

Association of fasting glucagon and proinsulin concentrations with insulin resistance

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The Relationship between Insulin Sensitivity and Cardiovascular
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Abstract

Aims/hypothesis Hyperproinsulinaemia and relative hyperglucagonaemia are features of type 2 diabetes. We hypothesised that raised fasting glucagon and proinsulin concentrations may be associated with insulin resistance (IR) in non-diabetic individuals.

Methods We measured IR [by a euglycaemic–hyperinsulinaemic (240 pmol min⁻¹ m⁻²) clamp technique] in 1,296

non-diabetic (on a 75 g OGTT) individuals [716 women and 579 men, mean age 44 years, BMI 26 kg/m² (range 18–44 kg/m²)] recruited at 19 centres in 14 European countries. IR was related to fasting proinsulin or pancreatic glucagon concentrations in univariate and multivariate analyses. Given its known relationship to IR, serum adiponectin was used as a positive control.

Results In either sex, both glucagon and proinsulin were directly related to IR, while adiponectin was negatively associated with it (all $p < 0.0001$). In multivariate models, controlling for known determinants of insulin sensitivity (i.e. sex, age, BMI and glucose tolerance) as well as factors potentially affecting glucagon and proinsulin (i.e. fasting plasma glucose and C-peptide concentrations), glucagon and proinsulin were still positively associated, and adiponectin was negatively associated, with IR. Finally, when these associations were tested as the probability that individuals in the top IR quartile would have hormone levels in the top quartile of their distribution independently of covariates, the odds ratio was ~2 for both glucagon ($p = 0.05$) and proinsulin ($p = 0.02$) and 0.36 for adiponectin ($p < 0.0001$).
Conclusions/interpretation Whole-body IR is independently associated with raised fasting plasma glucagon and proinsulin concentrations, possibly as a result of IR at the level of alpha cells and beta cells in pancreatic islets.

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Abbreviations

FFM fat-free mass
IFG impaired fasting glycaemia
IGR impaired glucose regulation
IGT impaired glucose tolerance
IR insulin resistance
NGT normal glucose tolerance

OR odds ratio
 RISC Relationship Between Insulin Sensitivity and Cardiovascular Disease Risk

Introduction

Day-long plasma glucagon concentrations are raised in patients with type 2 diabetes [1]. Impaired suppression of glucagon release can be detected in individuals with impaired glucose tolerance (IGT) and type 2 diabetes following intravenous [2] or oral glucose administration [3]. Fasting glucagon concentrations exert a tonic stimulatory influence on hepatic glucose output, which in the dog has been estimated to account for one-third of the fasting rate of glucose release [4]. Thus, under fasting conditions hyperglucagonaemia sustains glucose overproduction [4], and impaired glucagon suppression after oral glucose or a mixed meal contributes to the postprandial hyperglycaemia of type 2 diabetes [3]. Previous studies in postmenopausal women with IGT [5] or normal glucose tolerance (NGT) [6] have reported an inverse association between arginine-induced glucagon release and insulin sensitivity. Whether such a relationship extends to women and men of any age and body weight has not been determined.

Elevated fasting proinsulin concentrations, or their ratios to the fasting insulin level, have long been known to occur in states of impaired glucose regulation (IGR) [7–9] and to predict incident diabetes [10, 11]. This finding is generally interpreted as a sign of beta cell dysfunction, possibly related to defective processing of the pro-hormone, preferential release of newly synthesised proinsulin or the release of immature proinsulin-rich granules [12, 13]. Although hyperproinsulinaemia has been generally described in conditions characterised by insulin resistance (IR), whether it reflects, or is related to, IR in non-diabetic individuals has not been investigated.

In the present study, we have sought evidence for an independent association of fasting glucagon and proinsulin concentrations with IR (as a post hoc hypothesis) in a large cohort of non-diabetic men and women in whom insulin sensitivity was measured with the euglycaemic–hyperinsulinaemic clamp technique.

Methods

Study participants The Relationship Between Insulin Sensitivity and Cardiovascular Disease Risk (RISC) study is a prospective, observational cohort study whose rationale and methodology have been published [14]. In brief, participants were recruited from the local population at 19 centres

in 14 countries in Europe, according to the following inclusion criteria: men and women, age between 30–60 years and clinically healthy. Initial exclusion criteria were: treatment for obesity, hypertension, lipid disorders or diabetes, pregnancy, cardiovascular or chronic lung disease, weight change of ≥ 5 kg in the last 6 months, cancer (in the last 5 years) and renal failure. Exclusion criteria after screening were: arterial blood pressure $\geq 140/90$ mmHg, fasting plasma glucose ≥ 7.0 mmol/l, 2 h plasma glucose (on a 75 g OGTT) ≥ 11.0 mmol/l, total serum cholesterol ≥ 7.8 mmol/l, serum triacylglycerol ≥ 4.6 mmol/l and ECG abnormalities. Baseline examinations began in June 2002 and were completed in November 2004. The present analysis is based on the 1,296 individuals [716 women and 580 men, mean age 44 years, BMI 26 kg/m² (range 18–44 kg/m²)] who satisfied all criteria and whose clamp study (see below) passed the quality control check.

Insulin clamp A euglycaemic–hyperinsulinaemic clamp was performed in all individuals. Exogenous insulin was administered as a primed-continuous infusion at a rate of 240 pmol min⁻¹ m⁻² simultaneously with a variable 20% (w/v) glucose infusion adjusted every 5–10 min to maintain plasma glucose levels within 0.8 mmol/l ($\pm 15\%$) of the target glucose level (4.5–5.5 mmol/l). The clamp procedure was standardised across centres with the use of a demonstration video and operating instructions; the raw data from each clamp study were immediately transferred to the coordinating centre where they were underwent quality control scrutiny according to pre-set criteria.

Local Ethics Committee approval was obtained by each recruiting centre. Volunteers were given detailed written information on the study and signed a consent form.

Analytical determinations Samples were transported on dry ice at pre-arranged intervals to central laboratories. Plasma glucose was measured by the glucose oxidase technique. Plasma insulin, proinsulin and C-peptide were measured by a two-site time-resolved fluoroimmunoassay (AutoDELFIA Insulin kit; Wallac Oy, Turku, Finland) using monoclonal antibodies, with the following assay characteristics (for insulin, proinsulin and C-peptide, respectively): sensitivity 1–2, 0.3 and 5 pmol/l, within-assay variation 5, 6 and 5% and between-assay variation 5, 8 and 3.5%. The glucagon assay (developed in J. Holst's laboratory in Copenhagen, Denmark) is highly specific for the free C terminus of the molecule, and therefore specific for pancreatic glucagon, with the following assay characteristics: sensitivity < 1 pmol/l, within-assay CV $< 5\%$ at 20 pmol/l, between-assay CV $< 12\%$. Serum adiponectin was assayed by a novel, in-house time-resolved immunofluorimetric method, previously described in detail [15], which measures total circulating adiponectin (including high- and low-molecular-mass isoforms).

Data analysis Glucose tolerance was categorised into normal, impaired fasting glycaemia (IFG) and IGT according to the American Diabetes Association criteria [16]. IFG and IGT were grouped together as IGR ($n=152$ or 11.7% of the total cohort). Insulin sensitivity was expressed as the ratio of the M value, averaged over the final 40 min of the 2 h clamp and normalised for fat-free mass (FFM; measured by electrical bioimpedance [15]), to the mean plasma insulin concentration measured during the same interval [M/I , in units of $\mu\text{mol min}^{-1} \text{kg}_{\text{FFM}}^{-1} (\text{nmol/l})^{-1}$] [17].

Statistical analysis Data are reported as means \pm SD. Variables (BMI, M/I , hormone concentrations) with a skewed distribution (by a Shapiro–Wilk W test) are given as median and interquartile range and were logarithmically transformed for use in statistical testing. Association between two variables was tested by Spearman rank correlation ρ value. Differences across groups were tested by a Mann–Whitney or Kruskal–Wallis test. General linear models were used to test the simultaneous dependence of continuous variables on multiple parameters; results are presented as the partial regression coefficient. When multiple logistic regression was applied, results are given as odds ratio (OR) and 95% CIs. All multivariate models were adjusted for centre. Statistical analyses were carried out using JMP Version 3.1 (SAS Institute, Cary, NC, USA).

Results

Insulin sensitivity (as M/I) was higher in women than men [145 (interquartile range 82) vs 112 (70) $\mu\text{mol min}^{-1}$

$\text{kg}_{\text{FFM}}^{-1} (\text{pmol/l})^{-1}$, $p<0.0001$] as were fasting adiponectin concentrations [9.3 (4.9) vs 6.0 (3.1) mg/l , $p<0.0001$]. In contrast, fasting serum glucagon [9.0 (5.0) vs 7.0 (3.0) pmol/l] and proinsulin concentrations [6.0 (5.0) vs 5.0 (3.0) pmol/l] were higher in men than women ($p<0.0001$ for both). Glucagon [9.0 (6.0) vs 8.0 (4.0) pmol/l , $p<0.004$] and proinsulin [8.0 (7.0) vs 5.0 (4.0) pmol/l , $p<0.0001$] levels were also higher, and adiponectin was lower [6.3 (4.0) vs 7.9 (4.9) mg/l , $p<0.0001$], in IGR (i.e. IFG or IGT) individuals, who were also less insulin sensitive than NGT individuals [93 (73) vs 133 (86) $\mu\text{mol min}^{-1} \text{kg}_{\text{FFM}}^{-1} (\text{pmol/l})^{-1}$, $p<0.0001$].

In either sex, glucagon and proinsulin increased across sex-specific quartiles of IR, whereas adiponectin displayed the opposite trend ($p\leq 0.02$ for all; Fig. 1). In univariate analysis using continuous variables, both glucagon and proinsulin were negatively related to M/I , while adiponectin was positively associated with it (all $p<0.0001$; Table 1).

To rule out that these associations might be due to confounding, determinants of insulin sensitivity (i.e. sex, age, BMI and glucose tolerance status) as well as factors potentially affecting glucagon and proinsulin (i.e. fasting plasma glucose and C-peptide concentrations) were entered as covariates into multivariate models of the associations between insulin sensitivity and hormone levels. As shown by the partial correlation coefficients in Table 1, glucagon and proinsulin were still negatively associated, and adiponectin was positively associated, with M/I . From the multiple regression equation, fasting glucagon in an individual in the top quartile of IR is estimated to be ~15% higher than in an individual in the bottom quartile of IR. When the proinsulin:C-peptide ratio was used as the dependent variable (and fasting C-peptide concentration was removed as an

Fig. 1 Fasting concentrations of glucagon, proinsulin, adiponectin and insulin sensitivity (M/I) in 1,296 non-diabetic men (green) and women (red) stratified by sex-specific quartile of insulin sensitivity. Boxes are median, SD and 95% CIs

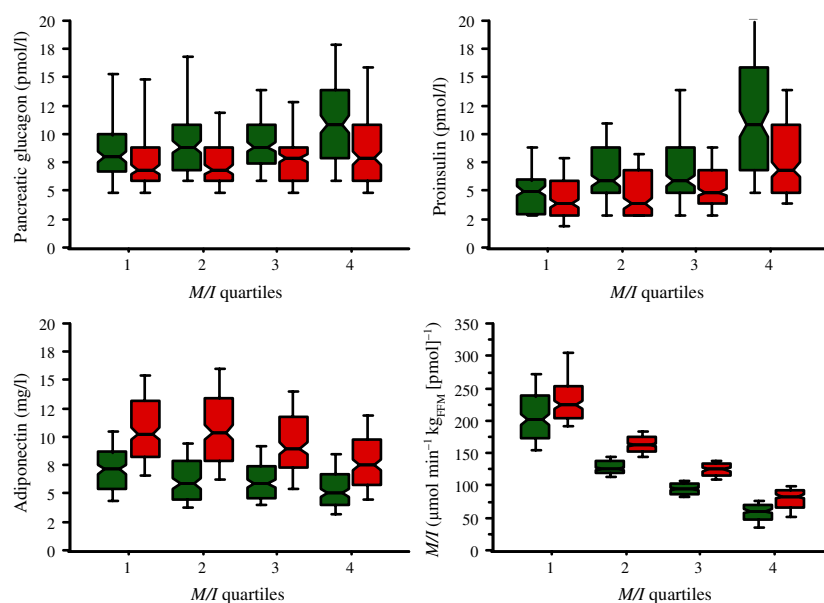


Table 1 Associations of insulin sensitivity (*M/I*) with fasting hormone levels

	Glucagon	Proinsulin	Proinsulin: C-peptide	Adiponectin
Univariate association ^a	-0.22	-0.44	-0.15	0.38
Multivariate association ^b	-0.05	-0.23	-0.12	0.19
Total explained variance	0.43	0.49	0.13	0.39

^a Spearman ρ ^b Partial *r* adjusted for centre, sex, age, BMI, fasting glucose and glucose tolerance

independent variable), the reciprocal relationship with *M/I* was still statistically significant.

These associations were also tested in terms of the probability that individuals in the top sex-specific quartile of IR (cf. Fig. 1) would have hormone levels in the top sex-specific quartile of their distribution. The results show that insulin-resistant individuals were more likely than more sensitive individuals to have raised glucagon and proinsulin concentrations [with an OR of ~ 2 for both glucagon ($p=0.02$) and proinsulin ($p=0.05$)] and lower adiponectin levels (OR of 0.36, $p<0.0001$) independently of co-variables (Fig. 2). Of interest, obesity (as the BMI) showed significant associations with relative hyperglucagonaemia, hyperproinsulinaemia and hypo adiponectinaemia as IR but independently of it.

Finally, individuals in the top quartile of fasting glucagon concentrations had a high chance of also having proinsulin concentrations in the top quartile ($\chi^2=14.5$, $p<0.0001$).

Discussion

The present analysis demonstrates that in a non-diabetic population IR is associated with raised fasting plasma pancreatic glucagon and proinsulin concentrations. Adiponectin, which was used as a positive control, showed the expected reciprocal association with IR. These associations were independent of factors, such as sex, age, BMI and glucose tolerance, known to affect insulin action. Also, the multivariate statistical models included fasting glucose and C-peptide concentrations: the former to account for the tonic influence of glucose on both insulin and glucagon release, the latter to adjust proinsulin and glucagon levels for the concomitant rate of insulin secretion. Finally, the relationships were statistically significant whether using continuous or categorical variables to best control for the non-normal distribution of most variables.

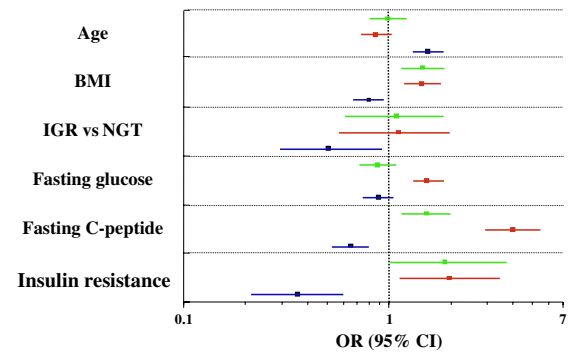


Fig. 2 Multivariate ORs for the association of IR (bottom sex-specific quartile of *M/I*) with fasting concentrations of plasma glucagon (green), proinsulin (red) and adiponectin (blue) in the respective top quartile of their distribution. ORs of continuous co-variables are calculated for 1 SD of the entire cohort

The physiological implication of these findings may be that IR extends to both alpha cells and beta cells in the islets of Langerhans. In alpha cells, IR translates into a reduced tonic inhibition of glucagon release, resulting in inappropriately (for the concomitant glucose levels) raised glucagon concentrations. In beta cells, IR translates into defective proinsulin to insulin secretory coupling, resulting in inappropriately (for the concomitant C-peptide and glucose levels) raised proinsulin concentrations.

In isolated rat pancreatic alpha cells, glucose, like arginine and tolbutamide, stimulates glucagon release by closing the K_{ATP} channel, an effect that is abolished by the K_{ATP} -channel opener, diazoxide, by N-type Ca^{2+} -channel blockers or by inhibitors of glycolysis and mitochondrial metabolism [18]. Therefore, the suppressive effect of glucose on glucagon secretion in intact islets and in vivo results entirely from paracrine signalling; as alpha cells are rich in insulin receptors, insulin is the foremost paracrine candidate (somatostatin being another potentially important mediator). The cellular mechanisms by which insulin exerts a tonic inhibition on alpha cell function involve, but are not limited to, activation of K_{ATP} channels [19]. Our finding of an independent association of IR with relative fasting hyperglucagonaemia in non-diabetic individuals implies such a physiological control mechanism, but does not rule out that the feedback may be mediated by changes in circulating nutrients. For example, IR is associated with raised plasma concentrations of NEFAs and branched-chain amino acids [20]. In the current database, NEFAs were not an independent correlate of plasma glucagon (data not shown), but aminoacidaemia was not measured. Also, the association of glucagon levels with IR might stand for an effect of glucagon on insulin sensitivity rather than the other way around. Although in human studies glucagon has not

been shown to interfere with insulin-mediated glucose uptake [21], recent experiments in dogs have shown that chronic hyperglucagonaemia impairs both hepatic and peripheral tissue glucose uptake [22].

The above considerations largely apply also to the observed association between IR and hyperproinsulinaemia. The cellular basis of this association is equally robust. Mice with beta cell-specific knockout of insulin receptors show a loss of first-phase insulin release reminiscent of typical human type 2 diabetes [23], and lack of functional insulin and IGF-1 receptors in beta cells leads to age-related reduction in beta cell mass, severe beta cell dysfunction and overt diabetes [24]. Together, genetic and physiological data provide evidence that insulin signalling in beta cells is important for competent beta cell functioning.

Of further interest is the independent association of obesity (as the BMI) with raised fasting glucagon and proinsulin concentrations after controlling for both IR and fasting C-peptide (Fig. 2). This previously unrecognised finding suggests that obesity per se imposes a secretory strain on the islet that is independent of the adaptive response to IR. We have previously shown that the insulin hypersecretion of obesity is only in part due to the concomitant IR: for the greater part, it is primary and reverses with weight loss [25, 26]. The current data are compatible with the view that such a stressful effect of obesity involves alpha cells as well as beta cells [12], and may underlie the strong predictivity of obesity for incident diabetes [27].

In summary, whole-body IR is independently associated with raised fasting plasma glucagon and proinsulin concentrations, possibly as a result of IR at the level of alpha cells and beta cells in pancreatic islets.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Reaven GM, Chen YD, Golay A, Swislocki AL, Jaspan JB (1987) Documentation of hyperglucagonemia throughout the day in nonobese and obese patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 64:106–110
2. Aronoff SL, Bennett PH, Unger RH (1977) Immunoreactive glucagon (IRG) responses to intravenous glucose in prediabetes and diabetes among Pima Indians and normal Caucasians. *J Clin Endocrinol Metab* 44:968–972
3. Mitrakou A, Kelley D, Mokan M et al (1992) Role of reduced suppression of glucose production and diminished early insulin release in impaired glucose tolerance. *N Engl J Med* 326:22–29
4. Cherrington AD (1997) Banting Lecture 1997. Control of glucose uptake and release by the liver in vivo. *Diabetes* 48:1198–1214
5. Ahrén B, Larsson H (2000) Islet dysfunction in insulin resistance involves impaired insulin secretion and increased glucagon secretion in postmenopausal women with impaired glucose tolerance. *Diabetes Care* 23:650–657
6. Ahrén B (2006) Glucagon secretion in relation to insulin sensitivity in healthy subjects. *Diabetologia* 49:117–122
7. Mako ME, Starr JI, Rubenstein AH (1977) Circulating proinsulin in patients with maturity onset diabetes. *Am J Med* 63:865–869
8. Yoshioka N, Kuzuya T, Matsuda A, Taniguchi M, Iwamoto Y (1988) Serum proinsulin levels at fasting and after oral glucose load in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 31:355–360
9. Porte D Jr, Kahn SE (1989) Hyperproinsulinemia and amyloid in NIDDM. Clues to etiology of islet beta cell dysfunction? *Diabetes* 38:1333–1336
10. Pradhan AD, Manson JE, Meigs JB et al (2003) Insulin, proinsulin, proinsulin:insulin ratio, and the risk of developing type 2 diabetes mellitus in women. *Am J Med* 114:438–444
11. Hanley AJ, D'Agostino R Jr, Wagenknecht LE, Saad MF, Savage PJ, Bergman R, Haffner SM; Insulin Resistance Atherosclerosis Study (2002) Increased proinsulin levels and decreased acute insulin response independently predict the incidence of type 2 diabetes in the insulin resistance atherosclerosis study. *Diabetes* 51:1263–1270
12. Rhodes CJ, Alarcon C (1994) What beta cell defect could lead to hyperproinsulinemia in NIDDM? Some clues from recent advances made in understanding the proinsulin processing mechanism. *Diabetes* 43:511–517
13. Alarcon C, Leahy JL, Schupp GT, Rhodes CJ (1995) Increased secretory demand rather than a defect in the proinsulin conversion mechanism causes hyperproinsulinemia in a glucose-infusion rat model of non-insulin-dependent diabetes mellitus. *J Clin Invest* 95:1032–1039
14. Hills SA, Balkau B, Coppack SW et al (2004) The EGIR-RISC study (The European Group for the Study of Insulin Resistance: Relationship between Insulin Sensitivity and Cardiovascular Disease Risk): I. Methodology and objectives. *Diabetologia* 47: 566–570
15. Frystyk J, Tarnow L, Hansen TK, Parving HH, Flyvbjerg A (2005) Increased serum adiponectin levels in type 1 diabetic patients with microvascular complications. *Diabetologia* 48:1911–1918
16. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2000) *Diabetes Care* 23(Suppl 1):S4–S19
17. Ferrannini E, Mari A (1998) How to measure insulin sensitivity. *J Hypertens* 16:895–906
18. Olsen HL, Theander S, Bokvist K, Buschard K, Wollheim CB, Gromada J (2005) Glucose stimulates glucagon release in single rat alpha-cells by mechanisms that mirror the stimulus-secretion coupling in beta-cells. *Endocrinology* 146:4861–4870
19. Gromada J, Franklin I, Wollheim CB (2007) Alpha cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocr Rev* 28:84–116
20. Groop LC, Ferrannini E (1993) Insulin action and substrate competition. *Baillieres Clin Endocrinol Metab* 7:1007–1032
21. Ferrannini E, DeFronzo RA, Sherwin RS (1982) Transient hepatic response to glucagon in man: role of insulin and hyperglycemia. *Am J Physiol* 242:E73–E81
22. Chen SS, Zhang Y, Santomango TS, Williams PE, Lacy DB, McGuinness OP (2007) Glucagon chronically impairs hepatic

- and muscle glucose uptake. *Am J Physiol Endocrinol Metab* 292:E928–E935
23. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR (1999) Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that of type 2 diabetes. *Cell* 96:329–339
 24. Ueki K, Okada T, Hu J, Liew CW et al (2006) Total insulin and IGF-1 resistance in pancreatic β cells caused overt diabetes. *Nat Gen* 38:583–588
 25. Ferrannini E, Natali A, Bell P, Cavallo-Perin P, Lalic N, Mingrone G (1997) Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). *J Clin Invest* 100:1166–1173
 26. Ferrannini E, Camastra S, Gastaldelli A et al (2004) Beta-cell function in obesity: effects of weight loss. *Diabetes* 53(Suppl 3):S26–S33
 27. Rana JS, Li TY, Manson JE, Hu FB (2007) Adiposity compared with physical inactivity and risk of type 2 diabetes in women. *Diabetes Care* 30:53–58