ASSOCIATIONOF PROINSULIN WITH CARDIOVASCULAR RISK IN NONDIABETIC SUBJECTS

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Background: There are data which suggest that high proinsulin concentration may be a better predictor for cardiovascular risk compared to insulin concentration. In this study we evaluated the relations between proinsulin and cardiovascular risk factors. We also evaluated the association between proinsulin and common carotid intima-media thickness (CIMT).
Methods: The study included 80 nondiabetic subjects (41 men and 39 women), free of coronary heart disease (CHD). Both intact proinsulin and insulin were measured by ELISA method. An oral glucose tolerance test (OGTT) was performed in all individuals.
Results: In univariate analysis intact proinsulin was associated positively with BMI, waist diameter and triglyceride in both sexes. Only in men proinsulin was positively associated with fibrinogen and negatively with HDL-cholesterol. After adjusting for BMI the correlations between intact proinsulin and cardiovascular risk factors and between fasting insulin and cardiovascular risk factors were no longer statistically significant. Fasting insulin and fasting intact proinsulin were not related to CIMT.
Conclusions: Data suggest that fasting insulin and fasting intact proinsulin do not predict cardiovascular risk in nondiabetic subjects.

Key words: Proinsulin; insulin; cardiovascular risk; intima-media thickness.

INTRODUCTION

Proinsulin is a precursor of insulin that is converted to insulin in the secretory vesicles of pancreatic beta cells under the action of two endopeptidases on C-terminal side: type 1 endopeptidase (prohormone convertase 3 – PC3, also known as PC1) and type 2 endopeptidase (prohormone convertase 2 – PC2). Type 1 endopeptidase (PC3) cleaves at the Arg 31–Arg 32 site, linking insulin B-chain to the C-peptide, leading to split-31,32 proinsulin and type 2 endopeptidase (PC2) cleaves at the Lys64-Arg65 site, linking the C-peptide to the insulin A-chain, leading to split-64,65 proinsulin. The C-terminal basic aminoacids left on the cleaved proinsulin are removed by carboxy peptidase H (CPH) leading to the formation of two proinsulin intermediates: des-31,32 proinsulin and des-64,65 proinsulin. Proinsulin is cleaved sequential, first by PC3 at 32, 33 sites and then by PC2 at 64, 65 sites to produce insulin and C-peptide[1-4.] Since this process is incomplete, some intact and partially processed proinsulins (split proinsulins) remain in the secretory vesicles and enter the circulation with insulin and C-peptide[5]. Proinsulin and its split products circulate in high concentra-tions in subjects with diabetes[6-10], gestational diabetes[11], IGT[12,13] and diabetic first-degree relative[9,14,15.] Proinsulin and proinsulin/insulin ratio are independent predictors for the development of diabetes[7,8,16]. Wareham *et al.*[8] showed that fasting 32,33 split proinsulin was a better predictor for diabetes risk than insulin or intact proinsulin. Hanley *et al.*[16] demonstrated that both low acute insulin response (AIR), determined during a frequently sampled intravenous glucose tolerance

test (FSIGTT) and high proinsulin independently predicted diabetes.

There are contradictory data regarding the correlation between hyperinsulinemia and cardiovascular risk[17-30]. In the earlier studies, insulin concentration was measured using radioimmunoassays (immunoreactive insulin – IRI) which fail to differentiate between insulin and proinsulin molecules (intact and split products). In present, proinsulin can be distinguished from insulin (true insulin) by using two-site immunoassays based on monoclonal antibodies[5,31-33]. Activity of proinsulin is about 10% of the biological activity of insulin[34,35]. Proinsulin-like molecules bind to the insulin receptor with a lower affinity than insulin and have a weaker biological effect[36]. Glauber *et al.*[37] found that subcutaneously injected proinsulin has prolonged pharmacokinetics in plasma and the hypoglycemic effects of proinsulin are mostly due to suppression of hepatic glucose output with little stimulation of glucose disposal and less hypoglycemia. After these assays became available, several groups have reported that proinsulin is associated with the cardiovascular risk[31,33,34,36,38-47]. The attempt to use proinsulin instead of long acting insulin had been associated with severe cardiovascular accidents which had let to the discontinuation of clinical trials[45].

In this study we evaluated both the correlation between intact proinsulin and cardiovascular risk factors and the correlation between intact proinsulin and intima media-thickness of the common carotid artery (CIMT) in nondiabetic subjects.

MATERIAL AND METHODS

The study included 80 healthy subjects (41 men and 39 women) recruited between February 2009 to January 2010 in Clinical Hospital Colentina, Department of Diabetes, Nutrition and Metabolic Diseases. All participants gave written informed consent before inclusion in the analyses. Subjects with diabetes, impaired glucose tolerance, impaired fasting glucose, currently smoking, history of cardiovascular diseases, use of drugs for dyslipidemia and hypertension, glucocorticoids, presence of viral hepatitis, renal disease, thyroid dysfunction, Cushing's syndrome, hematological disorder, systolic blood pressure (SBP) / diastolic blood pressure (DBP) ≥ 170/100 mmHg, triglyceride ≥ 400 mg/dl, total cholesterol ≥ 300 mg/dl were excluded.

Laboratory assay

The samples of blood were taken after 12 hours of overnight fasting. Insulin, intact proinsulin, total adiponectin, leptin and hs-CRP were measured by ELISA (DRG International, Inc.) on a Dynex analyzer. There was no cross-reactivity between insulin and intact proinsulin. Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, glucose and fibrinogen were measured using standard techniques. To estimate low-density (LDL) cholesterol we used Fridewald formula as follows: LDL-cholesterol = total cholesterol – HDL-cholesterol – triglycerides/5. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the product of the fasting plasma insulin value (in microunits per liter) and the fasting plasma glucose (in mg per deciliter) divided by 405. Oral glucose tolerance test was performed in all subjects. Impaired fasting glucose was defined as fasting plasma glucose level ≥ 100 mg/dl but < 126 mg/dl, impaired glucose tolerance was defined as 2h – postload glucose 140–199 mg/dl and fasting plasma glucose < 126 mg/dl and diabetes mellitus was defined as fasting plasma glucose ≥ 126 mg/dl or 2h – postload glucose ≥ 200 mg/dl.

Carotid artery ultrasound

CIMT was determined using a B-mode ultrasound scanner (Siemens Sonoline Sienna) and a 7.5 MHz linear probe with subjects in the supine positions. Longitudinal scan of both the right and left common carotid artery was recorded. Measurement of IMT was made on the near (anterior) and far (posterior) wall of the common carotid artery at 1 cm proximal to the bifurcation, in segments that are free of plaque. For each individual, the CIMT was determined as the average of near and far-wall measurement of both the left and right arteries.

Statistical analysis

Statistical analysis was performed using the program Graph Pad Instant 3. Continuous variables were tested for normality distribution with the use of Wilk-Shapiro test. Data normally distributed were expressed as mean±standard deviation (SD) and data skewed distributed were expressed as

median (interquartile range). Normally distributed variables were compared by unpaired t-test and not normally distributed variables were compared by Mann-Whitney test. Pearson's correlation coefficients were calculated, skewed variables were log transformed before evaluation. Values of $p<0.05$ were considered statistically significant.

RESULTS

Participating subjects were classified by BMI: 18 (9 men and 9 women) were defined as lean ($18.5<BMI<25.0$ kg/m^2), 34 (18 men and 16 women) were defined as overweight ($25.0<BMI<30.0$ kg/m^2) and 28 (14 men and 14 women) were defined as obese (BMI$\geq$30.0 kg/m^2). The clinical and biochemical characteristics of the subjects included in this study are presented in Table 1. Fasting insulin and fasting proinsulin were higher in obese subjects compared to non-obese subjects but the differences were not statistically significant. The differences were statistically significant when obese subjects were compared to lean subjects (obese *vs*. lean, proinsulin – 7.9 *vs*. 5.4 pmol/l, insulin – 13.9 *vs*. 6.4 μU/ml). We found no statistically significant differences between obese and lean subjects regarding proinsulin/ insulin ratio (obese *vs*. lean, 0.55 *vs*. 0.6, p=0.8).

In univariate analysis in men proinsulin was associated positively with BMI (r=0.41, p=0.0008), waist circumference (r=0.43, p=0.0002), triglyceride (r=0.34, p=0.001) and fibrinogen (r=0.28, p=0.047) and negatively with HDL-cholesterol (r=-0.31, p=0.003) and insulin was associated positively with BMI (r=0.47, p<0.0001), waist circumference (r=0.51, p<0.0001), triglyceride (r=0.32, p=0.0014) and hs-CRP (r=0.3, p=0.036) and negatively with HDl-cholesterol (r=-0.33, p=0.001) (Table 2). After adjusting for BMI the correlations between proinsulin and triglyceride, HDL-cholesterol and fibrinogen and between insulin and triglyceride, hs-CRP and HDL-cholesterol were no longer statistically significant. In women proinsulin was associated positively with BMI (r=0.39, p=0.002), waist diameter (r=0.4, p=0.0009), triglyceride (r=0.29, p=0.045) and insulin was associated positively with BMI (r=0.43, p<0.0001), waist diameter (r=0.48, p<0.0001) and triglyceride (r=0.29, p=0.04). After adjusting for BMI the correlations between proinsulin and triglyceride and insulin and triglyceride were no longer statistically significant (Table 2).

Pearson correlation coefficients among traditional cardiovascular risk factors, adiponectin, leptin, insulin, intact proinsulin and CIMT are shown in Table 3. We found no correlation among fasting insulin, fasting proinsulin and CIMT.

Table 1

Characteristics of study subjects

	Nonobese	**Obese**	**P**
Number	52	28	-
Sex (M/W)	27/25	14/14	-
Age (years)	55.4±8.7	53.9±9.6	0.76
BMI (kg/m^2)	27.2±2.4	33.7±2.8	**<0.0001**
Waist circumference (cm)	97.7±11.2	107.6±9.6	**<0.0001**
SBP (mmHg)	131.5±11.6	139.3±14.2	**0.016**
DBP (mmHg)	76.8±6.9	82.4±8.5	**0.042**
Total cholesterol (mg/dl)	217.7±21.3	238.1±46.5	0.51
HDL-cholesterol (mg/dl)	46.9±7.2	40.6±7.7	0.072
LDL-cholesterol (mg/dl)	129.3±37.8	140.1±38.1	0.22
Triglyceride (mg/dl)	111.4±35.3	168.9±59.7	**0.038**
Hs-CRP* (mg/l)	1.9 (0.95-3.1)	2.75 (1.6-6.3)	**0.045**
Fasting glucose (mg/dl)	82.1±13.5	91.3±7.9	**0.04**
HbA1c (%)	4.9±0.5	5.2±0.6	0.46
Fasting insulin* (μU/ml)	8.6 (5.4-16.7)	13.9 (9.2-23.5)	0.09
HOMA-IR*	2.2 (1.7-5.1)	3.5 (2.1-7.4)	0.25
Fasting proinsulin* (pmol/l)	5.7 (3.8-7.3)	7.9 (4.2-10.4)	0.13
Proinsulin/Insulin*	0.6 (0.3-0.9)	0.55 (0.2-0.75)	0.72
Adiponectin* (μg/dl)	5.6 (3.9-7.7)	3.8 (2-5.1)	0.19
Leptin* (ng/ml)	28.2 (15.6-41.3)	37.3 (16.6-60.9)	**0.047**
Fibrinogen (mg/dl)	307.2±56.9	336.6±61.7	0.51
CIMT (mm)	0.7±0.14	0.86±0.16	**0.029**

*log transformation because of the skewed distribution

Table 2

Correlation of insulin and intact proinsulin with anthropometric, haemodynamic and biochemical variables

	Fasting intact proinsulin				**Fasting insulin**			
	Men		**Women**		**Men**		**Women**	
	r	**P**	**R**	**P**	**r**	**p**	**R**	**P**
Age	0.04	0.76	0.08	0.64	0.1	0.46	0.07	0.69
BMI	**0.41**	**0.0008**	**0.39**	**0.002**	**0.47**	**<0.0001**	**0.43**	**<0.0001**
Waist circumference	**0.43**	**0.0002**	**0.4**	**0.0009**	**0.51**	**<0.0001**	**0.48**	**<0.0001**
Systolic blood pressure	0.07	0.68	0.09	0.55	0.15	0.31	0.12	0.4
Diastolic blood pressure	0.11	0.49	0.14	0.42	0.05	0.74	0.09	0.6
Total cholesterol	0.02	0.9	0.06	0.72	0.13	0.37	0.11	0.45
HDL-cholesterol	**-0.31**	**0.003**	-0.2	0.11	**-0.33**	**0.001**	-0.22	0.065
LDL-cholesterol	0.06	0.73	0.1	0.51	0.16	0.26	0.08	0.66
Triglyceride	**0.34**	**0.001**	**0.29**	**0.045**	**0.32**	**0.0014**	**0.29**	**0.04**
Hs-CRP*	0.19	0.21	0.16	0.35	**0.3**	**0.036**	0.23	0.07
Adiponectin*	-0.05	0.78	-0.03	0.82	-0.17	0.29	-0.2	0.19
Leptin*	0.03	0.88	0.07	0.75	0.08	0.62	0.11	0.46
Fibrinogen	**0.28**	**0.047**	0.23	0.09	0.14	0.31	0.17	0.2

*log transformation because of the skewed distribution

Table 3

Correlation among insulin, intact proinsulin and CIMT

Variable	**Carotid intima media-thickness**			
	Men		**Women**	
	r	**P**	**r**	**P**
Age	**0.47**	**<0.0001**	**0.38**	**0.002**
BMI	0.14	0.37	0.1	0.48
Waist circumference	0.1	0.52	0.07	0.73
Systolic blood pressure	**0.36**	**0.037**	**0.42**	**<0.0001**
Diastolic blood pressure	0.2	0.11	0.16	0.31
LDL-cholesterol	0.08	0.75	0.11	0.4
HDL-cholesterol	-0.06	0.8	-0.09	0.66
Triglycerides	0.18	0.18	0.2	0.15
Fasting glucose	0.09	0.68	0.05	0.8
Adiponectin	-0.16	0.3	-0.14	0.4
Leptin	0.13	0.32	0.19	0.18
Leptin/adiponectin	**0.34**	**0.042**	**0.32**	**0.047**
Hs-CRP	**0.39**	**0.027**	0.18	0.39
Fasting insulin	0.05	0.86	0.1	0.46
Fasting proinsulin	0.17	0.22	0.15	0.34
Proinsulin/Insulin	0.11	0.57	0.17	0.33

DISCUSSION

Previous studies demonstrated that proinsulin and des-31, 32 proinsulin correlated with many cardiovascular risk factors, in both diabetic and nondiabetic subjects[33,34,36,37,43]. In this cross-sectional study, which included subjects without cardiovascular diseases and glycoregulation disorders, we showed that proinsulin correlated positively with triglyceride and fibrinogen and negatively with HDL-cholesterol but after adjusting for BMI the associations were no longer statistically significant. Nagi *et al.*[33] showed in 51 type 2 diabetic subjects, significant relationships between immunoreactive insulin and triglyceride, total cholesterol, high density lipoprotein cholesterol, systolic blood pressure, diastolic blood pressure and body mass index but true insulin significantly correlated only with triglyceride, body mass index, systolic and diastolic blood pressure. Concentrations of 32-33 split proinsulin, correlated positively with triglyceride, total cholesterol, diastolic blood pressure and plasminogen activator inhibitor-1 (PAI-1) and negatively with HDL-cholesterol. Mohamed-Ali *et al.*[34] showed in 270 subjects with normal glucose tolerance that fasting and 2-h insulin concentrations significantly correlated with total and LDL-cholesterol, HDL-cholesterol and

triglycerides. Intact and des-31,32 proinsulin significantly correlated with total and LDL-cholesterol, HDL-cholesterol and triglycerides. Fasting insulin and intact proinsulin were significantly associated with fibrinogen. In multiple regression analyses des-31,32 proinsulin concentration was more strongly associated with HDL-cholesterol (negatively), LDL-cholesterol and triglycerides than fasting insulin concentrations. Haffner *et al.*[39] showed in 260 nondiabetic subjects from the San Antonio Heart Study that in multivariate analyses proinsulin was significantly associated with triglyceride concentrations and to systolic blood pressure, after adjusting for insulin. Haffner *et al.*[43] found in 423 nondiabetic subjects that the increased fasting proinsulin/insulin ratio was significantly associated with hypertension, low HDL-cholesterol, high triglycerides levels and impaired glucose tolerance. Also the authors showed that fasting proinsulin/insulin ratio increased significantly with the number of metabolic disorders. Jain *et al.*[40] and Panahloo *et al.*[41] found in type 2 diabetic patients that insulin treatment was associated with suppression of endogeneous insulin secretion, intact proinsulin and 32, 33 spil proinsulin and decreased activity of PAI-1. The authors showed that changes in intact proinsulin concentrations were positively correlated with those in PAI-1. Gray *et al.*[48] showed in 74 subjects (50 nondiabetic and 24 diabetic) who had survived a myocardial infarction between 6 and 24 months previously that proinsulin-like molecules and serum triglycerides are important determinants of PAI-1 activity. Jia *et al.*[31] found in 1196 Chinese subjects (44.57% men) that both insulin and proinsulin are associated with the clustering of cardiovascular risk factors.

There are studies which reported that proinsulin is associated with coronary heart disease (CHD)[32,36,38,42,49,50,51]. Lindahl *et al.*[38] found in a case-control study which included 194 nondiabetic subjects (67 cases of first acute myocardial infarction and 127 age and sex-matched controls) that high levels of proinsulin is an independent risk factor for acute myocardial infarction. Oh *et al.*[49] showed in a cross-sectional study which included 1456 nondiabetic subjects (554 men and 902 women) participants in the Rancho Bernardo Study that proinsulin was positively associated with CHD in both sexes. In the same study postchallenge insulin was significantly associated with CHD and only in women. Bävenholm *et al.*[51] showed a close association between proinsulin and coronary atherosclerosis in 62 nondiabetic men presenting with a first myocardial infarction before the age of 45. Four studies showed that proinsulin is a long-term predictor of CHD[32,36,42,52]. Yudkin *et al.*[36] showed in 1181 non-diabetic men, participants in the Caerphilly Study that concentrations of proinsulin-like molecules were a better predictor of the incidence of CHD than insulin during the 10–14 years follow-up. Zethelius *et al.*[32] found in a population-based cohort which included 874 men, aged 50 years at baseline from Sweden that increased proinsulin concentrations predict death and morbidity caused by CHD over a period of 27 years, independent of other major cardiovascular risk factors. Also, Zethelius *et al.*[42] showed in a population of 815 men, aged 70 years at baseline from Sweden that a low insulin-mediated glucose uptake measured by the euglycaemic insulin clamp technique and intact proinsulin predicted subsequent CHD, independent of traditional cardiovascular risk factors, over a follow-up period up to 10 years. The authors found no association between insulin concentrations and subsequent CHD. Alssema *et al.*[52] found in 604 subjects (48.34% men) from Hoorn Study (277 subjects with normal glucose metabolism, 208 subjects with impaired glucose metabolism and 119 subjects with newly diagnosed type 2 diabetes) that fasting proinsulin was associated with all-cause and CVD mortality over a period of 11 year independent of glucose tolerance status, insulin resistance and traditional cardiovascular risk factors.

To evaluate the associations between circulating levels of insulin markers (fasting insulin, non-fasting insulin and proinsulin) and CHD risk (defined as non-fatal myocardial infarction or coronary death), Sarwar *et al.*[53] reported a meta-analysis of 19 prospective studies in Western populations, involving about 3600 incident CHD cases. From all the studies included in the analysis 14 reported data on fasting insulin levels, 8 reported data on non-fasting insulin levels and 3 reported data on proinsulin levels. The authors suggest that proinsulin levels are strongly associated with CHD risk than are fasting and non-fasting insulin levels, but these data should be evaluated in larger studies.

In a study which included 272 nondiabetic subjects (94 cases with first-ever stroke and 178 age and sex- matched controls) Lindahl *et al.*[50] found that high levels of proinsulin is an independent predictor of first-ever stroke in women and in the entire study population. There

are data about the association between proinsulin and subclinical atherosclerosis evaluated by intima-media thickness (IMT). In this small, cross-sectional study we found no association among intact proinsulin, insulin, proinsulin/insulin ratio and CIMT. Haffner *et al.*[54] showed in 985 nondiabetic subjects from the Insulin Resistance Atherosclerosis Study (IRAS) a weak correlation between proinsulin and IMT in the common carotid artery (CCA) and internal carotid artery (ICA). The correlation between proinsulin and IMT was stronger than the correlation between insulin and IMT and became non-significant after adjustment for cardiovascular risk factors, especially PAI-1. Bokemark *et al.*[46] showed in 391 clinically healthy 58-year old men that proinsulin was associated with carotid artery IMT after adjusting for smoking, apo B, blood pressure, triglycerides, cross-reacting plasma insulin and C-peptide. Kronberg *et al.*[44] showed in 3857 nondiabetic subjects (1859 men and 1998 women) from the Tromsø Study that proinsulin/insulin ratio (PIR) was significantly associated with carotid artery plaque size in women.

A possible mechanism considered to mediate the association between proinsulin and CHD is disturbed fibrinolysis[54]. Schneider *et al.*[55] demonstrated that proinsulin augment endothelial cell PAI-1 expression, independent of its conversion to insulin and its interactions with the insulin receptors. Gray *et al.*[56] showed in 132 male subjects that concentrations of proinsulin-like molecules and serum triglycerides are stronger determinants of PAI-1 activity than plasma insulin sensitivity in both type 2 diabetic subjects and non-diabetic subjects with and without myocardial infarction. Also, the concentration of proinsulin could act indirectly as a marker of an underlying metabolic disturbance[50]. Palaniappan *et al.*[57] found in a prospective study including 714 subjects participants in the Insulin Resistance Study (IRAS) that waist circumference was the best metabolic syndrome predictor and proinsulin was associated with a significantly increased risk of the metabolic syndrome.

Our results showed that nondiabetic obese subjects had increased proinsulin levels which suggest the presence of beta-cell failure. Also in univariate analysis we found that intact proinsulin was positively associated with triglyceride in both sexes and in men was positively associated with fibrinogen and negatively with HDL-cholesterol. In present it is difficult to compare the results of the studies which evaluated the correlations between proinsulin and cardiovascular risk because of the different characteristics of the subjects included (differences in age, sex, presence or absence of diabetes and cardiovascular diseases). Further prospective studies are required to evaluate the proatherogenic properties of proinsulin.

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