Free fatty acids normalize a rosiglitazone-induced visfatin release

Dominik G. Haider,1 Friedrich Mittermayer,1 Georg Schaller,1 Michaela Arthwohl,2 Sabina M. Baumgartner-Parzer,2 Gerhard Prager,3 Michael Roden,4 and Michael Wolzt1,2

1Department of Clinical Pharmacology, 2Division of Endocrinology and Metabolism, Department of Internal Medicine III, and 3Department of Surgery, Medical University of Vienna; and 4Medical Department of Internal Medicine, Hanusch Hospital, Vienna, Austria

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Haider, Dominik G., Friedrich Mittermayer, Georg Schaller, Michaela Arthwohl, Sabina M. Baumgartner-Parzer, Gerhard Prager, Michael Roden, and Michael Wolzt.

and release is increased in obese subjects (3) and plasma concentrations increased in patients with type 2 diabetes mellitus (7) indicates that visfatin release may represent a nutrient sensor reaction. Consequently, interventions affecting insulin sensitivity, such as altered FFA concentrations or treatment with PPARγ agonists, could influence circulating concentrations of visfatin. On the other hand, changes in visfatin concentrations may synergistically sensitize tissue for insulin, an action described for PPARγ agonists. We have therefore hypothesized that rosiglitazone and FFA can alter visfatin plasma concentrations and have tested this assumption in a double-blind, randomized, placebo-controlled trial. Additionally, human adipocytes were isolated and exposed to rosiglitazone and FFAs to detect dose or time dependencies of visfatin release and to assess signaling pathways involved.

MATERIALS AND METHODS

Study population. The study protocol was approved by the Ethics Committee of the University of Vienna and conforms with the principles of the Declaration of Helsinki, including present revisions and the Good Clinical Practice guidelines.

Sixteen healthy male subjects from whom informed consent was obtained before enrollment were included in this double-blind, randomized, placebo-controlled, parallel-group study. Subject characteristics are shown in Table 1. All of the subjects claimed not to have ingested any prescribed medications or over-the-counter drugs from 2 wk before screening until the study was complete. All subjects were given a complete health examination (including physical examination, ECG, and laboratory screening) ±14 days before the first study day. Subjects were studied after overnight fasting. Studies were conducted in a quiet room with an ambient temperature of 22°C with complete resuscitation facilities.

Experimental setup. Rosiglitazone (8 mg/day, n = 8) or placebo (n = 8) were administered in single daily oral doses for 3 wk. On the last trial day, an intravenous infusion of triglycerides/heparin was administered for 360 min to increase circulating FFA concentrations. Venous blood samples for determination of insulin, glucose, visfatin, and FFA plasma concentrations were drawn before and at timed intervals during triglyceride/heparin infusion. Blood for measurement of FFAs was collected into vials containing tetrahydrolipstatin to avoid in vitro lipolysis, which could have resulted in artificially high FFA concentrations (21). Plasma was separated after centrifugation and stored at −80°C until further analysis. The intravenous infusion of the lipid emulsion (20% Intralipid, 1.5 ml/min; Pharmacia Upjohn, Vienna, Austria) plus heparin (bolus: 200 IU; constant infusion rate: 0.2 IU·kg⁻¹·min⁻¹; Baxter, Vienna, Austria) was administered to achieve systemic FFA concentrations typical for severely insulin-resistant subjects (30, 38). Heparin was added to enhance the breakdown of triglycerides to FFAs in plasma (30, 39).

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Table 1. Subject characteristics before and after 3-wk treatment with placebo or rosiglitazone

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Rosiglitazone</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>28±4</td>
<td>30±5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.0±2.6</td>
<td>25.5±1.6</td>
</tr>
<tr>
<td>Visfatin, ng/ml</td>
<td>0.5±0.0</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Insulin, μU/l</td>
<td>7.2±1.5</td>
<td>7.2±2.4</td>
</tr>
<tr>
<td>FFA, μmol/l</td>
<td>337±177</td>
<td>377±177</td>
</tr>
</tbody>
</table>

Data are given as means ± SD; n = 8/group. BMI, body mass index; FFA, free fatty acids. *P < 0.05 vs. before treatment; †P < 0.05 between groups.

Biochemical assays. Venous blood glucose concentrations were determined by routine laboratory methods. Plasma concentrations of FFAs were measured enzymatically, as described previously (31). Insulin concentrations were determined using a double-antibody RIA (Diagnostics Systems Laboratories, Webster, TX). Visfatin was measured with an ELISA assay from Phoenix Peptides (Karlsruhe, Germany).

Cell culture experiments. Subcutaneous fat was obtained from three lean subjects (1 male and 2 females, 36 ± 5 yr) undergoing elective surgery. Adipocytes were isolated and cultured according to the protocol published by Seboek et al. (33). In brief, after removal, adipose tissue was transferred into phosphate-buffered saline (PBS) solution containing 20 mg/ml bovine serum albumin (BSA). Adipose tissue was dissected from fibrous material and blood vessels and was digested for 90 min in PBS containing 20 mg/ml BSA and 250 U/ml collagenase type Clostridium histolyticum (Biochrom, Berlin, Germany). The dispersed tissue was filtered through nylon mesh (pore size 150 μm) and was centrifuged for 10 min at 200 g. Sedimented cells were resuspended in erythrocyte lysis buffer containing 0.154 mol/l NH₄Cl, 10 mmol/l KHCO₃, and 0.1 mmol/l EDTA for 10 min. The cells were then washed with PBS and resuspended in Dulbecco’s modified Eagle’s medium-nutrient mix F-12 (DMEM-F-12) (1:1) containing 15 mmol/l l-glutamine (Life Technologies, Karlsruhe, Germany) supplemented with 1.125 g/l NaHCO₃, 10% fetal calf serum, and 50 μg/ml gentamicin. Cells were seeded in six-well culture plates (Becton Dickinson, Heidelberg, Germany) at a density of 150,000 cells/well. On the following day, when most cells were attached to the plates, stromal cells were further cultured after being washed twice with PBS in a defined serum-free medium to induce differentiation into adipocytes. The adipogenic medium consisted of DMEM-F-12 supplemented with 1.125 g/l NaHCO₃, 50 μg/ml gentamicin, 10 μg/ml transferrin, 100 mmol/l cortisol, 66 mmol/l insulin, and 200 pmol/l triiodothyronine. During the first 2 days, 20 μmol/l 3-isobutyl-1-methylxanthine were also added to the adipogenic medium. Medium was exchanged every 48 h. Within 16 days of culture, stromal preadipocytes differentiated into adipocytes, which were grown to 95% confluence. All buffers and media were adjusted to a pH of 7.4.

Cells were incubated for 24 h with rosiglitazone (20, 60, 180, and 600 ng/ml; Alexis, San Diego, CA) and a synthetic FFA mixture (FFA-mix: stearic acid, C₁₈:0; oleic acid, C₁₈:1ω9c; linoleic acid, C₁₈:2ω6c; γ-linolenic acid, C₁₈:3ω6; arachidonic acid, C₂₀:4ω6) (1), and different coinoculation experiments with rosiglitazone and FFA were performed. After a preincubation period of 20 h, rosiglitazone was also coincubated for 4 h with DL-β-hydroxybutyrate (500 mM; Sigma, St. Louis, MO) (37), the phosphaditylinositol (PI) 3-kinase inhibitor Wortmannin (100 nM) (5), or a protein kinase B inhibitor (Akt; 10 μM) (the last two from Alexis) (25). Cell culture supernatants of the medium were stored and batch analyzed.

Western blot analysis. Adipocytes were grown to 95% confluence and then incubated for 24 h with 600 ng/ml rosiglitazone in the presence or absence of the PI 3-kinase inhibitor Wortmannin (100 nM) (5) or an Akt inhibitor (10 μM) (25). Cells were solubilized in Tris-buffer (20 mM, pH 8.2) containing 1% Nonidet P-40, 140 mM NaCl, 2 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (all from Sigma). Lysates were kept on ice for 30 min during the reaction with the SDS sample buffer. Proteins were separated on 12–15% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were cut, and strips were incubated with monoclonal antibodies (MAbs) for p85 and phospho-Akt (both from Santa Cruz Biotechnology, Santa Cruz, CA) as measurements for PI 3-kinase and Akt expression, respectively. Strips were washed, and binding of primary MAb was confirmed as described by Jakits et al. (17). Each lane was loaded with 10 μg of protein. Film exposure time was 40 s for all experiments. The expression levels of p85 and phospho-Akt were quantified by densitometry (version 1.1; TotalLab, Newcastle upon Tyne, UK).

Statistical analysis. Because of the skewed distribution of the parameters under study, nonparametric tests were used. Between-group comparisons were performed using the Mann-Whitney U-test. Within groups, effects were tested by Friedman’s ANOVA and the Wilcoxon signed-rank test, respectively. Pearson’s correlation was used for calculation of associations between variables. All calculations were performed using the Statistica software package (release 5.1: StatSoft, Tulsa, OK). P ≤ 0.05 was considered significant. Values are expressed as means ± SD unless indicated otherwise.

RESULTS

Effect of rosiglitazone treatment. Outcome parameters were comparable between groups before treatment with rosiglitazone or placebo (Table 1). Rosiglitazone treatment for 3 wk increased mean circulating visfatin by 283% (P < 0.01) and reduced mean FFA concentrations by 50% (P < 0.01; Table 1). Fasting insulin was not affected by rosiglitazone treatment. Infusion of triglycerides/heparin increased mean FFA concentrations by 1700% (Fig. 1) and circulating insulin to a maximum of 31.9 ± 20.3 μU/l (P < 0.05) during placebo conditions but had no effect on plasma visfatin concentrations. The increase of systemic FFA by infusion of triglycerides/heparin was smaller in subjects receiving rosiglitazone (P < 0.05 vs. placebo). However, the rosiglitazone-induced elevation of circulating visfatin was reduced to baseline during triglyceride/heparin infusion to concentrations seen in controls (Fig. 1). No correlations were detectable between body mass index (BMI) and changes of visfatin induced by rosiglitazone treatment. Furthermore, no association between changes of visfatin during FFA infusion and BMI was demonstrable in this group.

In subjects receiving placebo, there was no relationship between BMI and changes in visfatin over 3 wk or during FFA infusion.

Isolated adipocytes. Incubation of adipocytes with rosiglitazone caused a time- and concentration-dependent visfatin release into the supernatant medium. Rosiglitazone concentrations of 600 ng/ml significantly increased supernatant visfatin concentrations by ~13% after 18 h (P = not significant) and by ~40% after 24 h (P < 0.05; Fig. 2). Incubation of adipocytes with FFA mix had no effect on supernatant visfatin (data not shown), but coincubation of FFA mix with rosiglitazone completely prevented the rosiglitazone-induced visfatin release (P < 0.05; Fig. 3). Consistently, addition of FFA mix to adipocytes preincubated for 20 h with rosiglitazone abrogated the continued visfatin release (Fig. 3). To confirm signal transduction by rosiglitazone, protein expression of p85 (for PI 3-kinase) and phospho-Akt (for Akt) was assessed by Western
blotting. Expression of p85 and phospho-Akt were upregulated 1.7- and 4.5-fold by rosiglitazone, respectively. This increase was mitigated by the inhibition of PI 3-kinase 1.3-fold and by Akt inhibition 2.3-fold in coincubation experiments. Inhibitors alone had no effect on basal p85 and phospho-Akt expression. Likewise, inhibitors of PI 3-kinase or Akt had no effect alone (data not shown) but mitigated the rosiglitazone-induced visfatin release during coincubation (Fig. 4). Again, incubation of PI 3-kinase or Akt inhibitors after 20 h of rosiglitazone preincubation reduced visfatin release into the supernatant medium (Fig. 4). Blockade of FFA mix oxidation with hydroxybutyrate had no effect on the action of rosiglitazone but partially prevented the inhibitory effect of FFA mix on rosiglitazone-induced visfatin secretion during coincubation (Fig. 4).

DISCUSSION

This study demonstrates that rosiglitazone treatment increases circulating visfatin concentrations in healthy humans and induces a release of visfatin from isolated adipocytes into the supernatant medium. This effect is counteracted by FFA and can be influenced in vitro by antioxidant strategies. Furthermore, visfatin secretion from adipocytes by rosiglitazone involves activation of PI 3-kinase and Akt.

FFA elevation alone had no effect on resting visfatin concentrations, and the inhibitory action of FFAs was only detectable during rosiglitazone-induced visfatin release. This is consistent with recent results (29) where plasma visfatin and adipocyte visfatin mRNA was unchanged by FFA infusion in healthy subjects. This secretion pattern suggests a potential nutritional sensor role for visfatin, as also demonstrated for other adipocytokines like leptin, adiponectin, and resistin (18). On the other hand, the effects of the acute and excessive elevation of FFAs in healthy subjects might differ from the insulin resistance pattern observed in type 2 diabetes (10). For instance, animal studies (6, 15, 19, 20, 26, 33, 34, 40) investigating the effect of TZD on lipid infusion or high-fat diet-induced insulin resistance have produced variable results. Interestingly, FFA-induced insulin resistance as assessed by a euglycemic clamp is not improved by pretreatment with rosiglitazone (10). Thus the systemic effect of rosiglitazone on plasma visfatin concentrations is not likely the result of altered insulin sensitivity, and the changes observed occurred in the absence of altered circulating insulin concentrations in healthy subjects.

The marked effect of TZD treatment on adipocyte visfatin secretion is also in good agreement with previous data (8)
demonstrating that rosiglitazone increases visfatin mRNA expression in animal adipocyte deposits. Furthermore, the smaller increases of circulating FFAs in subjects receiving rosiglitazone compared with placebo are similar to results obtained by others (10, 27). Consequently, the action of TZD on FFA handling as paralleled by release of the insulin-mimetic adipocytokine visfatin might contribute to the anti-diabetic drug effect. This may be further influenced by simultaneous changes in other adipocytokines, such as adiponectin (23). However, circulating visfatin concentrations in healthy subjects were much lower than in patients with diabetes mellitus type 2 (7), even when stimulated with rosiglitazone for 3 wk. In a recent study, 3-wk treatment of pioglitazone in patients with type 2 diabetes had no effect on visfatin mRNA expression or systemic release (14). Because visfatin mRNA expression and circulating levels are increased in patients with type 2 diabetes, it is unclear whether this condition overlaps a possible TZD effect as detected in our young and healthy cohort (3, 7, 14). Additionally, the increase compared with levels in diabetic patients appeared to be small (14). Subjects with type 2 diabetes under study in this open-label trial had comorbidity and were of a higher age, which might have influenced the result. In our study, healthy young males were investigated to exclude a potential influence of comorbidity or metabolic derangements. Although several results from clinical studies (13) suggest differences between rosiglitazone and pioglitaza-
zone, cell culture experiments did not confirm major differences regarding PPAR activation (22, 24, 28). However, the agonistic properties on these receptors and other actions might be more augmented for some TZDs (2, 11, 16). It is presently unclear whether the concentrations of visfatin achieved are able to cause pharmacological actions. Furthermore, the systemic anti-diabetic mechanisms of TZDs are not fully characterized yet, and the source of plasma visfatin is unclear. It is also unknown whether adipocytes are particularly sensitive to TZDs to release visfatin because tissue differences may exist.

Our results demonstrate that the effect of rosiglitazone on visfatin release from adipocytes depends on the PI 3-kinase/Akt pathway and confirm previous data on TZD-induced activation of these signal transduction proteins (36). The concentration and time required for visfatin release during rosiglitazone incubation indicates that upregulation of protein synthesis might represent an additional underlying mechanism of elevated visfatin plasma concentrations in response to TZDs. Enhanced visfatin release after 24 h of rosiglitazone incubation in vitro could be related to cell-specific mechanisms of TZD signal transduction. This would be compatible with data demonstrating increased protein release from isolated skeletal muscle cells upon continued exposure to pioglitazone or rosiglitazone (22). The increase of circulating visfatin in vivo after 3 wk of systemic TZD treatment may be due to the fact that not adipocytes alone but also other cells such as macrophages can release visfatin (9). The net contribution of adipocytes and other cells for this finding, however, is speculative and cannot be derived from the present study.

Visfatin gene expression and translational regulation data from humans are limited at present. However, visfatin secretion from isolated skeletal muscle cells upon continued exposure to pioglitazone or rosiglitazone indicates that upregulation of protein synthesis might represent an additional underlying mechanism of elevated visfatin plasma concentrations in response to TZDs. Enhanced visfatin release after 24 h of rosiglitazone incubation in vitro could be related to cell-specific mechanisms of TZD signal transduction. This would be compatible with data demonstrating increased protein release from isolated skeletal muscle cells upon continued exposure to pioglitazone or rosiglitazone (22). The increase of circulating visfatin in vivo after 3 wk of systemic TZD treatment may be due to the fact that not adipocytes alone but also other cells such as macrophages can release visfatin (9). The net contribution of adipocytes and other cells for this finding, however, is speculative and cannot be derived from the present study.

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