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• BRIEF REPORTS •

Significant association of insulin and proinsulin with clustering of cardiovascular risk factors

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Abstract

AIM: To investigate the association between true insulin and proinsulin and clustering of cardiovascular risk factors.

METHODS: Based on the random stratified sampling principles, 1196 Chinese people (533 males and 663 females, aged 35-59 years with an average age of 46.69 years) were recruited. Biotin-avidin based double monoclonal antibody ELISA method was used to detect the true insulin and proinsulin, and a risk factor score was set to evaluate individuals according to the number of risk factors.

RESULTS: The median (quartile range) of true insulin and proinsulin was 4.91 mIu/L (3.01-7.09 mIu/L) and 3.49 pmol/L (2.14-5.68 pmol/L) respectively, and the true insulin level of female subjects was significantly higher than that of male subjects (P = 0.000), but the level of proinsulin displayed no significant difference between males and females (P = 0.566). The results of covariate ANOVA after age and sex were controlled showed that subjects with any of the risk factors had a significantly higher true insulin level (P = 0.002 for hypercholesterolemia, P = 0.021 for high low-density lipoprotein cholesterol, P = 0.003 for low high-density lipoprotein cholesterol, and P = 0.000 for other risk factors) and proinsulin level (P = 0.001 for low high-density lipoprotein cholesterol, and P = 0.000 for other risk factors) than those with no risk factors. Furthermore, subjects with higher risk factor scores had a higher true insulin and proinsulin level than those with lower risk factor scores (P = 0.000). The multiple linear regression models showed that true insulin and proinsulin were significantly related to cardiovascular risk factor scores respectively (P = 0.000).

CONCLUSION: True insulin and proinsulin are significantly associated with the clustering of cardiovascular risk factors.

Key words: True insulin; Proinsulin; Cardiovascular diseases

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INTRODUCTION

Dyslipidemia, hypertension, hyperinsulinemia and obesity (special central obesity) have been recognized as potent risk factors for coronary heart disease in adults[1-3]. In fact, the clustering of the above cardiovascular risk factors often occurs in adults and has been termed syndrome X[4], deadly quartet[5], insulin resistance syndrome[6], and multiple metabolic syndrome[7]. Insulin resistance emerges as a common pathogenetic denominator underlying the above risk factor clustering[8]. In the earlier studies, insulin concentration was measured using radioimmunoassays with polyclonal antibodies[9], which cross-react with largely inactive insulin precursor molecules such as proinsulin (PI) and des-31, 32 proinsulin; hence it is called immunoreactive insulin (IRI). These proinsulin molecules (PI) can be distinguished from more biologically active true insulin (TI) molecules by using highly sensitive and specific two-site immunoassays based on monoclonal antibodies[10]. After these assays became available, several groups have reported that PI is more closely associated with coronary heart disease[11], stroke[12], and hypertension[13] than TI. It is possible that hyperinsulinemia in subjects with insulin resistance syndrome may reflect increased PI concentration rather than increased levels of insulin itself. To explore the relationship between TI versus PI and cardiovascular risk factor clustering, a population-based epidemiological investigation was conducted in Pizhou City located in the mid-east of China.

MATERIALS AND METHODS

Study design and population

The Pizhou district, a rural area of 2097 km^2, is situated in the north of Jiangsu Province of China, with a population of 1.52 million. From April 2001 to May 2001, a large cross-sectional, community-based epidemiological study was conducted. The people surveyed were adults aged between 35 and 59 years. Signed informed consents were obtained from all participants and the study was approved by the Nanjing Medical University Ethics Review Committee. A two-stage cluster-sampling scheme based on existing census divisions was used to randomly select (with probability proportional to size) 4 areas, each with a population from 300 to 350 subjects, and samples were stratified by sex and age group (5 years) to ensure representation of each part of the population. Among 1351 individuals investigated,

the response rate was 88.5%, and the random sample and random-sample responder populations closely reflected the actual distribution of age group and sex in Pizhou area. Compared with the figures available from the most recent census, the samples were generally found to be representative in terms of sex and age group profiles, geographical locations, marital status, socio-economic groups and education levels. Data on sex and age groups and geographical locations collected from non-respondents were compared with those of the samples surveyed and no significant difference was detected between them.

Anthropometric measurements

Anthropometric measurements were performed after participants removed their shoes and upper garments and donned an examining gown. Each measurement was performed twice and the average was used in the analysis. Height (HT) was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Weight (WT) was measured to the nearest 0.1 kg using a hospital balance beam scale. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2). The waist circumference (WC) was measured to the nearest 0.5 cm at the point of narrowing between the umbilicus and xiphoid process (as viewed from behind) and the waist circumference was used as a judgement of upper-body adioposity. Blood pressure was measured in the right arm with the participant seated and the arm bared. Three readings were recorded for each individual, and the average of the second and third reading was defined as the subject's blood pressure.

Laboratory measurements

Twelve hour fasting blood samples were drawn in the morning and the sera were stored at -70 ℃ immediately after centrifugation until assayed. All laboratory measurements were conducted at the Central Clinical Laboratory in the First Affiliated Hospital of Nanjing Medical University. Fasting blood glucose (FBG), fasting total cholesterol (TCH), fasting triglyceride (TG) and fasting high-density lipoprotein cholesterol (HDL-c) were determined by enzymatic procedures on an automated autoanalyzer (AU 2700, Olympus, Japan). The laboratory tests were monitored for precision and accuracy of glucose and lipid measurements by the agency's surveillance program. Measurements on agency-assigned quality control samples showed no consistent bias over time within or between surveys. Low-density lipoprotein cholesterol (LDL-c) was assessed by the Friedwald method[14]. The TI level was measured using a highly sensitive two-site sandwich ELISA[15]. The detection limit was 5.0 pmol/L. The specificity of the assay excluded intact, split (32-33) and des (31,32) proinsulin. There was some cross-reactivity with the less abundant split (65-66) proinsulin (30%) and des (64,65) proinsulin (63%). The PI level was measured in a similar manner using another sensitive two-site sandwich ELISA[16]. The detection limit in human serum was 0.25 pmol/L. There was no cross-reactivity with human insulin and human C-peptide. However, the four major proinsulin conversion intermediates reacted in various proportions of 65% to 99%. The between- and within-assay coefficients of variation were 6.8%, 7.8% for TI respectively and 6.7%, 7.8% for PI respectively. All measurements were performed in duplicate. The four monoclonal antibodies including OXI-005, HUI-018, PEP-001 and HUI-001 were kind gifts from Novo Nordisk, Bagsvaerd, Denmark.

Definition of risk factors

To investigate the relationship between TI versus PI and cardiovascular risk factor clustering, we set a risk factor score to rank individuals according to the number of the risk factors at the time of survey. The following 9 factors and cut-off points were used to build up this risk factor scores. Hypertension was defined when systolic blood pressure (SBP) was $\geqslant$R140 mmHg and/or diastolic blood pressure (DBP) $\geqslant$R90 mmHg or antihypertensive drugs were taken because of previous hypertension according to the 1999 WHO/ISH criteria[17]. Hyperglycemia was diagnosed based on the fasting serum glucose >6.1 mmol/L according to the American Diabetes Association (ADA) criteria[18] or when the patient had a history of diabetes mellitus. Hypercholesterolemia was defined as fasting total cholesterol $\geqslant$ 5.20 mmol/L. High LDL-c was defined as LDL-c $\geqslant$ 3.38 mmol/L. Low HDL-c was recognized as HDL-c$\leqslant$1.04 mmol/L. Hypertriglyceridemia was defined as fasting triglyceride $\geqslant$ 1.70 mmol/L[19]. High TG/low HDL was considered as the risk score and the cut-off point was triglyceride $\geqslant$1.70 mmol/L and HDL-c$\leqslant$1.04 mmol/L. Overall overweight was considered as BMI $\geqslant$ 25.0 kg/m^2 according to the WHO guidelines[20]. Visceral obesity was defined as waist circumference $\geqslant$85 cm in males and $\geqslant$80 cm in females[21]. The final risk factor scores varied from 0 to 5. 0; 1 indicates the exposure to any one risk factor; 2, 3, and 4 indicate exposure to any combination of 2, 3, and 4 risk factors respectively; 5 indicates exposure to any combination of 5 or more than 5 risk factors simultaneously.

Statistical analysis

All data analyses were performed using Statistical Package for Social Science (SPSS for Windows, version 10.0, 1999, SPSS Inc, Chicago, IL). Data of BMI, WC, age and blood pressure were normally distributed parameters and presented as mean±SD, whereas skewed data including fasting blood glucose, fasting lipid, fasting TI and fasting PI were logarithmically transformed before analysis and expressed as a median and quartile range. Intergroup comparisons were normally made with Student's *t* test, and analysis of covariance (ANCOVA) controlling the age and sex was used to determine the relationship between risk factors and TI versus PI. Stepwise multiple linear regression was used, *P* values of 0.05 and 0.10 were used as the criteria for entry and removal at each step respectively. $P<0.05$ was considered statistically significant.

RESULTS

Anthropometric and biochemical characteristics of study population

The anthropometric and biochemical characteristics of the Chinese population studied are displayed in Table 1. Comparison between males and females was carried out by unpaired *t* test. Due to skewness, FBG, CH, TG, HDL-c, LDL-c, TI and PI were logarithmically transformed before analysis. No significant difference was found between males and females regarding their age, FBG, lipid and PI. The SBP, DBP and WC were significantly higher in males than in females. However, BMI and TI were significantly higher in females than in males.

ANCOVA analysis of TI versus PI and risk factors

The logarithmically transformed values of TI and PI were dependent variables respectively, and either the presence or absence of risk factors was factor variable. The covariate ANOVA (ANCOVA) after adjustment for age and sex was conducted. The results (Tables 2, 3) showed that after the age and sex were controlled, the subjects with any of the above risk factors had a significantly higher TI and PI level than those with no risk factors. Furthermore, the subjects with higher risk factor scores had a higher TI and PI level than the subjects with lower risk factor scores.

Table 1 Anthropometric and biochemical characteristics of study population (mean±SD)

Variables	Male	Female	Total	*T*	*P*
AGE	46.78±7.93	46.62±7.79	46.69±7.85	0.360	0.719
SBP	126.26±19.92	122.23±20.54	124.03±20.36	3.413	0.001
DBP	81.09±12.47	77.23±10.79	78.95±11.73	5.737	0.000
FBG	4.48 (4.07-4.94)	4.42 (4.08-4.84)	4.58 (4.07-4.88)	0.492	0.623
CH	4.06 (3.50-4.71)	3.98 (3.45-4.59)	4.02 (3.48-4.63)	1.578	0.115
TG	0.83 (0.59-1.24)	0.77 (0.57-1.12)	0.79 (0.57-1.18)	1.957	0.051
HDL	1.04 (0.86-1.28)	1.07 (0.89-1.27)	1.06 (0.88-1.28)	-1.405	0.160
LDL	2.54 (2.09-3.02)	2.44 (2.05-2.88)	2.48 (2.06-2.95)	0.990	0.322
BMI	23.60±2.84	24.16±3.19	23.91±3.05	-3.128	0.002
WC	79.43±8.76	76.34±8.57	77.72±8.78	6.130	0.000
TI	4.24 (2.57-6.53)	5.45 (3.51-7.47)	4.91 (3.01-7.09)	-5.164	0.000
PI	3.39 (2.04-5.65)	3.58 (2.22-5.69)	3.49 (2.14-5.68)	-0.574	0.566

Table 2 ANCOVA analysis of TI and risk factors (age and sex are covariate)

Risk factors		N (M/F)	Median (Q_R)	*F*	*P*
Hypertension	Y	149/142	5.71 (3.78-7.86)	34.063	0.000
	N	384/521	4.60 (2.78-6.75)		
Hyperglycemia	Y	17/22	7.50 (4.86-9.82)	12.116	0.000
	N	516/641	4.82 (2.97-7.02)		
Obesity	Y	149/227	6.41 (4.39-8.46)	89.080	0.000
	N	382/436	4.24 (2.59-6.31)		
Visceral obesity	Y	137/214	6.37 (4.54-8.31)	83.062	0.000
	N	395/449	4.25 (2.58-6.42)		
High CH	Y	85/78	5.69 (3.66-8.19)	9.638	0.002
	N	448/585	4.76 (2.96-6.97)		
High LDL	Y	72/79	5.55 (3.19-7.84)	5.301	0.021
	N	460/579	4.79 (2.97-7.00)		
High TG	Y	66/74	6.94 (4.89-8.62)	39.522	0.000
	N	467/589	4.64 (2.82-6.76)		
Low HDL	Y	258/297	5.31 (3.19-7.57)	8.829	0.003
	N	275/366	4.63 (2.82-6.69)		
Risk factor score	0	134/180	3.81 (2.54-5.84)	21.136	0.000
	1	170/220	4.59 (2.79-6.63)		
	2	123/151	5.19 (3.19-7.16)		
	3	56/65	6.15 (4.23-7.92)		
	4	29/27	7.75 (5.53-9.45)		
	⩾5	18/15	7.81 (5.74-10.20)		

M/F, male/female; Y or N, presence or absence of risk factors.

Table 3 ANCOVA analysis of PI and risk factors (age and sex are covariate)

Risk factors		N (M/F)	Median (Q_R)	*F*	*P*
Hypertension	Y	149/142	4.24(2.49-6.66)	20.523	0.000
	N	384/521	3.34(2.02-5.31)		
Hyperglycemia	Y	17/22	11.20(7.54-17.52)	78.858	0.000
	N	516/641	3.43(2.04-5.36)		
Obesity	Y	149/227	4.70(2.93-7.46)	70.508	0.000
	N	382/436	3.13(1.84-4.72)		
Visceral obesity	Y	137/214	4.67(3.05-7.56)	69.619	0.000
	N	395/449	3.13(1.83-4.86)		
High CH	Y	85/78	4.72(2.89-7.01)	25.807	0.000
	N	448/585	3.36(2.02-5.32)		
High LDL	Y	72/79	4.35(2.62-6.65)	15.775	0.000
	N	460/579	3.40(2.04-5.39)		
High TG	Y	66/74	5.37(3.63-8.57)	54.298	0.000
	N	467/589	3.31(1.99-5.30)		
Low HDL	Y	258/297	3.76(2.38-5.96)	12.049	0.001
	N	275/366	3.29(1.95-5.31)		
Risk factor score	0	134/180	2.77(1.61-4.29)	27.290	0.000
	1	170/220	3.15(2.02-5.03)		
	2	123/151	3.97(2.39-6.03)		
	3	56/65	4.56(3.04-7.19)		
	4	29/27	6.18(3.83-11.39)		
	⩾5	18/15	6.89(4.45-12.30)		

M/F, male/female; Y or N, presence or absence of risk factors.

Table 4 Multiple stepwise linear regression analysis with TI as a dependent variable

Parameter	Unstandardized coefficients		Standardized coefficients (Beta)	*T*	*P*
	B	SE			
Constant	-1.677	1.520	— —	-1.103	0.270
Risk factor score	0.410	0.102	0.156	4.036	0.000
FBG	0.558	0.112	0.142	4.992	0.000
Sex	0.836	0.248	0.094	3.375	0.001
BMI	0.162	0.052	0.111	3.121	0.002
TG	0.196	0.093	0.063	2.108	0.035
AGE	-3.18E-02	0.016	-0.056	-2.003	0.045

Table 5 Multiple stepwise linear regression analysis with PI as a dependent variable

Parameter	Unstandardized coefficients		Standardized coefficients (Beta)	*T*	*P*
	B	SE			
Constant	-3.915	1.281	— —	-3.058	0.002
FBG	1.095	0.093	0.316	11.741	0.000
Risk factors score	0.326	0.091	0.141	3.596	0.000
BMI	0.165	0.043	0.128	3.856	0.000
AGE	-4.80E-02	0.013	-0.097	-3.632	0.000
TG	0.281	0.084	0.103	3.346	0.001
LDL	0.321	0.158	0.062	2.034	0.042

Multiple linear regression analysis for risk factors

Tables 4 and 5 show the multiple stepwise linear regression analyses of the relationship between the dependent variables of TI and PI respectively and the independent variables of age, sex, BMI, WC, SBP, DBP, FBG, lipid, and risk factor scores. When the risk factor scores were entered in the regression model before other variables, the results presented in Table 3 indicated that the risk factor scores, fasting blood glucose, sex, BMI, triglyceride and age were significantly associated with the true insulin concentration. Table 4 demonstrates that fasting blood glucose, risk factor scores, BMI, age, triglyceride, low-density lipoprotein cholesterol remained in the regression model and were significantly associated with the concentration of proinsulin.

DISCUSSION

PI is converted to insulin in the secretory granules of pancreatic β cells. Two endoproteolytic activities are responsible for this conversion. These activities correspond to the two endoprotease types PC1 and PC2, two members of the mammalian family of subtilisin-like proteases, which are related to the yeast kex2 gene products. Type 1 endoprotease (PC1) cleaves on the C-terminal side of the pair of basic amino acids Arg^{31}-Arg^{32} linking the B-chain and connecting peptide (C-peptide) and type 2 endoprotease (PC2) on the C-terminal side of Lys^{64}-Arg^{65} linking the C-peptide and the A-chain. It has been reported that C-terminal basic residues generated by such cleavages are then trimmed by carboxypeptidase[22], and PI is cleaved sequentially, first by PC1 which cleaves at the 32,33 sites and then by PC2 which cleaves at the 64,65 sites to produce mature insulin and C-peptide[23]. Under physiological conditions, only a small amount of intact and split PI is co-secreted with insulin from the pancreatic β-cells. However, in type-2 diabetes[24] and other pathological conditions[11-13], PI and PI split products could be markedly elevated. Thus, it is possible that hyperinsulinemia in subjects with insulin resistance syndrome (IRS) may reflect increased PI concentrations rather than increased levels of insulin itself. The disagreement in results could be attributed to the difference in laboratory methods and in the geological distribution of investigated populations. For these reasons, we studied a population-based sample of 1196 Chinese adults living in the Pizhou City, Jiangsu Province of China. So far no population-based epidemiological studies on the relationship between the clustering of cardiovascular risk factors and TI versus PI have been reported.

The median and quartile range of fasting TI concentration in this study was 4.91 mIu/L and 3.01-7.09 mIu/L in response to a fasting PI of 3.49 pmol/L and 2.14-5.68 pmol/L. The TI and PI concentrations reported here are lower than those reported previously in a population-based study of diabetes and cardiovascular diseases in Mexican Americans and non-Hispanic whites[25], which might be attributed to the difference in ethnicity and laboratory measurement. The statistical results of ANCOVA after the age and sex were adjusted indicate that the subjects with cardiovascular risk factors including hypertension, hyperglycemia, obesity, visceral obesity, dyslipidemia and risk factor clustering have both hyperinsulinemia and hyperproinsulinemia rather than either hyperinsulinemia or hyperproinsulinemia alone. In general, our results are in agreement with the previous results in diabetic subjects[26], young nondiabetic male survivors with myocardial infarction[27], hypertension subjects[13], and subjects with dyslipidemia[25]. The results of univariate and multivariate analyses reveal that the concentrations of TI and PI are closely associated with cardiovascular risk factor clustering independent of age, sex, BMI, WC, blood pressure, fasting blood glucose and lipid, and the results are in accordance with the cohort epidemiological results that cardiovascular diseases and all-cause mortality are increased in subjects with metabolic syndrome, even in the absence of baseline CVD and diabetes[28]. The age-adjusted prevalence of metabolic syndrome in Americans is similar in men (24.0%) and women (23.4%), and about 47 million US residents have metabolic syndrome based on 2000 census data[29]. Therefore, early identification, treatment, and prevention of metabolic syndrome presents a major challenge to the health care professionals facing an epidemic of overweight and sedentary lifestyle.

This study concludes that both TI and PI are elevated in serum when the risk factors are co-presented, which means that it is not the premature secretion of insulin but the total activity of β cells is promoted in this situation. However, the mechanisms by which TI contributes to the clustering of cardiovascular risk factors are incompletely understood, and

the defects in nonesterified fatty acid (NEFA) metabolism which have been implicated in the abnormal lipid and glucose metabolism, may characterize the clustering of cardiovascular risk factors[8]. It has been shown that PI is at least as strong as insulin and can independently increase the level of PAI-1 activity, thereby lowering fibrinolytic activity[27]. TI and PI are secreted together from the β cells and probably exert their biological effects in the body independently of each other, but this does not exclude the possibility of coinciding effects later in the causal path of cardiovascular risk factor clustering (e.g., PAI-1 activity).

In conclusion, TI and PI are closely associated with the clustering of cardiovascular risk factors, further studies are needed to investigate quantitatively the prognostic significance of these variables.

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