Effect of Experimental Elevation of Free Fatty Acids on Insulin Secretion and Insulin Sensitivity in Healthy Carriers of the Pro12Ala Polymorphism of the Peroxisome Proliferator–Activated Receptor-γ2 Gene

Norbert Stefan, Andreas Fritsche, Hans Häring, and Michael Stumvoll

The transcription of many genes involved in lipid metabolism is regulated by the peroxisome proliferator–activated receptor-γ (PPAR-γ). The Pro12Ala polymorphism in the PPAR-γ2 gene has been associated with reduced transcriptional activity in vitro and increased insulin sensitivity in vivo. Although PPAR-γ has been demonstrated in human β-cells, it is unknown whether the Pro12Ala polymorphism plays a role in insulin secretion. Moreover, it is also unknown if and how the effect of free fatty acids (FFAs) on insulin secretion and insulin sensitivity is modulated by the presence of this polymorphism. We therefore performed hyperglycemic clamps (8 mmol/l, 140 min, 5 g arginine bolus at min 120) in 10 healthy subjects with the (X/Ala) polymorphism and in 10 subjects without the polymorphism (Pro/Pro) basally and after 5 h infusion of Intralipid plus heparin. FFA concentrations increased from 473 ± 61 μmol/l to 1,732 ± 163 μmol/l in the Pro/Pro group and from 372 ± 46 μmol/l to 1,630 ± 96 μmol/l in the X/Ala group (P = 0.68). Basally, neither insulin sensitivity nor insulin secretion were significantly different between the two groups. During infusion of Intralipid, first-phase insulin secretion remained unchanged in both groups (P = 0.21). In the Pro/Pro group, second-phase insulin secretion remained unchanged (444 ± 67 vs. 471 ± 93 pmol/mnin the response to arginine from 5,007 ± 41 to 6,072 ± 732 pmol/min. In contrast, in the X/Ala group, there was a decrease of both second-phase insulin secretion (533 ± 58 to 427 ± 48 pmol/min, P = 0.02 vs. Pro/Pro) and in the response to arginine (from 7,518 ± 1,306 to 6,458 ± 1,040 pmol/min, P = 0.014 vs. Pro/Pro). The insulin sensitivity index decreased comparably in Pro/Pro and X/Ala (to 71 ± 8 vs. 74 ± 9% of basal, P = 0.8). In conclusion, these results provide evidence that the Pro12Ala polymorphism in the PPAR-γ2 gene might be involved in a differential regulation of insulin secretion in response to increased FFAs in humans. Diabetes 50:1143–1148, 2001

Lipid and glucose metabolism are inseparable in humans. On the molecular level, the peroxisome proliferator–activated receptor isoform-γ (PPAR-γ), a transcription factor, may represent a key mediator between development and metabolism of adipocytes, free fatty acids (FFAs), and glucose homeostasis (1,2). On the one hand, PPAR-γ regulates the transcription of genes involved in lipid metabolism (e.g., lipoprotein lipase, fatty acid transport proteins, acyl-CoA synthetase, and malic enzyme) (2); on the other hand, unsaturated FFAs are natural ligands of this receptor (3). Moreover, synthetic ligands of PPAR-γ, the thiazolidinediones, possess antidiabetic properties (4,5). PPAR-γ1 is expressed in many tissues, including skeletal muscle, whereas the splice variant PPAR-γ2 is mainly expressed in adipocytes. Expression of PPAR-γ has also been demonstrated in human β-cells (6). This indicates a potential significance of PPAR-γ for development and/or metabolism of β-cells.

Recently, a reduced transcriptional activity of the proline to alanine exchange in codon 12 (Pro12Ala) in PPAR-γ2 has been shown in vitro (7). In humans, an association of the Pro12Ala polymorphism with lower fasting insulin was observed and thought to indicate improved insulin sensitivity (7). Higher insulin sensitivity based on hyperinsulinemic clamps was also reported for severely obese subjects carrying the Ala allele (8). The prevalence of the Pro allele was higher in Japanese-Americans with type 2 diabetes (7) but was not different in Caucasian populations (9,10). Intriguingly, a significantly higher BMI was reported for subjects carrying the Ala allele (11,12). Moreover, this polymorphism appears to modify the relationship between leptin levels and adipose tissue mass in such a way that, for a given leptin level, the BMI is relatively lower in obese subjects carrying the Ala allele (13). Recently, a reduced risk for type 2 diabetes was demonstrated for carriers of the Ala allele (14). All of these observations cannot easily be incorporated into a unifying schema explaining the functional role of this polymorphism in human obesity and type 2 diabetes. The available data regarding the effect of Pro12Ala in humans were exclusively based on cross-sectional data. However, to understand the pathophysiological relevance of genetic variants, it is essential to also study gene-environment interactions.

One environmental factor important for glucose ho-
meostasis is overeating and obesity. FFAs are thought to represent an important factor linking excess fat mass to type 2 diabetes (15,16). They have been shown to influence both insulin secretion (17,18) and insulin action (19,20). Because they also represent a prime candidate for producing or mediating effects of PPAR-γ, we experimentally raised serum FFA levels in 10 healthy subjects with the X/Ala polymorphism and 10 healthy subjects without the Pro12Ala polymorphism (Pro/Pro). We used the hyperglycemic clamp technique (8 mmol/l, 140 min, 5 g arginine bolus at min 120) to assess insulin secretion and insulin sensitivity before and after a 5-h infusion of Intralipid plus heparin.

**RESEARCH DESIGN AND METHODS**

**Subjects.** From a large study performed in Tübingen, conducted in order to phenotype metabolic risk factors for type 2 diabetes, we recruited 10 subjects with the Pro12Ala polymorphism in the PPAR-γ2 gene (X/Ala) and 10 subjects without the polymorphism (Pro/Pro) matched for sex, age, BMI, and waist-to-hip ratio who underwent the Intralipid plus heparin protocol. Two members of the X/Ala group were homozygous (Ala/Ala) and eight were heterozygous for Pro12Ala (Pro/Ala). The selection and matching of the subjects were based on genotype, glucose tolerance, and demographic parameters. Their insulin sensitivity or insulin secretory capacity was unknown before the experiments. The Ala allele has a frequency of ~13% in this population.

In addition, seven subjects (two Pro/Ala and five Pro/Pro) underwent a saline control experiment. The characteristics of the subjects are shown in Table 1. The study protocol was approved by the ethical committee of the University of Tübingen. Before the study, informed written consent was obtained from all participants. All subjects had their medical history taken and underwent a physical examination, a routine blood test, and an electrocardiogram. They had been instructed to maintain their usual diet before the study. The subjects were of German origin, were unrelated, and had normal glucose tolerance according to World Health Organization criteria.

**Experimental design.** After an overnight fast, a hyperglycemic clamp was performed at around 7:00 a.m. Immediately after completion of the baseline saline control experiment. The characteristics of the subjects are shown in Table 1. The study protocol was approved by the ethical committee of the University of Tübingen. Before the study, informed written consent was obtained from all participants. All subjects had their medical history taken and underwent a physical examination, a routine blood test, and an electrocardiogram. They had been instructed to maintain their usual diet before the study. The subjects were of German origin, were unrelated, and had normal glucose tolerance according to World Health Organization criteria.

**Sampling and analytical procedures.** During the hyperglycemic clamps, blood was sampled at ~15, ~5, 2.5, 5, 7.5, 10, 60, 80, 100, 120, 125, 127.5, 130, and 140 min for determination of plasma insulin and C-peptide. Blood for determination of FFAs was sampled at ~15, ~5, 60, and 120 min of every hyperglycemic clamp and at 240, 360, and 420 min. Tubes were immediately placed on ice water. Serum insulin was determined by a microparticle enzyme immunoassay (Abbott Laboratories, Tokyo). Plasma C-peptide was determined by radioimmunoassay (Byk-Sangtec, Diethenbach, Germany). Blood glucose was determined bedside every 2.5–5.0 min using a HemoCue analyzer (HemoCue, Mission Viejo, CA). The samples for FFA determination were spun down with minimal delay and frozen at ~20°C until analysis. Total FFA concentrations were determined by an enzymatic method (NENFAC kit, Waco Chemicals, Neuss, Germany).

**Calculations and statistical analysis.** The insulin secretion rate (ISR) was calculated assuming constant kinetic parameters for C-peptide (rate constants and volume of distribution) adjusted for age, sex, BMI, and body surface area by deconvolution, as previously described (25). The different phases of insulin secretion were calculated as follows: first-phase ISR, sum of 2.5 and 5 min; second-phase ISR, mean of 80–120 min; ISR in response to arginine, sum of 122.5 and 125 min; first-phase C-peptide and insulin, mean of 2.5, 5, 7.5 and 10 min; second-phase C-peptide and insulin, mean of 80–120 min; C-peptide and insulin in response to arginine, mean of 122.5, 125, 127.5 and 130 min. For the ISR of the acute phases, only the 2.5-min (122.5) and 5-min (125) values were included because by 7.5 min (127.5), ISR had returned to the prestimulus value.

Insulin sensitivity was assessed as an insulin sensitivity index (ISI), calculated by dividing the average glucose infusion rate during 80–120 min of the hyperglycemic clamp by the average plasma insulin concentration during the same interval. A disposition index was calculated as product of ISI and the respective insulin secretion phase (C-peptide levels).

Unless stated otherwise, data are expressed as means ± SE. For comparisons between basal and 5 h, a paired Student’s t test was used (two-tailed). Baseline differences between X/Ala and Pro/Pro were assessed using an unpaired Student’s t test. To assess the influence of the presence of the Ala allele on the FFA effect, differences (5-h experiment minus baseline experiment) were compared by an unpaired Student’s t test (two-tailed) or by multivariate analysis of variance (MANOVA) with repeated measures design. The statistical software package JMP (SAS Institute Inc, Cary, NC) was used.

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**Table 1.**

<table>
<thead>
<tr>
<th>Saline group</th>
<th>X/Ala group</th>
<th>Pro/Pro group</th>
<th>P (X/Ala vs. Pro/Pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (M/F)</td>
<td>5/2</td>
<td>8/2</td>
<td>8/2</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30 ± 2</td>
<td>33 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 0.7</td>
<td>23.3 ± 0.6</td>
<td>23.3 ± 0.9</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.85 ± 0.02</td>
<td>0.85 ± 0.02</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>70 ± 0.8</td>
<td>72.8 ± 2.9</td>
<td>73.3 ± 4</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>39 ± 0.5</td>
<td>39.5 ± 5.5</td>
<td>38 ± 8</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.5 ± 0.1</td>
<td>4.4 ± 0.1</td>
<td>4.7 ± 0.09</td>
</tr>
<tr>
<td>Fasting FFAs (μmol/l)</td>
<td>498 ± 61</td>
<td>372 ± 46</td>
<td>473 ± 61</td>
</tr>
</tbody>
</table>

Data are means ± SD.
RESULTS
Free fatty acid concentrations and glucose concentrations during the hyperglycemic clamp. As shown in Fig. 1, in the saline experiment FFAs increased from \(\sim 498 \pm 61 \text{ mmol/l} \) by the end of the basal clamp to \(\sim 670 \pm 86 \text{ mmol/l} \) after 5 h \((P = 0.07)\). In the Pro/Pro group, FFAs increased from \(473 \pm 61 \text{ mmol/l} \) at baseline to \(1,732 \pm 163 \text{ mmol/l} \) after 5 h \((P < 0.001)\). In the X/Ala group, FFAs increased from \(372 \pm 46 \text{ mmol/l} \) to \(1,630 \pm 96 \text{ mmol/l} \) \((P < 0.001)\) after 5 h, which was not statistically different from the Pro/Pro group \((P = 0.68)\).

In the saline group the mean blood glucose concentration during the hyperglycemic clamp was \(8.58 \pm 0.14 \text{ mmol/l} \) at baseline and \(8.48 \pm 0.13 \text{ mmol/l} \) after 5 h. In the Pro/Pro and X/Ala group, the mean blood glucose concentration during the hyperglycemic clamp was \(8.58 \pm 0.2 \) and \(8.53 \pm 0.13 \text{ mmol/l} \) at baseline and \(8.54 \pm 0.22 \) and \(8.47 \pm 0.14 \text{ mmol/l} \) during the second clamp, respectively. There was no statistical difference between the baseline and the respective 5-h clamp or between any of the groups.

C-peptide levels, ISRs, and serum insulin concentrations. C-peptide levels, ISRs, and serum insulin concentrations are shown in Table 2. At baseline, C-peptide levels, ISRs, and insulin levels for first phase, second phase, and response to arginine were not significantly different between the Pro/Pro and X/Ala groups (Fig. 2).

After infusion of Intralipid, first-phase insulin secretion decreased comparably in both groups \((P = 0.12)\). Second-phase insulin secretion did not change significantly in Pro/Pro but decreased significantly in the X/Ala group. The change from baseline was significantly different between the two groups \((P = 0.02 \text{ for C-peptide})\). In response to arginine, insulin secretion did not change significantly in the Pro/Pro but decreased significantly in the X/Ala group. The change from baseline, however, was significantly different between the two groups \((P = 0.004 \text{ for C-peptide})\).

There was a relative increase in the C-peptide levels in the Pro/Pro group compared with the saline group for second phase \((P = 0.077)\) and the response to arginine \((P = 0.036)\) (Fig. 2).

The corresponding ISRs were analogous. The change in ISR during the Intralipid infusion is shown in Fig. 3. While there was an increase in the Pro/Pro group at the end of the clamp, there was a decrease in the X/Ala group.

DISCUSSION
The aim of the present study was to assess whether and how the Ala allele in codon 12 of PPAR-\(\gamma_2\) modulates the FFA effect on insulin secretion and insulin sensitivity. We studied subjects with a known genotype from a larger study in which risk factors for type 2 diabetes are being phenotyped in a German population. They were carefully

![FIG. 1. Total FFA levels during the Intralipid and saline infusions in subjects with Pro/Pro or X/Ala in PPAR-\(\gamma_2\).

### TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Pro/Pro</th>
<th>X/Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First phase</td>
<td>Second phase</td>
<td>Arginine</td>
</tr>
<tr>
<td>C-peptide (pmol/l)</td>
<td>1,333 ± 259</td>
<td>1,576 ± 189</td>
<td>4,328 ± 530</td>
</tr>
<tr>
<td>5 h</td>
<td>1,136 ± 171</td>
<td>1,309 ± 154</td>
<td>3,985 ± 561</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>-197 ± 201</td>
<td>-267 ± 79</td>
<td>-342 ± 192</td>
</tr>
<tr>
<td>(P)</td>
<td>0.40</td>
<td>0.02</td>
<td>0.15</td>
</tr>
<tr>
<td>ISR (pmol/min)</td>
<td>2,712 ± 638</td>
<td>426 ± 59</td>
<td>7,673 ± 1025</td>
</tr>
<tr>
<td>5 h</td>
<td>2,343 ± 363</td>
<td>351 ± 45</td>
<td>7,394 ± 1220</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>-369 ± 482</td>
<td>-75 ± 31</td>
<td>-279 ± 436</td>
</tr>
<tr>
<td>(P)</td>
<td>0.51</td>
<td>0.07</td>
<td>0.58</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>316 ± 133</td>
<td>170 ± 37</td>
<td>1,282 ± 302</td>
</tr>
<tr>
<td>5 h</td>
<td>237 ± 46</td>
<td>130 ± 26</td>
<td>1,114 ± 289</td>
</tr>
<tr>
<td>(\Delta)</td>
<td>-79 ± 103</td>
<td>-40 ± 15</td>
<td>-167 ± 72</td>
</tr>
<tr>
<td>(P)</td>
<td>0.5</td>
<td>0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Data are means ± SE. First phase, mean of 2.5–10 min (C-peptide, insulin), sum of 2.5 and 5 min (ISR); second phase, mean of 80–120 min; and arginine, mean of 122.5–130 min (C-peptide, insulin), sum of 122.5 and 125 min (ISR). *Pro/Pro vs. X/Ala < 0.05; †Pro/Pro vs. X/Ala < 0.01.
matched for factors known to influence insulin secretion and insulin sensitivity such as age, sex, BMI, and waist-to-hip ratio.

At baseline, the mean secretion parameters differed by as much as 40%. Due to the large variation within the group and the unpaired statistical analysis, these differences did not even approach statistical significance. This illustrates the difficulties one faces in studies associating a single gene polymorphism with a phenotype as complexly regulated as insulin secretion. Large numbers of subjects are required to detect differences that are probably much smaller than the biological variation. Furthermore, the matching process, which is crucial to eliminate factors obviously influencing the phenotype more than the actual polymorphism, such as BMI, can introduce a considerable bias. In contrast, a functional perturbation of the equilibrium by a metabolic challenge such as the lipid infusion may unmask subtle effects of a polymorphism in much smaller cohorts.

After the 5-h infusion of Intralipid, we observed a 20–30% decrease in insulin sensitivity compared with saline. This observation is consistent with many reports in the literature (rev. in 16). The decrease, however, was not different between Pro/Pro and X/Ala. Whereas the Intralipid infusion had no effect on first-phase insulin secretion compared with saline, it resulted in an increased second-phase insulin secretion in Pro/Pro. The effect, however, became statistically significant compared with the saline experiment only with the additional stimulation by arginine. This is consistent with hypersecretion of insulin during prolonged hyperglycemia in the presence of elevated FFAs (17,18).

The key finding of the present study was that in the subjects carrying the Ala allele, second-phase insulin secretion and insulin secretion in response to arginine decreased significantly after 5 h infusion of Intralipid, while both increased in Pro/Pro. This resulted in changes from baseline clamps going in opposite directions (Fig. 3). The contrary behavior suggests that differences in the transcriptional activity of PPAR-γ2 involved differential handling of the fatty acids, which ultimately resulted in alterations of insulin secretion. The fact that first-phase insulin secretion, which decreases early in the natural history of type 2 diabetes, was not different suggests that the Ala allele does not predispose to β-cell dysfunction.

The different behavior of second phase is more difficult to interpret. The second or late phase of glucose-stimulated insulin secretion is a reflection of different intracellular processes compared with first phase (26). While first
phase reflects the rapid release of insulin from the docked granule pool, second phase involves recruitment, translocation, and docking of a different pool of granules. Second-phase insulin secretion is often normal in conditions of impaired glucose tolerance when first phase is clearly reduced (27). It is possible that specific cellular mechanisms involved in second phase are affected by the lipid infusion and the polymorphism in a way that does not apply to first phase. The response to arginine might simply represent an amplification of any such scenario.

The underlying cellular and molecular mechanisms are unclear. Theoretically, two principle mechanisms are possible: there could be relevant expression of PPAR-γ in β-cells resulting in altered transcription of genes involved in fatty acid metabolism in the subjects with the Ala allele. In β-cells, the metabolism of fatty acids is tightly coupled to glucose-stimulated insulin secretion (28) by enzymes that, at least in adipose tissue, are under transcriptional control of PPAR-γ. To date, only expression of total PPAR-γ but not of specific isoforms has been determined for human β-cells (6).

Alternatively, any effect of altered expression of PPAR-γ-dependent genes could be mediated by adipose tissue in which this transcription factor is abundant. For example, alterations of the fatty acid composition as a result of differential metabolism of acyl-CoAs could be involved. Both chain length and degree of saturation have been shown to influence insulin secretion (29). In addition, minute changes in circulating levels of peptides, the release or expression of which in adipose tissue is under control of PPAR-γ, such as leptin (30) or tumor necrosis factor-α (31), may be involved. Both have been shown to influence insulin secretion in vitro (32,33). Interestingly, a 5-h infusion of Intralipid resulted in a 200% increase of PPAR-γ mRNA in adipose tissue in humans (34). It is thus possible that PPAR-γ-dependent cellular mechanisms are amplified by an Intralipid infusion. In addition, PPAR-γ is activated by a number of mono- and polyunsaturated fatty acids in vitro (3,35), and Intralipid contains significant proportions of unsaturated fatty acids (manufacturer’s information). Thus, it is possible that the FFAs originating from the Intralipid infusion or immediate metabolites acted as ligand for PPAR-γ2, resulting in differential gene expression in X/Ala compared with Pro/Pro. Insulin secretion could then be modulated again by any of the two mechanisms proposed above.

In general, effects of FFAs on insulin sensitivity are more pronounced than those on insulin secretion, and Pro12Ala appears to be important for insulin sensitivity. It is of note, therefore, that we detected a significant modulation of the FFA effect on insulin secretion but not on insulin sensitivity by the Ala allele. This may be explained by the methodology used. The hyperglycemic clamp, which was originally designed for determining insulin secretion (36), has clear limitations in assessing insulin sensitivity, although the correlation with the euglycemic clamp appears to be sufficient for most practical purposes (21). In the present study, insulin levels of only ~200 pmol/l (during second phase) were achieved compared with 400 pmol/l during a standard hyperinsulinenic clamp (insulin infusion of 40 mU/m²). Moreover, we cannot exclude a statistical type 2 error by which we would have missed a significant effect due to the small number of subjects studied. With the 10 subjects in both groups, we had an 80% power to detect only a difference of 50%. Furthermore, a more prolonged exposure to elevated FFAs might have produced a significant effect. Finally, our subjects were all lean (BMI ~23 kg/m²), and it is possible that an effect of the polymorphism on insulin sensitivity becomes evident only in obesity (8).

In conclusion, insulin secretion after Intralipid infusion decreased in subjects with the Pro12Ala polymorphism in PPAR-γ2 compared with control subjects, while insulin sensitivity was unaffected. This suggests that the Pro12Ala polymorphism in the PPAR-γ2 gene might be involved in a differential regulation of insulin secretion in response to increased FFAs in humans and underlines the functional importance of this polymorphism in the pathogenesis of type 2 diabetes.

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