Increased Insulin Clearance in Peroxisome Proliferator-Activated Receptor γ_2 Pro12Ala

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The Pro12Ala polymorphism of the peroxisome proliferator-activated receptor (PPARγ_2) is associated with reduced risk for type 2 diabetes. Although increased insulin sensitivity of glucose disposal and lipolysis has been reported, the exact mechanism by which the risk reduction is conferred is not clear. Because the conclusion of greater insulin sensitivity hinged upon lower insulin levels in some studies, it is possible that more efficient insulin clearance is involved. We therefore estimated insulin clearance during a euglycemic hyperinsulinemic clamp (insulin infusion rate divided by steady-state insulin concentration, 229 normal glucose tolerant [NGT] subjects), an oral glucose tolerance test (OGTT) (mean C-peptide divided by mean insulin concentrations, 406 NGT, 54 impaired glucose tolerant or mildly diabetic subjects), and a hyperglycemic clamp (120 minutes, 10 mmol/L, C-peptide divided by insulin in the steady-state, 56 NGT subjects). In the carriers of the Ala allele (prevalence ~24%), insulin clearance in all 3 protocols was significantly greater (~ 10%), than in controls. While the results from the euglycemic clamp reflect both hepatic and peripheral insulin clearance, those from the OGTT and the hyperglycemic clamp reflect mainly hepatic insulin extraction. Free fatty acids (FFA) during the steady state of the euglycemic hyperinsulinemic clamp were significantly lower in carriers of the Ala allele (26 ± 5 μmol/L) than in controls (46 ± 3 μmol/L, $P = .02$). In conclusion, the Pro12Ala polymorphism is associated with increased insulin clearance. This could be the result of reduced FFA delivery, which has been shown to improve hepatic insulin removal and sensitivity. Because PPARγ_2 is mainly expressed in adipose tissue, one of the main regulatory effects of the polymorphism may well be the more efficient suppression of (possibly intra-abdominal) lipolysis.

THE COMMON Pro12Ala polymorphism (20% to 25% prevalence in Caucasians) in the peroxisome proliferator-activated receptor (PPARγ_2) gene (first identified in 1997[1]) has been shown to be associated with reduced risk of type 2 diabetes.[2,3] PPARγ_2 is most abundantly expressed in fat tissue and plays a key role in adipocyte differentiation and lipid metabolism.[4,5] Many,[6-10] although not all studies,[11] indicated that the mechanism by which this polymorphism reduces the risk of type 2 diabetes involves greater insulin sensitivity.

The inference of greater insulin sensitivity in carriers of the Ala allele in a large population-based study was based on lower basal insulin levels.[7] In a small preliminary study from our laboratory with cases and controls matched one-for-one, we found no significant difference for the glucose infusion rates during the steady state of the hyperinsulinemic euglycemic clamp. However, when expressing the glucose infusion rate per unit insulin (so-called insulin sensitivity index) carriers of the Ala allele had significantly greater insulin sensitivity.[12] These observations suggested that insulin clearance from the circulation may be more efficient in carriers of the Ala allele.

Moreover, in a very recent report, knockout of a protein with a key role in hepatic internalization and degradation of receptor-bound insulin (CEACAM1) resulted in hyperinsulinemia, insulin resistance, and impaired glucose tolerance (IGT).[13] This strongly indicates that impairment of insulin clearance can be a primary event secondarily inducing insulin resistance and need not necessarily be a byproduct of defective insulin action.

In the present series of studies, we examined data from the Tübingen family study to specifically address the question whether carriers of the Pro12Ala polymorphism have greater insulin clearance compared with wild-type controls. For this purpose, we initially looked at insulin concentrations during the steady state of a standard euglycemic hyperinsulinemic clamp produced by a constant insulin infusion based on body weight (N = 229). This approach, however, assumes the complete or near complete suppression of endogenous insulin secretion. Under physiologic conditions, hepatic insulin elimination contributes to total insulin clearance to a higher proportion than in the hyperinsulinemic euglycemic clamp, because endogenous insulin passes the liver before reaching the systemic circulation. Therefore, we additionally analyzed data from hyperglycemic clamps (10 mmol/L, N = 56) and oral glucose tolerance tests (N = 460) using the C-peptide/insulin ratio as an estimate for insulin clearance.

MATERIALS AND METHODS

Subjects

In the Tübingen Family Study for type 2 diabetes, primarily normal glucose tolerant (NGT) subjects with (and without) family history for type 2 diabetes were recruited and metabolically characterized. In the present analysis, we report data from oral glucose tolerance tests (OGTT), euglycemic hyperinsulinemic clamps (N = 228), and hyperglycemic clamps (N = 56). In our population, the prevalence of the Ala allele in PPARγ_2 is approximately 23%. We mainly present data from NGT subjects (N = 406), but a reasonably sized set of OGTT data was available from IGT and mildly diabetic (fasting glucose < 7.8 mmol/L)

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Table 1. Characteristics of Subjects Undergoing the Hyperinsulinemic Euglycemic and the Hyperglycemic Clamp

	Hyperinsulinemic Euglycemic Clamp			Hyperglycemic Clamp		
	Pro/Pro	X/Ala	*P*	Pro/Pro	X/Ala	*P*
N (M/F)	177 (85/92)	52 (28/24)	.46*	35 (16/19)	21 (10/11)	.9*
Family history of diabetes (N)	83 (48%)	18 (45%)	.09*	20 (57%)	7 (33%)	.08*
Age (yr)	32 ± 1	32 ± 1	.9	33 ± 2	35 ± 2	.5
BMI (kg/m^2)	24.7 ± 0.3	24.4 ± 0.6	.7	26.4 ± 1.2	23.2 ± 0.6	.054
Waist-hip ratio	0.84 ± 0.01	0.84 ± 0.01	.9	0.85 ± 0.01	0.84 ± 0.02	.8
Body fat (%)	24 ± 1	23 ± 1	.26	NA	NA	
Fasting serum glucose (mmol/L)	4.87 ± 0.03	4.97 ± 0.06	.12	4.83 ± 0.11	4.82 ± 0.11	.9
Fasting serum insulin (pmol/L)	44 ± 2	42 ± 3	.8	61 ± 10	41 ± 4	.14

Abbreviation: NA, not available.
*χ^2 test.

subjects (N = 54) for additional analysis. The characteristics of the subgroups undergoing the different protocols are shown in Tables 1 and 2. The subjects of both the euglycemic and the hyperglycemic clamp are different, but both groups are contained in the OGTT analysis. Data from subgroups of the subjects have been included in previous publications.[12,14-17] In the hyperglycemic clamp database, the genotype distribution is not representative of our population because carriers of the polymorphism were actively recruited for a previous analysis.[14] The genotype distribution of the 460 subjects undergoing the OGTT (76.8% Pro/Pro, 21.7% Pro/Ala, 1.5% Ala/Ala) was in Hardy-Weinberg-equilibrium ($P = .92$, χ^2 test). The subjects were not related. The protocols were approved by the local ethical committee, and after explaining the nature of the study, all subjects gave informed written consent.

OGTT

After a 10-hour overnight fast, subjects ingested a solution containing 75 g dextrose, and venous blood samples were obtained at 0, 30, 60, 90, and 120 minutes for determination of plasma glucose, plasma insulin, and plasma C-peptide. Stages of glucose tolerance were classified according to World Health Organization (WHO) criteria.[18]

Hyperinsulinemic Euglycemic Clamp

After the baseline period, subjects received a primed insulin infusion at a rate of 1.0 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} for 2 hours as previously described.[8] Blood was drawn every 5 to 10 minutes for determination of blood glucose, and the infusion rate of exogenous glucose (GIR) was adjusted appropriately to maintain the baseline glucose level. Arterialized blood samples were obtained at 0 and 120 minutes for determination of serum insulin and free fatty acid (FFA) concentrations.

Hyperglycemic Clamp

After an overnight fast, an intravenous glucose bolus was given to instantaneously increase blood glucose to 10 mmol/L. Subsequently, a glucose infusion was adjusted to maintain blood glucose at 10 mmol/L.[19] Samples for insulin and C-peptide determinations were taken at −30, −15, 0, 2.5, 5, 7.5, 10, 20, 40, 60, 80, 100, and 120 minutes.

Analytical Procedures

Blood glucose was determined using a bedside glucose analyzer (glucose-oxidase method; YSI, Yellow Springs Instruments, Yellow Springs, CO). Plasma insulin was determined by microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan), plasma C-peptide by radioimmunoassay (Byk-Sangtec, Dietzenbach, Germany), and serum FFA concentrations with an enzymatic method (WAKO Chemicals, Neuss, Germany). The Pro12Ala polymorphism in exon B of the PPARγ_2 gene was determined by polymerase chain reaction (PCR) and subsequent restriction enzyme analysis with BstU-I as reported previously.[8]

Calculations

The insulin sensitivity index (ISI, in μmol $\cdot$ kg^{-1} $\cdot$ min^{-1} $\cdot$ pmol^{-1} $\cdot$ L) for systemic glucose uptake was calculated as mean infusion rate of exogenous glucose (GIR) necessary to maintain euglycemia during the last 60 minutes of the euglycemic clamp divided by the steady-state serum insulin concentration. The metabolic clearance rate of insulin (MCR_{INS}) during the steady state of the euglycemic clamp was calculated as insulin infusion rate divided by the steady-state insulin concentration as previously described.[20]

Table 2. Characteristics of Subjects Undergoing OGTT

	NGT			IGT		
	Pro/Pro	X/Ala	*P*	Pro/Pro	X/Ala	*P*
N (M/F)	313 (118/195)	93 (55/38)	.6*	41 (17/24)	13 (9/4)	.5*
Family history of diabetes (N)	144 (48%)	35 (38%)	.09*	27 (69%)	10 (77%)	.6*
Age (yr)	35 ± 1	34 ± 2	.4	42 ± 2	45 ± 2	.6
BMI (kg/m^2)	26.0 ± 0.3	24.9 ± 0.5	.08	27.9 ± 1.1	30.4 ± 2.8	.3
Waist-hip ratio	0.84 ± 0.01	0.84 ± 0.01	.9	0.89 ± 0.02	0.92 ± 0.03	.3
Body fat (%)	27 ± 1	25 ± 1	.07	30 ± 2	34 ± 4	.20
HbA_{1c} (%)	5.1 ± 0.02	5.2 ± 0.04	.4	560 ± 0.1	6.1 ± 0.2	.14
Fasting serum glucose (mmol/L)	4.84 ± 0.03	4.85 ± 0.06	.9	5.50 ± 0.13	5.76 ± 0.29	.4
Fasting serum insulin (pmol/L)	50 ± 2	44 ± 3	.12	75 ± 1	83 ± 2	.7

*χ^2 test.

Table 3. Results From the Hyperinsulinemic Euglycemic Clamp

	Pro/Pro	X/Ala	*P*	*P* Adjusted
Steady-state insulin (pmol/L)	414 ± 7	384 ± 15	.06	.03*
Fasting FFAs (μmol/L)	426 ± 15	449 ± 27	.4	.4†
Steady-state FFAs (μmol/L)	46 ± 5	26 ± 3	.02	.02*
Δ-FFA (μmol/L)	399 ± 17	414 ± 31	.67	.53‡
Glucose infusion rate (mg/kg/min)	8.7 ± 0.2	9.0 ± 0.4	.55	.6*
Insulin sensitivity index ($\mu mol\ kg^{-1} \cdot min^{-1} \cdot pmol^{-1} \cdot L$)	0.096 ± 0.004	0.108 ± 0.006	.088	.16§
MCR_{ins} (mL/kg/min)	15.3 ± 0.3	16.5 ± 0.5	.045	.022†

*Adjusted for BMI; †adjusted for BMI, age, and WHR; ‡adjusted for BMI, age, and sex; §adjusted for BMI, percent body fat, age, and WHR.

In the hyperglycemic clamp, insulin sensitivity was estimated by calculating an insulin sensitivity index analogously to the euglycemic clamp as glucose infusion rate divided by plasma insulin concentrations in the second phase of insulin secretion.

Estimates for insulin clearance were obtained during the OGTT and the hyperglycmic clamp by dividing C-peptide concentrations by insulin concentrations as previously described.[21] Mean C-peptide and insulin was used during the entire OGTT and during the last 40 minutes of the hyperglycemic clamp. Because ratios are sensitive to a non-zero intercept bias when plotting C-peptide against insulin, we also compared insulin concentrations adjusted for respective C-peptide concentrations, ie, used the insulin residuals from the regression line predicted by C-peptide values.

Statistical Analysis

Data are mean ± SEM unless otherwise stated. Differences were assessed by analysis of variance for 3 groups. In addition, comparison between 2 groups (subjects heterozygous and homozygous for the polymorphism were pooled, X/Ala) were analyzed using an unpaired Student's *t* test on log-transformed data adjusted for covariates (age, body mass index [BMI], percent body fat, waist-to-hip ratio [WHR], ISI as appropriate). Adjustments were made for independently correlating variables as determined by stepwise multiple linear regression analysis. The nonparametric Wilcoxon test was used for non-normally distributed parameters. A *P* value of less than .05 was considered to be statistically significant. The statistical software package JMP (SAS Institute, Cary, NC) was used for the statistical analyses.

RESULTS

Hyperinsulinemic Euglycemic Clamp

As shown in Tables 1 and 2, there were only small differences in anthropometric characteristics between the genotype groups concerning BMI and body fat. Insulin sensitivity was slightly greater in X/Ala, but the difference did not reach statistical significance. The metabolic clearance of insulin as obtained during the hyperinsulinemic euglycemic clamp experiment was significantly greater in subjects with the Pro12Ala polymorphism. This difference remained significant after adjusting for covariates (Table 3, Fig 1). For analysis of FFA concentrations, data from 178 subjects were available. While FFA concentrations at baseline were not different, FFAs were signficantly lower at the end of the clamp (Table 3, Fig 2). This suggests more sufficient suppression of lipolysis in the carriers of the polymorphism.

Hyperglycemic Clamp

During the second phase of insulin secretion, glucose and C-peptide concentrations were not significantly different between the groups (Table 4). The significant difference in the second phase insulin concentration and ISI disappeared upon adjusting for covariates. In contrast, the ratio mean C-peptide over mean insulin was significantly greater in carriers of the polymorphism, and the difference held up to adjusting for covariates (Table 4). To avoid a possible non-zero intercept

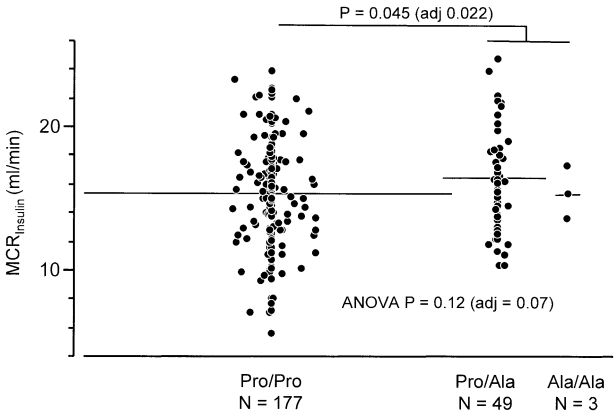

Fig 1. Insulin clearance (insulin infusion rate divided by steady-state insulin concentration) during the euglycemic hyperinsulinemic clamp.

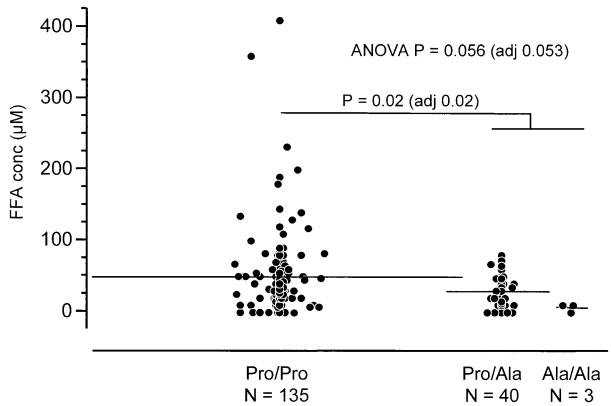

Fig 2. Suppressed FFA concentrations during the euglycemic hyperinsulinemic clamp.

Table 4. Results From the Hyperglycemic Clamp

	Pro/Pro	X/Ala	*P*	*P* Adjusted
Steady-state insulin (80-120 min; pmol/L)	389 ± 59	226 ± 26	.045	.5*
Steady-state C-peptide (80-120 min; pmol/L)	2,767 ± 191	2,319 ± 125	.097	.23†
Glucose infusion rate (mg/kg/min)	37.2 ± 3.4	38.8 ± 3.9	.8	.9*
Insulin Sensitivity Index (μmol · kg^{-1} · min^{-1} · pmol^{-1} · L)	0.15 ± 0.02	0.20 ± 0.03	.037	.23†
C-peptide/insulin ratio (80-120 min, molar ratio)	36 ± 1	40 ± 1	.006	.03†

*Adjusted for BMI and age; †adjusted for BMI.

bias caused by analysis of this ratio, we adjusted mean insulin for mean C-peptide (in addition to the other covariates). This resulted in significantly lower insulin concentrations in X/Ala (data not shown). Provided that the Pro12Ala polymorphism does not alter elimination kinetics of C-peptide, this clearly indicates that during the hyperglycemic clamp, insulin clearance is also greater in carriers of the polymorphism.

OGTT

With the exception of mean insulin in the NGT carriers of the polymorphism, glucose, insulin, or C-peptide concentrations were not significantly different between the groups (Table 5). In contrast, the ratio mean C-peptide over mean insulin was significantly greater in both NGT and IGT carriers of the polymorphism even after adjusting for covariates (Table 5). Moreover, simply adjusting mean insulin also for mean C-peptide (rather than using a ratio), in addition to the other covariates, resulted in significantly lower insulin concentrations in X/Ala (data not shown). This suggests that during this near-physiologic stimulation, insulin clearance is also greater in carriers of the polymorphism.

DISCUSSION

In all 3 sets of experiments, insulin clearance was significantly greater in carriers of the Ala allele compared with controls. Insulin clearance as measured by the euglycemic clamp is the sum effect of hepatic and peripheral insulin removal. The C-peptide/insulin ratios during the OGTT and the hyperglycemic clamp (or the insulin concentrations adjusted for C-peptide concentrations, respectively) more accurately reflect hepatic insulin extraction. In the present analysis, a significant difference in insulin-stimulated glucose uptake was not consistently detected, and in some subanalyses, disappeared upon adjustment for BMI. While insulin sensitivity in carriers of the Ala allele always tends to be greater in our population, the difference becomes significant only in certain subsets. This observation clearly contrasts with the clearcut difference in insulin clearance seen in the present studies. Considering the consistency across the different experimental procedures (euglycemic clamp, OGTT, hyperglycemic clamp), this difference in insulin clearance represents the most striking finding about the Pro12Ala polymorphism in PPARγ_2 in our population. It strongly suggests that alterations in insulin clearance may be closely related to the primary mechanism of action of the Pro12Ala polymorphism in PPARγ_2.

Differences in insulin clearance may be secondary to differences in (mainly hepatic) insulin sensitivity or, as suggested by the CEACAM1 knockout model,[13] a primary event. The liver-specific mouse knock-out model of the insulin receptor (LIRKO), a model of primary hepatic insulin resistance, is characterized by extreme hyperinsulinemia as a result not only of hypersecretion, but also of reduced or absent insulin clearance.[22] In humans, hepatic insulin resistance resulting in inadequate suppression of glucose production by insulin was recently identified as the single most important determinant of glucose tolerance in a Scandinavian population (among insulin-stimulated glucose disposal and early insulin response to oral glucose).[23] Endogenous glucose production was not measured in this study. In Pima Indians, however, insulin suppression of glucose production was 40% more efficient in carriers of the Ala allele, while insulin-stimulated glucose uptake was not different (Leslie Baier, personal communication).

Both hepatic insulin resistance and decreased insulin clearance can be the result of increased FFA delivery to the liver.[24] In vitro, FFAs reduced insulin binding, degradation, and action.[25] In animals in vivo, elevation of FFAs substantially decreased insulin clearance.[26,27] Moreover, human obesity, a condition characterized by excessive FFA turnover,[28] is usually associated with reduced insulin clearance.[29-31] Finally, in dogs[32-34] and humans,[35] insulin-induced suppression of endogenous glucose production is prevented by experimental elevation of FFA. Therefore, it is possible that the greater insulin clearance in carriers of the Ala allele mirrors reduced portal FFA availability.

In fact, we observed significantly lower insulin-suppressed FFA concentrations in carriers of the polymorphism, suggest-

Table 5. Results From the OGTT

	NGT				IGT			
	Pro/Pro	X/Ala	*P*	*P* Adjusted	Pro/Pro	X/Ala	*P*	*P* Adjusted
Mean glucose (mmol/L)	6.16 ± 0.06	6.15 ± 0.12	1.0	.7*	9.8 ± 0.3	11.1 ± 1.1	.13	.12*
Mean insulin (pmol/L)	318 ± 13	254 ± 16	.01	.029†	450 ± 54	448 ± 160	1.0	.6†
Mean C-peptide (pmol/L)	2,395 ± 50	2,202 ± 16	.055	.18‡	2,681 ± 160	2,728 ± 501	.9	.7‡
Mean C-peptide/mean insulin (molar ratio)	9.0 ± 0.2	10.0 ± 0.3	.01	.04†	7.5 ± 0.4	8.8 ± 0.9	.14	.03†

*Adjusted for BMI and age; †adjusted for BMI and WHR; ‡adjusted for BMI, age, and WHR.

ing that one effect of the Ala allele is the more efficient suppression of lipolysis (including intra-abdominal depots, which drain directly into the portal vein). Consistent with this interpretation, we previously reported greater sensitivity to insulin-suppressed lipolysis in carriers of the Ala12 allele based on isotopically determined rate of appearance of glycerol as an index for lipolysis (data of 40 of the 178 subjects with FFA data were contained in that report).[16] Interestingly, after a 12-week experimental induction of intra-abdominal obesity in dogs, insulin clearance was reduced by 50%.[21] Thus, it appears possible that intra-abdominal fat depots are of particular importance.

Not only FFAs, but also peptide hormones released from adipose tissue, have been reported to influence hepatic insulin sensitivity and possibly removal. For example, adiponectin renders the liver more sensitive to the suppressive effect of insulin on glucose production in vitro[36] and in vivo.[37] Because adiponectin release is under transcriptional control of PPARγ,[38,39] it represents a plausible candidate for mediating the effect of the Pro12Ala polymorphism on hepatic insulin sensitivity and clearance.

Nevertheless, our results are also compatible with an alternative hypothesis. Although PPARγ is mainly expressed in adipose tissue, a direct effect of this polymorphism on hepatic insulin binding, action, internalization, and degradation cannot be excluded. In such a scenario, the Ala allele would primarily cause enhanced hepatic insulin clearance. In analogy to the concept raised by the CEACAM1-knockout experiment,[13] improvement of peripheral insulin sensitivity would be a consequence. Thus, greater insulin sensitivity of peripheral glucose and fatty acid metabolism in carriers of the Ala allele would be a secondary event.

It is necessary to point out that our determinations of insulin clearance are based on a number of assumptions and may therefore be inaccurate to some extent. During the hyperinsulinemic euglycemic clamp, complete or near complete suppression of endogenous insulin secretion is assumed, which may not necessarily be the case. Nevertheless, it is a very commonly used procedure for measuring insulin clearance.[20] Using the C-peptide over insulin ratio also has shortcomings, such as interindividual variability of C-peptide clearance. It appears very unlikely, however, that the Pro12Ala polymorphism influences C-peptide elimination.

Finally, both calculations of the metabolic clearance rate of insulin and C-peptide over insulin ratios require a metabolic steady state. This can be assumed for the second hour of the hyperinsulinemic euglycemic clamp and the last 40 minutes of the hyperglycemic clamp, but not during the OGTT. Insulin and C-peptide are cosecreted equimolarly and for both, a 2-compartmental model with similar distribution spaces can be assumed in our experimental setting. Due to the longer half-life of C-peptide, the C-peptide/insulin ratio will be lower when insulin secretion increases and higher when insulin secretion decreases.[40,41] Therefore, changes of this ratio during the OGTT may not necessarily reflect changes in insulin clearance. The use of mean values over the entire OGTT reduces a possible systematic error. Moreover, these limitations apply to all genotypes alike, and because there is no significant difference in insulin secretion, it seems to be very unlikely that the observed difference in the C-peptide/insulin ratio is entirely attributable to the different half-lives of the 2 peptides. Nevertheless, the relatively large number of subjects studied and the consistency across the 3 different protocols conveys confidence in the overall picture.

In conclusion, we found significantly greater insulin removal from the circulation in carriers of the Pro12Ala polymorphism in PPARγ_2 and significantly lower FFA concentrations during hyperinsulinemia. Increased insulin clearance could reflect increased hepatic insulin removal and sensitivity secondary to decreased FFA delivery, which has been shown to strongly interfere with hepatic insulin removal. Because PPARγ_2 is mainly expressed in adipose tissue, it is possible that the main regulatory effect of the Ala allele is the more efficient suppression of (possibly intra-abdominal) lipolysis. It is possible that PPARγ-mediated adipocytokine release is involved.

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