to each tube and incubated for a further 24 h. Antibody-bound UII was precipitated using a goat anti-rabbit antisera and normal horse serum. Using a gamma counter, the amount of bound 125I-UII was measured as pg/mL. Urinary UII was concentration calibrated by urine creatinine and expressed as the ratio of urinary UII concentrations to grams of urinary creatinine (ng/g).

Body Composition Measurement
Every participant’s body composition was analyzed by Bioelectrical Impedance (BIA, Tanita, Japan). The collected data included body fat (% and kg), lean body mass (kg), muscle mass (kg) and visceral fat rating (1–5 grade; 1–12 is considered healthy, whereas ≥13 corresponds to excessive abdominal fat and indicates central obesity). The measurements were carried out by skilled staff who were experienced in collecting these measurements.

Statistical Analysis
Data are presented as means ± standard deviation (SD) or median (25–75% quartile). Independent Student’s t-test was used to test differences for numerical variables for normal distribution between two groups. Non-parameter test was used to test the difference for non-normal distribution. The chi square test was used to compare categorical variables and nominal variables. Non-normal distributed data, such as urinary NGAL, RBP4, urinary albumin excretion and so on, were transferred to the natural logarithm transformation (Ln) for NAGL, LnRBP4 and Ln urinary albumin excretion (the natural logarithm of urinary NAGL, RBP4, urinary albumin excretion) for Pearson’s correlated analysis, and then multiple linear regression was carried out. All data were analyzed by using the statistical package SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).
RESULTS
Clinical Characteristics of Participants
The clinical characteristics and biochemical data of the control subjects and diabetic patients are summarized in Table 1. Notably, patients with diabetes had higher levels of systolic blood pressure, diastolic blood pressure, FBG, HbA1c, blood UII, urinary NGAL, urinary RBP4 and urinary UII levels, higher body fat and a higher visceral fat rate than did normal controls ($P < 0.05$), whereas serum irisin levels, lean body mass and muscle body mass were significantly lower ($P < 0.05$) in diabetic patients. Furthermore, we measured these participants’ urinary irisin and found that the urinary irisin levels were significantly lower than serum irisin levels. The urinary irisin levels in diabetic patients were significantly lower than that of normal controls ($53.2 \pm 37.8$ vs $93.2 \pm 37.9$ ng/mL, $z = -2.472$, $P = 0.02$; Table 1).

Subgroup Analysis of Diabetic Patients
We classified diabetic patients into two subgroups: (i) diabetes with normal albuminuria; and (ii) diabetic nephropathy. Circulating irisin was significantly decreased in two subgroups of diabetic patients, whereas circulating UII was increased in patients with diabetic nephropathy. There was higher urinary UII, higher serum creatinine, Ln urinary NGAL/creatinine, Ln urinary RBP4/creatinine, Ln urinary albumin excretion, and a higher body fat and visceral fat rate (grade) in patients with diabetic nephropathy, whereas there was lower urinary irisin in patients in the two diabetic subgroups. Furthermore, there was lower lean body mass and muscle body mass, and a higher body fat and visceral fat rate in diabetic patients with normal albuminuria and diabetic nephropathy compared with the normal controls. There was no sex dimorphism in circulating irisin in the diabetic patients (Table 2).

Table 1 | Comparison of clinical parameters between diabetic patients and normal controls

| Variables                  | Normal controls $n = 40$ | Diabetic patients $n = 71$ | P-value |
|----------------------------|--------------------------|----------------------------|---------|
| Age (years)                | 58 ± 14                  | 63 ± 12                    | 0.057   |
| Sex (male/female)          | 22/18                    | 42/29                      | 0.693   |
| SBP (mmHg)                 | 120.6 ± 10.1             | 128 ± 19                   | 0.030   |
| DBP (mmHg)                 | 68.4 ± 12.6              | 73 ± 12                    | 0.030   |
| HbA1c (%)                  | 5.9 ± 0.4                | 84 ± 20                    | 0.000   |
| FBG (mmol/L)               | 5.3 ± 0.6                | 8.0 ± 3.0                  | 0.000   |
| Hb (g/L)                   | 147.0 ± 11.0             | 1320 ± 174                | 0.000   |
| Alb (g/L)                  | 40.2 ± 5.1               | 360 ± 5.0                  | 0.010   |
| Sodium (mmol/L)            | 138.3 ± 2.0              | 1360 ± 3.5                 | 0.541   |
| Potassium (mmol/L)         | 3.8 ± 0.3                | 4.0 ± 1.0                  | 0.472   |
| Chloride (mmol/L)          | 103.8 ± 2.7              | 101 ± 4.6                  | 0.356   |
| Lnc acid (mmol/L)          | 315.6 ± 88.1             | 349 ± 110                  | 0.246   |
| Serum creatinine (umol/L)  | 75.8 ± 15.7              | 1400 ± 1370                | 0.040   |
| Urea (mmol/L)              | 7.1 ± 2.1                | 7.6 ± 4.3                  | 0.050   |
| eGFR (mL/min/1.73 m²)      | 849 ± 15.2               | 682 ± 32                   | 0.030   |
| Serum UII (pg/mL)          | 40.1 ± 13.3              | 540 ± 180                  | 0.030   |
| Serum irisin (ng/mL)       | 4270 ± 95.0              | 1800 ± 239                 | 0.000   |
| Urinary irisin (ng/mL)     | 913.3 ± 37.9             | 532 ± 37.8                 | 0.020   |
| Urinary UII (ng/g)         | 1530 ± 66.1              | 190 ± 81.2                 | 0.040   |
| Ln urinary NGAL/cr (mg/g)  | 1.5 ± 0.56               | 3.0 ± 1.2                  | 0.0004  |
| Ln Urinary RBP/cr (mg/g)   | 4.1 ± 0.6                | 6.3 ± 1.2                  | 0.000   |
| Body fat (%)               | 263 ± 48                 | 334 ± 39                   | 0.001   |
| Body fat (kg)              | 170 ± 41                 | 245 ± 7.8                  | 0.001   |
| Lean body mass (kg)        | 467 ± 65                 | 437 ± 83                   | 0.035   |
| Muscle mass (kg)           | 441 ± 42                 | 413 ± 79                   | 0.04    |
| Visceral fat rate (grade)  | 130.4 ± 40               | 135.4 ± 43                 | 0.001   |

Alb, albumin; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; Hb, hemoglobin; HbA1c, hemoglobin A1c; Ln, the natural logarithm transformation; NGAL/cr, neutrophil gelatinase-associated lipocalin/creatinine; RBP/cr, retinol binding protein/creatinine; SBP, systolic blood pressure; UII, urotensin II.
negatively related with muscle mass ($r = -0.399$, $P = 0.026$) in diabetic patients. We did not observe a significant correlation between irisin and age, sex, triglyceride, HDL-ch, body mass index, body fat, and uric acid (Table 3).

**Correlation Analysis of Urinary Irisin and Urinary UII, and Biomarkers of Renal Injury**

Bivariate correlation analysis showed that the Ln of urinary irisin calibarated with creatinine was positively correlated with urinary UII calibrated with creatinine ($r = 0.426$, $P = 0.011$). We did not observe a significant correlation between urinary irisin with other markers of renal tubular injury, such as NGAL and RBP4 (Table 4).

**Multiple Regression Analysis of Circulating Irisin with Other Parameters**

A multivariate linear regression model was used to study which kinds of clinical and biochemical factors were independently associated with circulating irisin levels. In our model, eGFR, hemoglobin and LDL-ch were excluded from the linear model. Our model showed that HbA1c and circulating UII were independently associated with circulating irisin in diabetic patients (Table 5).

**DISCUSSION**

Irisin has been identified as a novel myokine released into the blood by cleavage and shedding of the membrane fraction of FNDC5 in response to activation of peroxisome proliferator-activated receptor-$\gamma$ coactivator-1z (PGC-1z). Recent studies showed that circulating irisin levels were significantly lower in type 2 diabetic patients. In our current study, we found that circulating irisin concentrations were decreased in diabetic patients. Previous studies also showed that UII levels were increased in diabetic patients. Furthermore, we first showed that circulating irisin had a negative correlation with circulating UII in diabetic patients. How can we explain this phenomenon? Recently, Wang et al. reported that UII could inhibit the glucose transport in skeletal muscle in diabetic mice and aggravated insulin resistance. As a result, it is reasonable to speculate that lower levels of circulating irisin in type 2 diabetic patients observed in the present study might be secondary to UII-induced insulin resistance and skeletal muscle atrophy so as to decrease irisin.
from skeletal muscle. Furthermore, the present results showed that there was higher body fat and visceral fat rate, and lower muscle mass in diabetic patients, and that circulating irisin was positively associated with muscle mass whereas circulating UII was negatively associated with muscle mass in diabetic patients. These results support our hypothesis. However, when circulating UII was not increased in diabetic patients with normal albuminuria, the circulating irisin had already decreased in these patients. This could hint at other factors affecting circulating irisin level besides UII.

In order to verify our hypothesis, at the next step we need to culture skeletal muscle cells with UII treated in vitro and investigate whether UII can inhibit skeletal muscle cells to release irisin or not, then we will verify that the expression of UII and UII receptor are negatively associated with expression of FNDC5 in skeletal muscle in a diabetic animal model.

We first investigated the urinary irisin in our current study. Usually, only substances of molecular weight lower than 70 kDa can be filtrated from glomeruli. Irisin is a 12 kDa soluble secreted form by cleavage and shedding of FNDC5, and can be filtrated freely from glomeruli. Urinary irisin levels were significantly lower in the present participants than those of circulating irisin, and the urinary irisin levels in the diabetic patients were significantly lower than those of the normal controls. These results show that urinary irisin mainly comes from glomeruli filtration with a short half-life and non-secreted by renal tubular epithelial cells. Furthermore, at present there is no evidence to verify that renal tubular epithelial cells can secrete irisin, except myocytes cells or adipose tissues. Different from urinary irisin, urinary UII concentrations were significantly higher in diabetic patients, which means that urinary UII mainly comes from renal tubular epithelial cell besides UII filtrated from glomeruli. We found that urinary UII levels were associated with markers of renal tubular injury, such as NGAL and RBP4 (data not shown), but urinary irisin did not have such an association, although it was positively associated with urinary UII concentration.

Recently, some studies showed that irisin might be associated with the protection of endothelial function. Furthermore, irisin, in pharmacological concentrations could increase cell proliferation in mouse hippocampal neuronal cells. Colaianni et al. reported that irisin directly enhanced osteoblasts to differentiation. Whether irisin possess the potential functions to protect the kidney cells in diabetes is worth investigating.

In contrast, there might be other factors that affect irisin levels. The correlation of circulating irisin levels with metabolism disorders in human subjects remains controversial. Our present study found that FBG and HbA1c were negatively associated with circulating irisin levels, and these were consistent with the study of Choi et al.

Recent studies suggested that circulating irisin might also be associated with renal function in humans. Some authors reported a positive correlation between eGFR and circulating irisin in diabetic patients. In our current study, we also observed that irisin is negatively associated with serum creatinine and positively correlated with eGFR in diabetic patients.
Furthermore, in our current study, we found a negative correlation of circulating irisin with early markers of kidney injury, such as microalbuminuria, but we have no evidence to verify that irisin can protect the kidney from injury. The mechanisms underlying a significant reduction in circulating irisin in late-stage chronic kidney disease (CKD) could be multifactorial. First, CKD, especially late stage CKD, is characterized by progressive muscle wasting. It is possible that reduced muscle mass could partially account for reduced irisin expression and secretion in late-stage CKD. Second, studies in cellular models showed that uremic toxins, such as indoxyl sulfate, might negatively regulate the expression of irisin precursor FNDC5 and irisin secretion. It is possible that accumulating uremic toxins at late-stage CKD might impair expression and secretion of irisin from myocytes and adipocytes, and result in a lower level of circulating irisin. Besides previous novel factors, such as inflammation mediators, oxidative stress biomarkers, advanced glycation end-products and uremic toxins associated with circulating irisin, the present results show that UII might be the important adjusting factor for irisin in diabetic patients.

There were several limitations to our current study. The sample size of this cross-sectional study was relatively small. In the present clinical study provides correlated evidence, but does not address the cause-effect relationship in type 2 diabetes.

In summary, the present results provide clinical evidence of an association between irisin and UII in diabetic patients. HbA1c and circulating UII are independent determinants of circulating irisin. Our results show that UII and high glucose might inhibit the release of irisin from skeletal muscle in diabetic patients.

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DISCLOSURE
The authors declare no conflict of interest.

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