Regulation of transforming growth factor-β1 by insulin in prediabetic African Americans

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Insulin resistance has been associated with kidney disease even in the absence of diabetes; however, pathways linking insulin resistance to kidney disease are unclear. The purpose of this study was to determine if transforming growth factor (TGF)-β1, a key cytokine associated with kidney disease, responds to circulating levels of glucose and/or insulin. Urinary TGF-β1 levels were measured in 249 young adult African Americans (mean age 40) at baseline, after an oral glucose tolerance test and after a euglycemic hyperinsulinemic clamp procedure. Baseline urinary geometric mean TGF-β1 levels were somewhat lower in those with normal compared with the impaired glucose tolerance. The urinary TGF-β1 level increased by 56% followed by a 23% decrease in the normal glucose tolerance group, changes that were significant and corresponded to the changes in the plasma glucose and insulin concentrations. The impaired tolerance group showed little change in the urinary TGF-β1 level following glucose ingestion. All participants had a significant increase in urinary TGF-β1 level after steady-state hyperinsulinemia, with sustained euglycemia during the clamp procedure in both of the groups. At baseline, there was a significant correlation between the urinary TGF-β1 level and urinary albumin excretion. Thus our results suggest that insulin contributes to increased TGF-β1 production and possible early renal injury in prediabetic young African Americans.

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Diabetes is a leading cause of end-stage kidney disease in the United States.[1] African Americans develop diabetic nephropathy at a disproportionately greater rate compared with Caucasians.[2] The Pathological characteristics of diabetic nephropathy include thickening of glomerular and tubular membranes, and subsequent accumulation of extracellular matrix components resulting in fibrosis. Transforming growth factor (TGF)-β1 is a tissue cytokine with fibrogenic properties.[3] A convincing body of experimental studies have demonstrated high levels of TGF-β1 in kidney cells of diabetic rodents,[4] and attenuation of matrix deposition in the renal tissue of diabetic rodents with exposure to anti-TGF-β1 antibodies.[5,6]

Although experimental studies have developed considerable evidence implicating an increase in TGF-β1 activity as a key mediator in the pathogenesis of diabetic nephropathy,[7] there are limited data on the role of TGF-β1 in the pathogenesis of diabetic nephropathy in humans. In a small sample of patients undergoing cardiac catheterization, the concentration of TGF-β1 in samples from the renal vein compared with samples from the aorta was elevated in diabetic patients but not in non-diabetic patients, indicating greater local renal production of TGF-β1 in the diabetic patients.[8] The net renal production of TGF-β1 across the renal vascular bed was associated with increased urinary levels of TGF-β1. In another clinical study, urinary TGF-β1 level was measured in healthy non-diabetic volunteers following a constant glucose infusion to achieve sustained hyperglycemia. Following 120 min of modest hyperglycemia (200–225 mg/dl), there was a marked increase in urinary TGF-β1 level without a corresponding increase in the plasma TGF-β1 concentration.[9] Together, these reports indicate that both diabetes and episodes of hyperglycemia are associated with upregulation of TGF-β1 activity in the kidney. The purpose of this study was to determine whether renal TGF-β1 production, estimated by urinary TGF-β1 level, responds to modest increases in plasma insulin. Our study was designed to test the hypothesis that urinary TGF-β1 levels are greater in prediabetic African Americans, and that urinary TGF-β1 levels increase following glucose and/or insulin exposure.

RESULTS

The study cohort consisted of 249 participants, who were stratified as having normal glucose tolerance (NGT; $N = 176$) and impaired glucose tolerance (IGT; $N = 73$) based on their fasting blood glucose and oral glucose tolerance test (OGTT) results according to the American Diabetes Association criteria.[10] Table 1 provides the clinical characteristics of the study participants. The mean age of the cohort was 40 years and there was no significant age difference between the NGT and IGT groups. In the whole study sample, 62% were females and 51% were self-identified cigarette smokers. The mean body mass index values for both NGT and IGT groups were in the obese range (body mass index $\geqslant 30\,kg/m^2$). However, the IGT group had a significantly higher mean body mass index value than the NGT group. The mean systolic and diastolic blood pressure (BP) values of the cohort were in the normotensive range and there was no significant difference between the NGT and IGT groups.

Table 2 presents the means and standard deviations of plasma glucose and insulin measurements during the OGTT, for both the NGT and IGT groups. The two groups had significantly different fasting and 2-h OGTT glucose levels, consistent with our classification system. Insulin levels at 2-h post glucose ingestion were also significantly different between the two groups. In the NGT group, the plasma insulin levels peaked between 30–60 min and subsequently decreased at 2 h post glucose ingestion. In the IGT group, however, the plasma insulin levels remained elevated at 2 h after glucose ingestion.

Table 2 also shows the results of the insulin-clamp procedure. The mean clamp glucose level was similar to the basal glucose level in both NGT and IGT groups, indicating that sustained euglycemia was achieved during clamp hyperinsulinemia. The achieved steady-state hyperinsulinemia (mean clamp insulin) was somewhat higher in the IGT group than in the NGT group. The glucose infusion rate, which quantifies the insulin-mediated glucose uptake, was higher in the NGT group than in the IGT group. The insulin sensitivity index (M/I, where M is the mean glucose infusion rate and I is the mean plasma insulin concentration) derived from the insulin clamp showed a clear separation of insulin sensitivity according to the glucose tolerance state; the NGT group had significantly higher M/I than the IGT group.

Table 3 presents the geometric means and interquartile ranges of urinary TGF-β1 level (in picograms per milligram creatinine) and urinary albumin excretion (UAE, in milligrams per gram creatinine) during the OGTT and insulin-clamp procedure, for both the NGT and IGT groups. Figure 1 is a graph of the estimated geometric means and 95% confidence intervals for the urinary TGF-β1 level during the OGTT and insulin clamp, for the two groups. There were significant differences between the NGT and IGT groups in the overall pattern of urinary TGF-β1 level during the OGTT ($P = 0.038$), but not during the insulin clamp ($P = 0.404$).

In analyses that adjusted for age, sex, smoking, alcohol use, obesity, hypertension, and UAE, the NGT group had an estimated 56% increase in urinary TGF-β1 level at 2 h post glucose ingestion compared with baseline ($P = 0.001$) and a subsequent 23% decrease from 2 to 4 h post glucose ingestion ($P = 0.025$). The IGT group showed a 2% decrease from baseline to 2 h post glucose ingestion ($P = 0.902$) and a 10% increase from 2 to 4 h post glucose ingestion ($P = 0.598$). The NGT and IGT groups were significantly different with regard to the change from baseline to 2 h post glucose ingestion ($+56\%$ versus -2%, $P = 0.019$) and marginally so with regard to the change from 2 to 4 h post glucose ingestion (-23% versus $+10\%$, $P = 0.096$). For UAE, the NGT group had 68% increase at 2 h post glucose ingestion compared with baseline ($P = 0.001$) and a subsequent increase of 99% from 2 to 4 h post glucose ingestion ($P = 0.001$). The IGT group had 67% increase at 2 h post glucose ingestion compared with baseline ($P = 0.012$) and a 38% increase from 2 to 4 h post glucose ingestion ($P = 0.064$). There were no significant differences between the NGT and IGT groups in terms of change in UAE from baseline to 2 h ($P = 0.970$) and from 2 to 4 h post glucose ingestion ($P = 0.077$).

Following the insulin-clamp procedure, there was a significant increase in urinary TGF-β1 level in both NGT (42%, $P = 0.008$) and IGT (95%, $P = 0.001$) groups, compared with baseline (after adjustment for age, sex, smoking, alcohol use, obesity, hypertension, and UAE). The magnitude of these increases, however, was not statistically different between the two groups ($P = 0.200$). Despite the marked increase in urinary TGF-β1 following the exposure to euglycemic hyperinsulinemia, the post-clamp UAE values in both NGT and IGT groups were essentially unchanged

Table 1 | Characteristics of study subjects

Characteristic (mean ± s.d.)	All (N=249)	NGT (N=176)	IGT (N=73)	P[a]
Age (years)	40 ± 3.4	40 ± 3.3	40 ± 3.6	0.89
Sex, n *(%)*				0.32
Male	94 (38)	70 (40)	24 (33)	
Female	155 (62)	106 (60)	49 (67)	
Smoking, n *(%)*				0.04
No	123 (49)	79 (45)	44 (60)	
Yes	126 (51)	97 (55)	29 (40)	
BMI (kg/m^2)	30.9 ± 7.2	30.3 ± 7.0	32.2 ± 7.5	0.06
Obesity, n *(%)*				0.21
Normal weight	55 (22)	44 (25)	11 (15)	
Overweight	73 (29)	51 (29)	22 (30)	
Obese	121 (49)	81 (46)	40 (55)	
SBP (mm Hg)	125 ± 20	124 ± 21	126 ± 18	0.46
DBP (mm Hg)	73 ± 13	74 ± 14	73 ± 11	0.70
Hypertension[b], *n* (%)	79 (32)	54 (31)	25 (34)	0.65

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; SBP systolic blood pressure.
[a]*P*-values are based on Fisher's exact test for categorical variables and analysis of variance for continuous variables.
[b]Hypertension is defined as ⩾140/90 or on antihypertensive medication.

Table 2 | Glucose, insulin, and insulin sensitivity measurements during the OGTT and insulin clamp

Measurements during the OGTT (mean ± s.d.)	NGT (*N*=176)	IGT (*N*=72)	*P*[a]
Glucose OGTT baseline (mg/dl)	98 ± 8	102 ± 11	0.001
Glucose OGTT 30 min (mg/dl)	155 ± 34	165 ± 29	0.025
Glucose OGTT 60 min (mg/dl)	154 ± 35	186 ± 32	0.001
Glucose OGTT 120 min (mg/dl)	105 ± 24	158 ± 16	0.001
Insulin OGTT baseline (μU/ml)	9 ± 8	11 ± 8	0.06
Insulin OGTT 30 min (μU/ml)	72 ± 46	75 ± 64	0.71
Insulin OGTT 60 min (μU/ml)	78 ± 55	92 ± 61	0.08
Insulin OGTT 120 min (μU/ml)	40 ± 36	92 ± 57	0.001
Measurements during the insulin clamp (mean ± s.d.)	**(*N*=168)**	**(*N*=67)**	***P*[a]**
Basal glucose[b] (mg/dl)	88 ± 7	92 ± 7	n/a[b]
Basal insulin (μU/ml)	8 ± 7	11 ± 10	0.021
Mean clamp glucose (mg/dl)	87 ± 9	91 ± 8	0.001
Mean clamp insulin (I) (μU/ml)	73 ± 27	81 ± 28	0.05
Mean glucose infusion rate (M) (mg/kg/min)	6.3 ± 2.7	4.9 ± 2.4	0.001
M/I × 100	10.3 ± 6.8	7.4 ± 5.6	0.002

Abbreviations: IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test.
[a]*P*-values are based on analysis of variance.
[b]For subjects with fasting glucose greater than 100 mg/dl, the basal glucose level was fixed at 100 mg/dl for the clamp.
To convert glucose to SI units, multiply by 0.05551. To convert insulin to SI units, multiply by 7.175.

Table 3 | Urinary TGF-β1 level and UAE during the OGTT and insulin clamp

During the OGTT (geometric means and interquartile ranges)	NGT (*N*=176)	IGT (*N*=73)	*P*[a]
Urinary TGF-β1 (pg per mg creatinine)			
Baseline	0.8 (0.4, 1.9)	1.3 (0.8, 2.7)	0.02
2 h	1.3 (0.6, 3.3)	1.3 (0.6, 3.0)	0.76
4 h	1.0 (0.4, 2.5)	1.4 (0.7, 3.4)	0.04
UAE (mg per g creatinine)			
Baseline	0.9 (0.3, 3.1)	0.8 (0.3, 2.7)	0.84
2 h	1.5 (0.5, 3.6)	1.4 (0.5, 3.2)	0.73
4 h	2.9 (1.0, 7.3)	1.9 (0.6, 6.1)	0.06
During the insulin clamp (geometric means and interquartile ranges)	**NGT (*N*=171)**	**IGT (*N*=68)**	***P*[a]**
Urinary TGF-β1 (pg per mg creatinine)			
Baseline	1.1 (0.5, 2.6)	1.0 (0.6, 1.9)	0.53
Post-clamp	1.6 (0.7, 3.8)	1.9 (0.7, 3.6)	0.39
UAE (mg per g creatinine)			
Baseline	1.3 (0.3, 4.4)	1.8 (0.6, 6.6)	0.23
Post clamp	1.6 (0.6, 4.2)	1.7 (0.6, 4.0)	0.71

Abbreviations: IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; TGF-β1, transforming growth factor; UAE, urinary albumin excretion.
[a]*P*-values are based on *t*-tests for the log-transformed TGF-β1 measurements.

compared with baseline ($P = 0.22$ and $P = 0.92$, respectively). There was no significant difference between the NGT and IGT groups in terms of change in UAE from baseline to post hyperinsulinemic clamp ($P = 0.573$).

The changes in urinary TGF-β1 level during the insulin clamp were further analyzed according to three insulin sensitivity levels based on the *M/I* values (<5 = resistant, 5–7.5 = intermediate, and >7.5 = sensitive). The increase in the urinary TGF-β1 level at the end of the clamp compared with baseline appeared to be greatest in the insulin-resistant group (2.25-fold increase, $P = 0.001$), intermediate in the intermediate group (79% increase, $P = 0.020$), and lowest in the insulin sensitive group (33% increase, $P = 0.062$). However, despite the trend of greater increase in urinary TGF-β1 level following hyperinsulinemia with insulin resistance, analyses compare the change in slope did not detect a statistically significant difference between the three insulin sensitivity groups ($P = 0.114$).

As shown in Table 4, there were also significant correlations between baseline urinary TGF-β1 level and UAE in both OGTT and the clamp study, supporting the role of urinary TGF-β1 level in early renal injury. The correlation coefficient for UAE measured in the two baseline urine samples in OGTT and the clamp study was 0.505 ($P = 0.001$), indicating a high level of consistency within subjects for measurements of UAE.

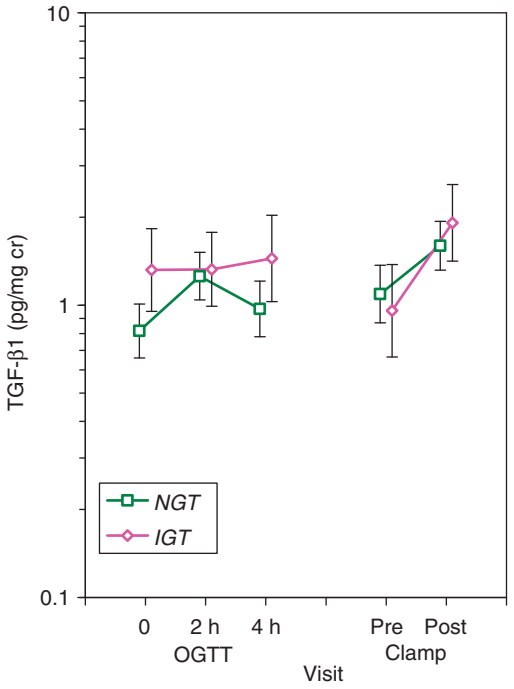

Figure 1 | The estimated geometric means and 95% confidence intervals for urine transforming growth factor (TGF)-β1 for the normal glucose tolerance (NGT) group (box connected by green line) and the impaired glucose tolerance (IGT) group (diamond connected by pink line). The values during the oral glucose tolerance test (OGTT) are plotted on the left side and the values before and after the insulin clamp on the right side.

Table 4 | Correlations between baseline urinary TGF-β1 level and urinary albumin excretion (UAE)

Baseline urinary TGF-β1 and UAE	Correlation[a]	*P*
OGTT baseline	0.209	<0.001
Clamp baseline	0.317	<0.001

Abbreviations: OGTT, oral glucose tolerance test; TGF-β1, transforming growth factor-β1; UAE, urinary albumin excretion.
[a]Correlations are Pearson's correlations between urinary TGF-β1 and UAE values. Both urinary TGF-β1 and UAE are corrected for creatinine and log-transformed.

DISCUSSION

Data from this study indicate that following insulin exposure there is an increase in urinary TGF-β1 in young adult African Americans without clinical diabetes. Participants with NGT had somewhat lower baseline urinary TGF-β1 levels than those with IGT and responded to the glucose challenge with an increase followed by a decrease in urinary TGF-β1 level. Participants with IGT had no significant changes in the urinary TGF-β1 level in response to the oral glucose challenge. During the insulin clamp, both NGT and IGT groups had significant increases of comparable magnitude in urinary TGF-β1 level following the exposure to hyperinsulinemia without change in plasma glucose concentration. Together, these findings suggest that insulin has a role in upregulating the TGF-β1 production. Baseline urinary TGF-β1 level was also correlated significantly with UAE, a valid marker of renal injury. There was no increase in UAE following the insulin clamp, indicating that the increase in urinary TGF-β1 following the insulin clamp was not due to leakage of circulating TGF-β1 into the urine.

Experimental studies have implicated TGF-β1 in the pathogenesis of diabetic nephropathy.[7,11] In kidneys of diabetic rodents, there is evidence of upregulation of renal TGF-β1, its receptors, and intracellular signaling pathway resulting in collagen deposition and tissue fibrosis.[4–6,12–18] In diabetic mice, treatment with neutralizing anti-TGF-β1 antibodies prevented the development of renal hypertrophy, mesangial expansion, and renal function deterioration.[6]

Limited human data are available on TGF-β1 in type 2 diabetes and diabetic nephropathy. Yamamoto *et al.*[19] examined TGF-β1 expression in renal tissues from six patients with advanced diabetic glomerulosclerosis compared with three normal controls, three cases of minimal change disease, and three cases of thin basement membrane disease. They reported that renal tissue from diabetic patients had markedly higher levels of TGF-β1 expression compared with normal controls, whereas that from patients minimal change disease and thin basement membrane disease had similar levels of TGF-β1 expression as normal controls. In a study of 44 type 2 diabetic patients and 28 non-diabetic controls, Pfeiffer *et al.*[20] reported a higher plasma TGF-β1 level in the diabetic patients compared with non-diabetic controls. There was also a significant correlation between plasma TGF-β1 and hemoglobin A1c level. In a subsequent study of similar size, the same group reported that type 2 diabetic patients with nephropathy had a higher circulating level of an activated form of TGF-β1 than type 2 diabetic patients without nephropathy.[21] Sharma *et al.*[8] measured plasma TGF-β1 concentration in the aorta and renal vein during elective cardiac catheterization in 14 type 2 diabetic and 11 non-diabetic patients. In diabetic patients, the concentration of TGF-β1 in the renal vein was greater than in the aorta, whereas in non-diabetic patients, the TGF-β1 concentration in the renal vein was lower than in the aorta. In addition, they reported greater urinary TGF-β1 level in diabetic patients. These results indicated that there was increased local renal production of TGF-β1 in type 2 diabetes that could be estimated by the urine levels. Sato *et al.*[22] reported higher urinary TGF-β1 level in 57 diabetic patients compared with 20 healthy controls. In addition, they found that higher urinary TGF-β1 levels were associated with higher hemoglobin A1c levels and greater mesangial expansion on renal biopsy. Data from our study extend these findings to the prediabetic condition, with detection of increased urinary TGF-β1 levels in those with only IGT.

Glucose exposure has been suggested as a key mediator in upregulating TGF-β1 activity. *In vitro* studies have shown an increase in TGF-β1 expression and bioactivity in renal cells when exposed to high glucose concentration.[23–27] In diabetic rodents, the expression of TGF-β1 mRNA and protein in

renal tissues was also reported to increase within 3 days after the onset of overt diabetes with hyperglycemia.[4] In a previous clinical study, normal volunteers were exposed to steady-state hyperglycemia using a glucose-clamp procedure.[9] After 120 min of steady-state hyperglycemia (200–250 mg/dl), there were significant increases in urinary TGF-β1 level in the normal (non-diabetic) individuals. In this acute study, the increase in urinary TGF-β1 level seemed to be in response to the prolonged hyperglycemia. However, during hyperglycemia, there was also a progressive and substantial increase in endogenous insulin production. In these normal individuals, the mean insulin concentration reached 98 μU/ml at the end of the glucose clamp. Despite the substantial acute increase in urinary TGF-β1, there was no increase in plasma TGF-β1. In this study, the NGT group had an increase followed by a decrease in urinary TGF-β1 level during the OGTT, corresponding to the changes in plasma glucose and insulin concentrations.

Hyperinsulinemia associated with insulin resistance is characteristic of type 2 diabetes and also of prediabetes. In our study, both NGT and IGT groups had a significant increase in urinary TGF-β1 level in response to the steady-state hyperinsulinemia with the plasma glucose concentration clamped at the fasting level of less than 100 mg/dl. The marked increase in urinary TGF-β1 cannot be simply explained by increased urinary leakage, as UAE was essentially unchanged after the insulin clamp in both NGT and IGT groups. These results indicate that, in humans, insulin has a more direct role in upregulating TGF-β1 activity. Our findings are consistent with a previous study by Ellis *et al.*[28] on 46 type 1 diabetic patients. They reported a significant correlation between urinary TGF-β1 level and exogenous insulin dose but not glycemic control, also suggesting that insulin stimulates TGF-β1 activity.

Our data show some association between insulin resistance and increase in urinary TGF-β1 level following hyperinsulinemia. This observation suggests that increased TGF-β1 response to insulin may occur in patients before the onset of clinically evident type 2 diabetes. Romano *et al.*[29] used the Bergman minimal model approach to measure insulin sensitivity in obese women. These investigators found an association between the plasma TGF-β1 level and *M/I*, which is consistent with our finding that insulin resistance is associated with a greater increase in urinary TGF-β1 level. Yener *et al.*[30] measured plasma TGF-β1 levels in women with a history of gestational diabetes (a prediabetic state), type 2 diabetes, and in healthy controls. They reported significantly higher plasma TGF-β1 levels in women with gestational diabetes than in the normal women and highest plasma TGF-β1 levels in women with type 2 diabetes. These previous reports also indicate a relationship between insulin resistance and increased TGF-β1 activity.

African Americans have a greater prevalence of end-stage kidney disease compared with Caucasians.[2] A study by Suthanthiran *et al.*[31] examined the clinical risk factors for renal disease in African and Caucasian Americans. In both normotensives and hypertensives, serum TGF-β1 levels were higher in African-Americans compared with Caucasians. These authors reported significantly stronger associations in African Americans than in Caucasians between serum TGF-β1 level and renal disease risk factors, including BP, metabolic syndrome, and microalbuminuria. Although serum TGF-β1 levels are contributed by platelets and may have high variability due to sample preparation, the observations are consistent with our finding that urinary TGF-β1 levels are increased in African Americans.

While our study is based on a cohort of relatively young adult African Americans without overt diabetes or nephropathy, it is the largest available clinical study on TGF-β1 in an ethnic group at higher risk for type 2 diabetes and diabetic nephropathy. We observed considerable variability within urinary TGF-β1 measurements. The Pearson's correlation coefficient between the two fasting urinary TGF-β1 measurements before the OGTT and insulin-clamp procedure was only $r = 0.334$ ($P < 0.05$). Based on the variability in urinary TGF-β1, we are interpreting with caution the significance of the difference in baseline urinary TGF-β1 between the NGT and IGT groups. A limitation of our study is that we did not measure TGF-β1 levels in plasma. In our previous study,[9] no changes were observed in plasma TGF-β1 concentration despite a marked increase in urinary TGF-β1 level following sustained hyperglycemia and endogenous hyperinsulinemia. However, that study was conducted on a small sample of Caucasian subjects who were acutely exposed to hyperglycemia. It is possible that chronic relative hyperinsulinemia in the IGT group in this study could have caused increases in plasma TGF-β1 level that we failed to detect.

Data from this study show higher urinary TGF-β1 level in the prediabetic state and an increase in urinary TGF-β1 level following an increase in plasma insulin level. These data suggest that insulin upregulates TGF-β1 activity, and that both insulin and TGF-β1 contribute to the pathogenesis of diabetic nephropathy. Our data also indicate that the process may be operational at an early phase, before the onset of clinically evident diabetes.

MATERIALS AND METHODS

Subjects

Participants in this study were drawn from a cohort of healthy young adult African Americans enrolled in a longitudinal study of BP and risks for cardiovascular and renal injury. The participants were all self-identified African Americans recruited from urban Philadelphia and were previously examined between 1994 and 1999. Exclusion criteria at that time included known type 1 and 2 diabetes, polycystic ovarian syndrome, and chronic kidney disease. The participants were re-enrolled for this project between August 2001 and July 2007. Individuals who developed type 2 diabetes subsequent to the previous enrollment, as well as participants found to be diabetic on re-examination, were excluded from the data analyses. Written informed consent was obtained from each participant at the time of re-enrollment on an institutionally approved protocol and consent form.

Procedures

Each participant was examined on two separate visits, 4–8 weeks apart. Instructions were given to collect a timed overnight urine sample on the morning of both visits. Clinical assessment at both visits consisted of anthropometric measurements (height and weight), BP and fasting blood samples. An oral glucose tolerance test (OGTT) was performed on the first visit and a euglycemic hyperinsulinemic clamp was performed on the second visit. Body mass index was calculated as weight (kg) divided by height squared (m^2). The BP measurements were obtained on each subject after a 10-min rest period in a seated position using auscultation with a mercury column sphygmomanometer. The average of two successive readings of systolic and diastolic BP (Korotkoff phase V) was used as the BP value of each visit.

The OGTT was conducted after a 12-h overnight fast. The fasting blood sample was obtained for plasma insulin and glucose concentrations before the ingestion of 75 g of glucose solution (Glucola; Ames Diagnostics, Elkhart, IN, USA). Blood samples were then obtained at 30, 60, and 120 min post-ingestion and assayed for plasma insulin and glucose concentrations. Plasma glucose concentration was analyzed with the glucose oxidase technique (YS Model 27; Glucostat, Yellow Springs, OH, USA). Plasma insulin concentration was determined with a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corp, Los Angeles, CA, USA). Coefficients of variation for intra- and inter-assay variability for glucose and insulin assays were $<5\%$.

The euglycemic hyperinsulinemic clamp was carried out as described previously.[32–34] In brief, all subjects were required to have a 12-h overnight fast before the insulin clamp procedure. Blood samples were obtained for baseline plasma glucose and insulin concentration. Hyperinsulinemia was established with a primed constant infusion of insulin (Eli Lilly, Indianapolis, IN, USA) at a concentration of 1000 mU/ml in normal saline according to the method of Rizza *et al.*[33] The primed infusion rate was sufficient to achieve steady-state hyperinsulinemia at 80–120 μU/ml above fasting insulin levels with the aim of suppressing hepatic glucose production. Hyperinsulinemia was maintained for 120 min, during which time the plasma glucose concentration was maintained at the fasting (baseline) level using a variable infusion of 20% dextrose in water (Abbott Lab, Abbott Park, IL, USA). For participants with elevated fasting glucose greater than 110 mg/dl, the baseline glucose level at 100 mg/dl was set as the target euglycemic level. The glucose infusion rate was adjusted by the negative feedback equation of DeFronzo *et al.*,[32] according to the plasma glucose level sampled every 10 min. The calculated mean value of M (in mg/kg min) during the final 60 min of the clamp procedure was the measure of insulin-mediated glucose uptake, or insulin sensitivity. M was adjusted for the level of steady-state hyperinsulinemia in each case by dividing M by I during the final 60 min of the clamp procedure to derive the M/I (in mg/kg min $\times 100$).

A timed overnight urine sample was collected from each participant at both visits. The volume and collection time were recorded for each urine sample. On the first visit, each participant was asked to void before ingesting the glucose load for the OGTT. Two additional urine samples were then collected at 2 h (2-h OGTT) and 4 h after the glucose ingestion. On the second visit, each participant was asked to void before starting the insulin clamp. One additional urine sample was collected at the end of the insulin-clamp study. All urine samples were assayed for albumin, creatinine, and TGF-β1. Urine albumin concentration was analyzed by enzyme-linked immunosorbent assay using an Albuwell kit (Exocell, Philadelphia, PA, USA) according to the manufacturer's instructions. Urine creatinine concentration was assayed using a NOVA analyzer (NOVA Biomedical, Waltham, MA, USA). Urine albumin excretion was computed in micrograms per minute and in milligrams per gram creatinine.

TGF-β1 measurements

TGF-β1 in urine was assayed by a method previously described and developed by Siva and Dunn.[35] This assay uses a sandwich enzyme-linked immunosorbent assay (Quantikine kit for human TGF-β1 immunoassay; R&D Systems, Minneapolis, MN, USA). In brief, a 10-ml aliquot of urine stored at $-80^{\circ}C$ in a polypropylene tube without preservatives was thawed overnight in a refrigerator and centrifuged gently to remove sediments. Duplicates of 2-ml aliquot from each urine sample were made acidic ($pH<2$) by the addition of hydrochloric acid to activate latent TGF-β1 to the active form. After at least 15 min, the urine samples were adjusted to pH 7.3 ± 0.3 with sodium hydroxide. The duplicates were concentrated to less than 200 μl using a Centricon filter unit (YM-10; Millipore, St. Charles, MO, USA) and the final volume of each sample was adjusted to 200 μl to achieve a concentration factor of 10 using a buffer from the Quantikine kit for human TGF-β1 immunoassay (R&D System). Corrections were made for urine concentration by measuring net urine creatinine, and values were expressed as TGF-β1 in picograms per milligram creatinine. The correlation coefficient with standards is >0.98 and the lowest detectable limit for TGF-β1 measurement is 0.7 pg/ml. The reliability of this assay for urinary TGF-β1 is high with an intra- and inter-assay coefficients of variation of $2.5 \pm 3.0\%$ and $5.6 \pm 4.2\%$, respectively. The recovery of fortified TGF-β1 added to the urine samples was 94%. No interference from TGF-β2 was observed.

Statistical analysis

The cohort was stratified into two groups of different glucose tolerance status, NGT, and IGT, according to the American Diabetes Association criteria[10] based on their fasting glucose and OGTT results. To avoid over-classification of IGT based on a single isolated elevated fasting blood glucose level close to the threshold of 100 mg/dl, the NGT group was defined as having a fasting blood glucose level less than 126 mg/dl and 2 h OGTT less than 140 mg/dl, whereas the IGT group was defined as having a fasting blood glucose level less than 126 mg/dl and 2 h OGTT between 140 and 200 mg/dl. For additional analyses, the cohort was also stratified into three groups based on the M/I derived from the insulin clamp, with <5 indicating insulin resistance and >7.5 corresponding to insulin sensitivity.

Because of the extreme skewness in the distribution of urinary TGF-β1 values, the analyses were performed after log transformation and results are expressed in terms of geometric means. The main analyses included all repeated measurements for each subject (up to three values for the OGTT, and up to two values for the insulin clamp) and were based on mixed-effects linear regression. This is an extension of ordinary linear regression that appropriately accounts for the non-independence (within-subject correlation) of the repeated measures and the unequal number of observations contributed by each subject. The main results presented are from a model that also controlled for age, sex, smoking, alcohol use, obesity status, hypertension, and urine albumin excretion (UAE). All analyses were carried out in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

DISCLOSURE

All the authors declared no competing interests.

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