Glucagon secretion in relation to insulin sensitivity in healthy subjects

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Short title: Glucagon secretion and insulin sensitivity

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Abstract

Aims/hypothesis: The study evaluated whether glucagon secretion is regulated by changes in insulin sensitivity under normal conditions. *Methods:* A total of 155 healthy women with normal glucose tolerance (aged 53-70 years) underwent a glucose-dependent arginine stimulation test for evaluation of glucagon secretion. Arginine (5g) was injected iv under fasting conditions (plasma glucose 4.8±0.1 mmol/l) and after raising the glucose to 14.8±0.1 mmol/l and 29.8±0.2 mmol/l. The acute glucagon response to arginine during the three glucose levels were estimasted (AGR_1, AGR_2, AGR_3) as was the suppression of baseline glucagon by raising glucose. All women also underwent a 2 h euglycemic hyperinsulinemic clamp study for estimation of insulin sensitivity. *Results:* Insulin sensitivity displayed a normal distribution with a mean of 73.2±29.3 (SD) nmol glucose kg^{-1} min^{-1}/pmol insulin l^{-1}. When relating the variables obtained from the arginine test to insulin sensitivity, insulin resistance was associated with 1)increased AGR and 2)increased suppression of glucagon levels by glucose. For example, the regression between insulin sensitivity and AGR_2 was r=-0.38 ($P<0.001$) and between insulin sensitivity and suppression of glucagon levels by 14.8 mmol/l glucose was r=0.36 ($P<0.001$). Insulin sensitivity also correlated negatively to insulin secretion; multivariate analysis revealed that changes in insulin sensitivity and insulin secretion were independently related to changes in glucagon secretion.

Conclusions/interpretation: Insulin resistance is adapted by increasing the glucagon response to arginine and by increasing the suppression of glucagon levels by glucose. Hence, not only the islet beta cells but also the alpha cells seem to undergo compensatory changes during development of insulin resistance.

Key words: Arginine, glucagon, glucose tolerance, insulin sensitivity, glucose tolerance

Abbreviations: AGR=acute glucagon response, AIR = acute insulin response, BMI = body mass index, IGT = impaired glucose tolerance,

Introduction

It is known that type 2 diabetes is associated with insulin resistance and defective insulin secretion [1-3]. It is, however, also known that type 2 diabetes is associated with elevated levels of glucagon [4-6] and defective suppression of glucagon secretion [4,7,8]. Also impaired glucose tolerance (IGT) is associated with impaired suppression of glucagon secretion [9-11]. The relative hyperglucagonemia is most likely of importance for the postprandial hyperglycaemia in diabetes due to increased hepatic glucose production [5,6,12]. In fact, Shah and collaborators have demonstrated that also in the absence of insulin resistance, glucose tolerance is critically dependent on a normal suppression of glucagon secretion [13]. Hence, defective regulation of glucagon secretion is in addition to defective insulin secretion, a hallmark of the islet dysfunction accompanying type 2 diabetes.

The defective regulation of glucagon secretion in type 2 diabetes, which is associated with insulin resistance, raises the issue of the relation between glucagon secretion and insulin resistance *per se*. It is well known that insulin resistance is adaptively accompanied by increased insulin secretion [for review see 3]. In a previous study we observed that also glucagon secretion is inversely related to insulin sensitivity [10]. Whether insulin resistance is adapted by increased glucagon secretion or defective suppression of glucagon secretion was, however, not possible to conclude from the results. In the present study, we have therefore examined the relation between insulin sensitivity versus basal and stimulated glucagon levels and suppression of glucagon secretion by glucose in a large number of women with normal glucose tolerance.

Subjects and methods

Subjects. A total of 155 women with normal glucose tolerance were included in the study. They were recruited from a health screening study in Malmö, Sweden, on the basis of a normal glucose tolerance [14]. All subjects were healthy and none was taking any medication known to affect carbohydrate metabolism. Thirty-four women were taking estrogens. All women underwent a standardized 75g 2h oral glucose tolerance test. The test was undertaken after an overnight fast and the subjects spent the 2h in a semirecumbent position. All subjects had normal values of fasting and 2h glucose (Table 1).

Glucagon secretion. Glucagon secretion was determined with the intravenous arginine stimulation at three glucose levels (fasting, 14 and >25 mmol/l) as previously described [15,16]. After an overnight fast, intravenous catheters were inserted into antecubital veins in both arms. One arm was used for the infusion of glucose and the other arm for intermittent sampling. The sampling catheter was kept patent by slow infusion of 0.9% saline when not in use. Baseline samples were taken at –5 and –2 min. A maximally stimulating dose of arginine hydrochloride (5g) was then injected intravenously over 45s. Samples were taken at 2, 3, and 5 min. A variable-rate 20% glucose infusion was then initiated to raise and maintain blood glucose at 13-15 mmol/l. Blood glucose was determined every 5 min bedside and the glucose infusion adjusted to reach the desired blood glucose level in 20-25 min. New baseline samples were taken, then arginine (5g) was again injected and new 2, 3, 4 and 5 min samples were taken. A 2.5-h resting period was then allowed, whereafter new baseline samples were obtained and a high-speed (900 ml/h) 20% glucose infusion during 25-30 min was used to raise blood glucose to >25 mmol/l, as determined bedside. At this blood glucose level, new baseline samples were taken and arginine (5g) injected followed by final 2, 3, 4 and 5 min samples.

Insulin sensitivity. Insulin sensitivity was determined with the euglycemic-hyperinsulinemic clamp [17]. After an overnight fast, intravenous catheters were inserted into antecubital veins in both arms. One arm was used for infusion of glucose and insulin. The contralateral arm was used for intermittent sampling, and the catheter was kept patent with slow infusion of 0.9% saline. A primed constant infusion of insulin (Actrapid^{R} 100U/ml, Novo Nordisk, Bagsvaerd, Denmark) with a constant infusion rate of 0.28 nmol m^{-2} body surface min^{-1} was started. After 4 min, a variable rate 20% glucose infusion was added and its infusion rate was adjusted manually throughout the clamp procedure to maintain the blood glucose level at 5.0 mmol/l. Blood glucose was determined every 5 min. Samples for analysis of the achieved insulin concentrations were taken at 60 and 120 min.

Analyses. Blood glucose concentration was determined bedside by the glucose dehydrogenase technique with a Hemocue (Hemocue AB, Ängelholm, Sweden) during the euglycemic-hyerpinsulinemic clamp and with an Accutrend (Boehringer Mannheim Scandinavia AB, Bromma, Sweden) during the arginine test. Blood samples for glucagon, insulin and glucose from the arginine study and for insulin from the clamp study were immediately frozen and serum or plasma frozen at -20°C. Serum glucagon and insulin were analyzed with double-antibody radioimmunoassay technique. Samples for analysis of glucagon were obtained in prechilled test tubes containing 0.084ml ethylene-diamine-tetraacetate (0.34 mol/l) and aprotinin (250 kallikrein inhibiting U/ml blood, Bayer AG, Leverkusen, Germany). For the assay, guinea pig anti-human glucagon antibodies specific for pancreatic glucagon, ^{125}I-glucagon as tracer, and glucagon standard were used (Linco Res., St Charles, Mo, USA). For insulin assay, guinea pig anti-human insulin antibodies, mono-^{125}I-tyr-human insulin and

human insulin standard were used (Linco). Plasma glucose concentrations were analyzed using the glucose oxidase method. All samples were analysed in duplicate.

Calculations and statistics. Data are presented as mean±SEM unless otherwise stated. For the determination of glucagon secretion, the acute glucagon response to arginine was determined as the mean of the 2 to 5-min samples minus the mean prestimulus glucagon concentration at fasting (AGR_1), at 13-15 mmol/l (AGR_2) and at >28 mmol/l glucose (AGR_3). The slope between AGR_1 and AGR_2 was calculated as a measure of the alpha-cell sensitivity of glucose to suppress arginine-stimulated glucagon secretion. Also the maximal AGR suppression was calculated (AGR_3 minus AGR_1) as was the suppression by glucose of basal glucagon levels at 13-15 mmol/l glucose (baseline $suppression_1$) and at >28 mmol/l (baseline $suppression_2$). Finally, the rapidity of the glucagon response to arginine was calculated as the increase in glucagon levels at min 2 after arginine in percent of the maximal increase at fasting ($rapidity_1$), 13-15 mmol/l glucose ($rapidity_2$) and at >28 mmol/l glucose ($rapidity_3$). Conversely, for insulin levels, the acute insulin response to arginine at fasting glucose (AIR_1), at 13-15 mmol/l glucose (AIR_2) and at >28 mmol/l glucose (AIR_3) were determined. Finally, for the calculation of insulin sensitivity, a steady-state condition was assumed during the second h of the clamp. Insulin sensitivity was calculated as the glucose infusion rate per kg body weight during the second hour divided by the mean of the insulin levels at 60 and 120 min during the clamp (i.e., nmol glucose kg body $weight^{-1}$ min^{-1}/pmol insulin l^{-1}). Statistical analyses were performed with the SPSS for Windows system (Chicago, Il, USA). Differences in responses to arginine at different glucose levels were estimated by paired t-test. Pearson´s product moment correlation coefficients were obtained to estimate linear correlation among variables. Linear multiple regression was used to assess the indepndent effect of several variables; the stepwise forward method was used.

Results

Study group characteristics. Table 1 shows the characteristics of the 155 women participating in the study. They were all healthy and had normal values of fasting and 2h glucose during an OGTT.

Glucose-dependent arginine-stimulation test. When arginine was injected iv after an overnight fast (plasma glucose 4.8±0.1 mmol/l) there was a sharp and rapid increase in circulating glucagon in all subjects with the peak observed after 3 min, the AGR_1 being 102±3.6 ng/l (Fig. 1). After the first arginine administration, glucose was administered intravenously which raised the glucose level to 15.2±0.2 mmol/l. This reduced baseline glucagon from 64±1.4 to 48±1.4 ng/l ($P<0.001$). A new arginine administration resulted again in a rapid and marked increase in circulating glucagon, again with peak level at 3 min after administration. However, the AGR_2 was only 55.4±2.1 ng/l, which was a reduction from AGR_1 by 44.2±1.5% ($P<0.001$). Finally, glucose was administered again, which raised plasma glucose to 29.8±0.3 mmol/l. This reduced further baseline glucagon to 39.3±1.1 ng/l. When arginine was injected at this higher glucose, there was again an increase in circulating glucagon, although the increase was slower than at lower glucose levels. Thus, the increase in glucagon levels peaked at 4 min after administration. AGR_3 was only 39.2±1.6 ng/l, which was equivalent to a reduction by 60.0±1.4% of AGR_1 ($P<0.001$). Fig. 1 also shows the AGRs and AIRs as functions of the plasma glucose levels. Table 2 displays the glucagon secretory characteristics of glucose-dependent arginine-stimulation test, i.e., shows how glucagon secretion responds to raise in circulating glucose in subjects with normal glucose tolerance. It is seen that glucose suppresses both baseline glucagon levels and the glucagon response to arginine. Futhermore, also the rapidity of the glucagon response to arginine was suppressed by glucose, i.e., the time from arginine injection to peak of glucagon. Hence, both the baseline

and stimulated glucagon secretion as well as the rapidity of the responsiveness of glucagon secreton are suppressed by glucose.

Relation between glucagon secretion and insulin sensitivity. In all women, a euglycemic hyperinsulinemic clamp was undertaken to establish the insulin sensitivity. It was found (Fig. 2) that insulin sensitivity displayed a normal distribution and a broad range. To explore whether insulin sensitivity correlated to the glucagon responses during the arginine stimulation test, linear correlation was undertaken between the M/I value during the clamp with the variables obtained from the arginine test (Table 2). It was found that there was a significant and negative correlation between insulin sensitivity and AGR (AGR_1, AGR_2 as well as AGR_3), showing that the highest glucagon response to arginine was seen in the most insulin resistant subjects. Also the reduction in baseline glucagon by raising the glucose levels correlated negatively with insulin sensitivity, showing that subjects with insulin resistance were those that exhibited the strongest suppressibility of baseline glucagon to glucose. In contrast, the slope of AGR or the rapidity of the glucagon response to arginine seemed uninfluenced by insulin sensitivity. To illustrate the relation between variables with insulin sensitivity, the subjects were divided into quartiles of insulin sensitivity. Table 3 shows fasting glucose and insulin as well as BMI in these quartiles; as expected the most insulin resistant subjects had higher insulin levels and higher BMI; however, it should be emphasized that all subjects had normal glucose tolerance with normal fasting glucose. Fig. 2 shows that fasting glucagon, the AGR_2 and the suppression of baseline glucagon levels when raising glucose to 14 mmol/l (baseline $suppression_1$) displayed a progressive increase along a reducing insulin sensitivity.

Relation of changes in glucagon secretion to insulin secretion. Two adaptive processes in glucagon secretion were thus evident in relation to changes in insulin sensitivity: an increased glucagon response to arginine and an increased suppression of baseline glucagon to glucose along reduced insulin sensitivity. To examine whether these two processes are dependent on changes in insulin secretion, multivariate analysis was undertaken. The results were similar when introducing the islet hormone responses from either first, second or third arginine challenge; to avoid repeating data only the results from the second arginine bolus are reported here. In a first analysis, the AGR_2 was used as the dependent variable and insulin sensitivity and AIR_2 (i.e., the insulin response to arginine at 15 mmol/l glucose, which is a parameter also increased by insulin resistance) were used as independent variables. It was found that both these variables independently contributed to the AGR_2, with a combined r-value of 0.48 ($P<0.001$). Similarly, when suppression of baseline glucagon by glucose at 13-15 mmol/l (baseline $suppression_1$) was used as the dependent variable and insulin sensitivity and AIR_2 were used as independent variables, it was found that both these variables independently contributed to the suppression of glucagon by glucose with a combined r-value of 0.45 ($P<0.001$). In contrast, adding also fasting (or 2h) glucose to the models did not change these conclusions.

Relation of changes in glucagon secretion to glucose, BMI and age. The 2h glucose value correlated to AGR (correlation to AGR_2 $r=0.36$; $P<0.001$) as well as to suppression of glucagon by glucose at 13-15 mmol/l (baseline suppression; $r=0.21$; $P=0.012$). Also BMI correlated to AGR (to AGR_2 $r=0.21$; $P=0.028$), but not to glucagon suppression. However, neither 2h glucose or BMI correlated to AGR or glucagon suppression in a model where also insulin sensitivity was introduced. It should be emphasized, however, that all subjects were normal glucose tolerant and therefore that the variation in 2h glucose was small. Age did not correlate to glucagon secretion.

Discussion

It has been documented that insulin resistance is adapted by a compensatory increase in insulin secretion [3,18-21]. This concept is of important for the understanding of the key role of the islet beta cells for the development of IGT and type 2 diabetes: if the beta cell compensation to insulin resistance fails, glucose homeostasis deranges which will result in impaired glucose tolerance and type 2 diabetes [3]. We, and other, have, however, demonstrated that also glucagon secretion is important for the development of IGT and type 2 diabetes, in view of the hyperglucagonaemia and increased glucagon secretion which accompany these conditions [4-12]. This raises the issue of the relation between insulin resistance and glucagon secretion under normal conditions. Is there, like for insulin secretion, a compensation in glucagon secretion in insulin resistance, and may failure of the alpha cells to adapt to insulin resistance be a mechanism of glucose intolerance?

In this study we have examined the glucagon secretion as judged from the glucose-dependent arginine stimulation test in relation to insulin sensitivity as judged from the euglycemic hyperinsulinemic clamp technique in a large number of healthy women with normal glucose tolerance. The glucose-dependent arginine-stimulation test was initially developed for the careful analysis of beta cell function since it displays both the basal and maximal insulin secretion as well as glucose sensitivity of the beta cells [15,16]. However, since arginine stimulates glucagon secretion as well and since glucose suppresses alpha cell secretion the test is useful also for characterization of alpha cell function. Indeed, we have previously used this test to show that increased glucagon secretion contributes to future worsening of glucose intolerance and that this is seen independent from changes in insulin secretion [22]. A potential limitation is that changes in glucagon secretion are inferred from changes in glucagon levels. However, it is unlikely that the large and rapid changes in glucagon levels

seen after challenging with glucose and arginine would be secondary to other processes than glucagon secretion, such as glucagon disposal.

We show here that glucose suppresses glucagon levels and that arginine markedly stimulates glucagon secretion by an effect which is suppressed by raising the glucose level; both the absolute increase in glucagon levels by arginine and the rapidity by which arginine stimulated glucagon secretion were reduced by glucose. The main findings of this study were, however, that two processes in alpha cell function are different when comparing subjects with insulin resistance versus those with high insulin sensivity: first there is an increased glucagon response to arginine and second there is an increased suppression of baseline glucagon by glucose in insulin resistance. The finding of insulin resistance is associated with increased glucagon response to arginine confirms a previous study (10). That study showed an inverse relation between the glucagon respnse to arginine in relation to insulin sensitivity with an r-value of 0.5. In the present study, it was also demonstrated that insulin resistance is associated with augmented glucagon suppression by glucose, i.e., the direct action of glucose, not only arginine, on glucagon secretion seems to be perturbed by insulin resistance.

It should be emphasized that the women studied here all had normal glucose tolerance, and therefore that the changes in glucagon secretion reported here should not be regarded as secondary to changes in glycaemia. Instead, the changes should be regarded as being adaptations to development of insulin resistance. Hence, islet compensation to insulin resistance does not involve insulin secretion only but also changes in glucagon secretion. The mechanisms of the alpha cell adaptation to insulin resistance remains to be established. Several adaptive responses to insulin resistance might be possible to mediate the responses. These potential mediators include free fatty acids as well as adipokines released from

adipocytes in relation to insulin sensitivity as well as neural reflexes activated by insulin resistance and signalling to the islets, in analogy to discussions on mechanisms of changes in insulin secretion during insulin resistance [23]. One intriguing aspect is also whether the changes in glucagon secretion are secondary to insulin resistance in the glucagon producing alpha cells. It is known that insulin inhibits glucagon secretion [24,25] and, therefore, resistance in the alpha cells to insulin would augment glucagon secretion [26], which would explain the higher glucagon response to arginine in the arginine test. On the other hand, insulin secretion is increased in insulin resistance, which would counterbalance a defective insulin action also in the alpha cells. Also, the augmented suppression by glucose would not be explained by such a mechanism. Interestingly, subjects with IGT and type 2 diabetes have reduced suppression by glucose of glucagon secretion [4-12]. Whether failure of the augmenting action of glucose to suppress glucagon levels in insulin resistance represents the mechanism initiating IGT remains to be studied.

In conclusion, this study has demonstrated that in healthy subjects with normal glucose tolerance, insulin resistance is associated with increased fasting glucagon levels, augmented glucagon response to arginine and an augmented suppression by glucose of glucagon levels. This suggests that islet adaptation to insulin resistance involves not only increased insulin secretion but also changes in glucagon secretion.

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Table 1. Characteristics of the 155 women participating in the study

Variable (unit)	Mean±SD	Range
Age (years)	62±2	53-70
Body weight (kg)	67.0±10.0	44-102
BMI (kg/m^2)	25.7±3.7	17.0-35.3
Fasting glucose (mmol/l)	4.8±0.5	3.7-5.7
2h glucose (mmol/l)	5.9±1.0	4.6-6.4
Fasting insulin (pmol/l)	71±31	16-301
Fasting glucagon (ng/l)	64±22	20-143
Insulin sensitivity (nmol glucose kg^{-1} min^{-1}/pmol insulin l^{-1})	73.2±29.3	5.2-147.9

Table 2 Estimates of glucagon values during the glucose-dependent arginine stimulation test in subjects with normal glucose tolerance (n=155) and the correlation of the estimates to insulin sensitivity (M/I) as determined by the euglycemic hyperinsulinemic clamp. Means±SEM are shown; P value indicate probability level of random estimate of regression.

	Means±SEM	Correlation to insulin sensitivity (r-value)
AGR_1 (ng/l)	102±3.6	-0.28 (P<0.001)
AGR_2 (ng/l)	55.4±2.1	-0.38 (P<0.001)
AGR_3 (ng/l)	39.3±1.1	-0.31 (P<0.001)
$Slope_{AGR}$ (ng glucagon/mmol glucose)	-4.9±0.3	0.088 (ns; P=0.296)
Maximal suppression of AGR (ng/l)	63.3±2.7	0.181 (P=0.031)
Peak response to $arginine_1$ (ng/l)	137±3.8	-0.31 (P<0.001)
Peak response to $arginine_2$ (ng/l)	89.9±2.7	-0.41 (P<0.001)
Peak response to $arginine_3$ (ng/l)	64.1±1.8	-0.32 (P<0.001)
Baseline $glucagon_1$ (ng/l)	64.7±1.8	-0.36 (P<0.001)
Baseline $glucagon_2$ (ng/l)	45.1±1.4	-0.27 (P<0.001)
Baseline $glucagon_3$ (ng/l)	35.5±1.4	-0.22 (P=0.008)
Suppression of baseline $glucagon_2$ (ng/l)	15.7±0.8	0.36 (P<0.001)
Suppression of baseline $glucagon_3$ (ng/l)	25.7±0.9	0.42 (P<0001)
Rapidity of glucagon $response_1$ (%)	108±11	-0.034 (ns; P=0.681)
Rapidity of glucagon $response_2$ (%)	55±11	-0.19 (P=0.021)
Rapidity of glucagon $response_3$ (%)	40±13	-0.121 (ns; P=0.151)

Table 3 Fasting glucose and insulin and BMI as well as insulin sensitivity in four subgroups of the study population being quartiles of insulin sensitivity. Means±SD are shown.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Number of subjects	39	39	38	39
Fasting glucose (mmol/l)	5.2±0.9	5.0±0.8	4.9±0.8	4.9±0.6
Fasting insulin (pmol/l)	105±36	62±20	58±18	56±18
BMI (kg/m^2)	28.8±3.5	26.1±2.6	23.9±2.7	24.0±3.3
Insulin sensitivity (nmol glucose kg^{-1} min^{-1}/pmol insulin l^{-1})	37.7 ±12.0	63.1±6.0	82.6±6.5	110.2±16.8

Legends to the figures

Fig. 1 Glucagon levels before and after intravenous administration of arginine (5g) under fasting conditions (plasma glucose 4.8±0.1 mmol/l; upper left panel) or after raising the glucose levels to 15.2±0.2 mmol/l (upper middle panel) or 29.8±0.3 mmol/l (upper right panel) in 155 healthy women with normal glucose tolerance. Lower panels show the estimated acute glucagon response (AGR; left panel) or acute insulin response (AIR; right panel) to arginine versus the glucose levels during the glucose-dependent arginine stimulation test. Means±SEM are shown.

Fig. 2 Left upper panel: distribution of insulin sensitivity as determined by the euglycemic hyperinsulinemic clamp in the 155 women with normal glucose tolerance. Other panels show fasting glucagon (upper right panel), the acute glucagon response to iv arginine (5g) after raising the glucose level to 15.2±0.2 mmol/l (AGR_2; lower left panel) and the suppression of baseline glucagon after raising the glucose level to 15.2±0.2 mmol/l (lower right panel) versus insulin sensitivity in the subjects divided in quartiles of insulin sensitivity. Means±bidirectional SEM are shown.

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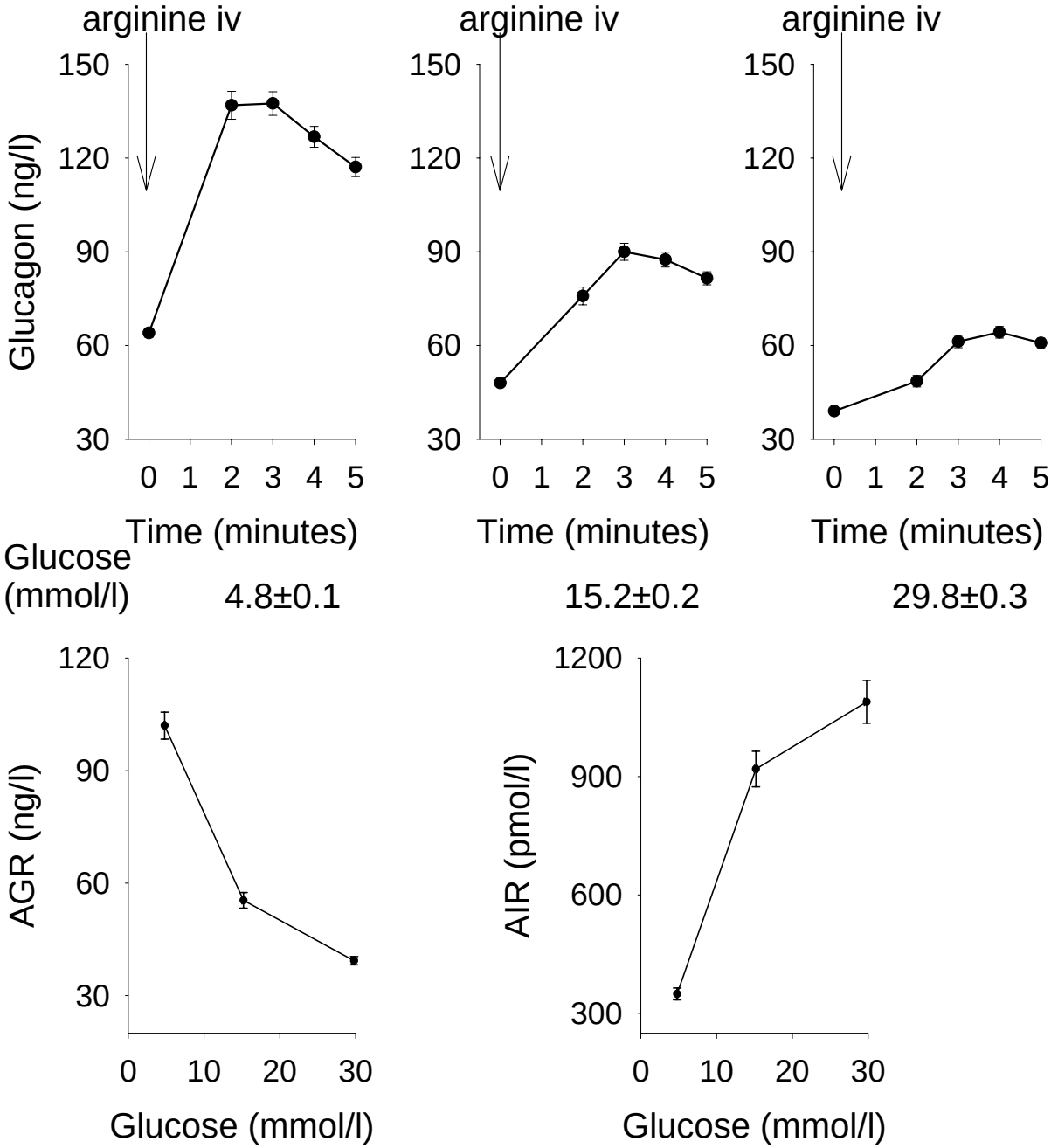
arginine iv
arginine iv
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Glucagon (ng/l)
150
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Time (minutes)
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Time (minutes)
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29.8±0.3
AGR (ng/l)
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Glucose (mmol/l)
AIR (pmol/l)
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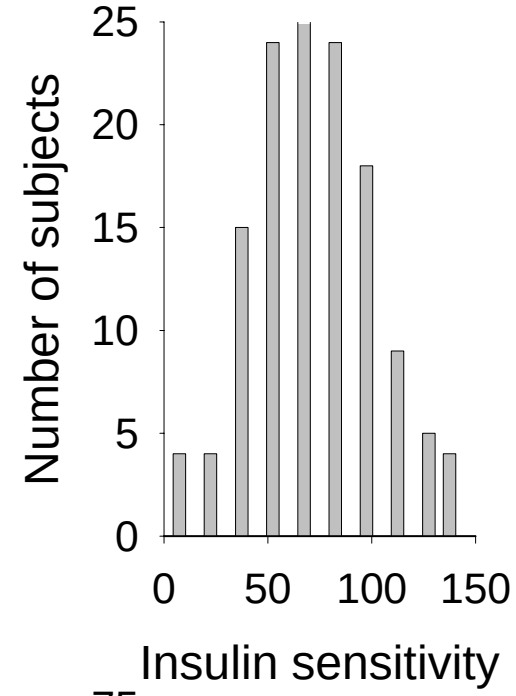
Glucose (mmol/l)


Number of subjects
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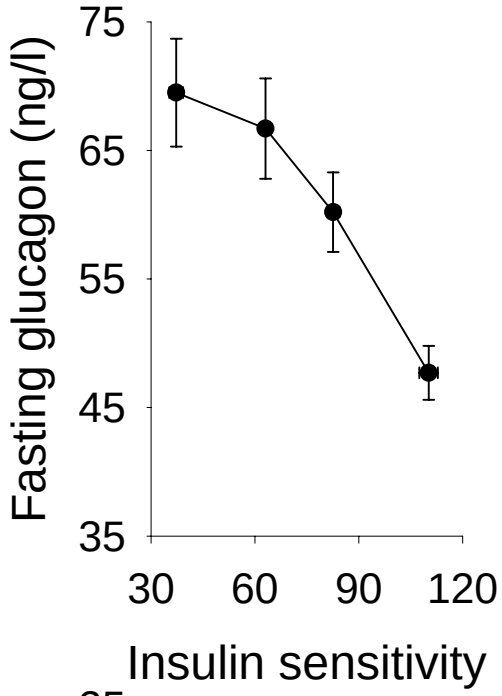
Insulin sensitivity


Fasting glucagon (ng/l)
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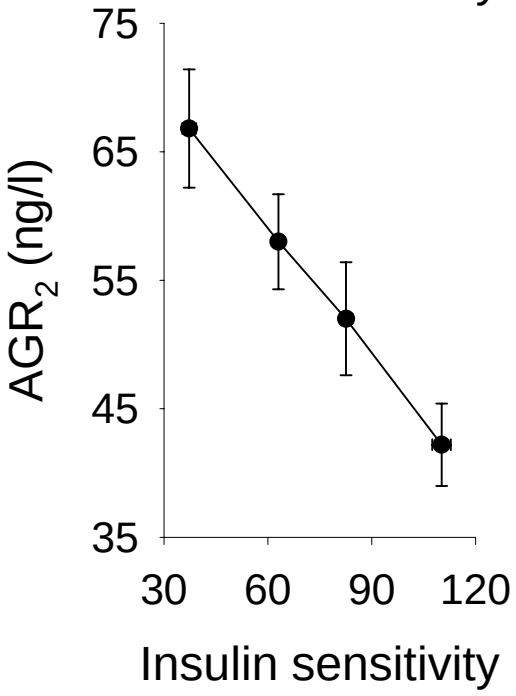
Insulin sensitivity


AGR2 (ng/l)
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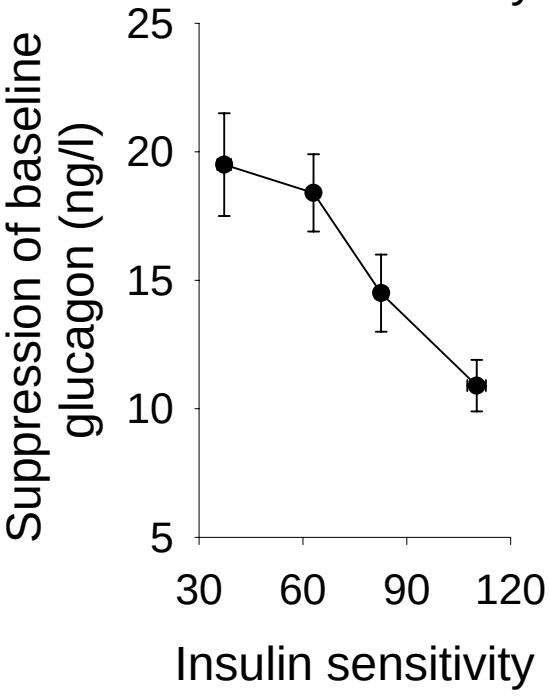
120
Insulin sensitivity


Suppression of baseline glucagon (ng/l)
25
20
15
10
5
30
60
90
120
Insulin sensitivity