Troglitazone Treatment Increases Plasma Vascular Endothelial Growth Factor in Diabetic Patients and Its mRNA in 3T3-L1 Adipocytes

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CBP, cAMP response element binding protein; DMEM, Dulbecco’s modified Eagle’s medium; PPAR-γ, peroxisome proliferator-activated receptor-γ; SU, sulfonylurea; TZD, thiazolidinedione; VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

Troglitazone is one of the thiazolidinediones, a new class of oral antidiabetic compounds that are ligands of peroxisome proliferator-activated receptor-γ. This study on vascular endothelial growth factor (VEGF), also known as vascular permeability factor, was prompted by our clinical observation that the characteristics of troglitazone-induced edema were very similar to those caused by vascular hyperpermeability. When Japanese diabetic patients were screened for plasma VEGF, we found levels to be significantly (P < 0.001) increased in troglitazone-treated subjects (120.1 ± 135.0 pg/ml, n = 30) compared with those treated with diet alone (29.2 ± 36.1 pg/ml, n = 10), sulfonylurea (25.8 ± 22.2 pg/ml, n = 10), or insulin (24.6 ± 19.0 pg/ml, n = 10). Involvement of troglitazone in increased VEGF levels was further supported by the plasma VEGF levels in five patients before treatment (20.2 ± 7.0 pg/ml), after 3 months of troglitazone treatment (83.6 ± 65.9 pg/ml), and 3 months after discontinuation (28.0 ± 11.6 pg/ml). We further demonstrated that troglitazone, as well as rosiglitazone, at the plasma concentrations observed in troglitazone-treated subjects. We found that cross-sectional as well as longitudinal measurements strongly suggest that troglitazone-induced edema is the most likely cause of neovascularization and hyperpermeability in diabetic proliferative retinopathy. Although increased VEGF may be beneficial for subjects with macroangiopathy and troglitazone is currently not available for clinical use, vascular complications, especially diabetic retinopathy, must be followed with great caution in subjects treated with thiazolidinediones.

Vascular endothelial growth factor (VEGF) is an angiogenic and mitogenic substance that seems to be active in vascular endothelial cells (15–17). VEGF plays an important role in tumor growth and in the metastatic process (18). VEGF is also known as vascular permeability factor (VPF), based on its ability to induce microvascular leakage; VEGF is at least four orders of magnitude more potent than histamine (19,20). Importantly, VEGF/VPF is currently considered the most likely cause of neovascularization and hyperpermeability in diabetic proliferative retinopathy (21–24).

RESEARCH DESIGN AND METHODS

Subjects. A total of 30 patients with type 2 diabetes who had already been treated with troglitazone 400 mg daily for 3–6 months were randomly recruited from Yamaguchi University Hospital and affiliated hospitals (troglitazone group; n = 30). Before administration of troglitazone, 23 patients had
TABLE 1
Characteristics of the enrolled subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diet</th>
<th>SU</th>
<th>Insulin</th>
<th>Troglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>(M/F)</td>
<td>5/5</td>
<td>6/4</td>
<td>4/6</td>
<td>12/18</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.1 ± 10.1</td>
<td>63.1 ± 9.4</td>
<td>61.3 ± 8.4</td>
<td>55.9 ± 13.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>62.1 ± 7.5</td>
<td>52.3 ± 7.4</td>
<td>55.1 ± 7.7</td>
<td>52.9 ± 8.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.7 ± 3.5</td>
<td>21.6 ± 3.4</td>
<td>24.8 ± 3.8</td>
<td>22.1 ± 3.9</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>9.4 ± 7.6</td>
<td>8.3 ± 6.1</td>
<td>9.2 ± 5.8</td>
<td>10.9 ± 7.7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.6 ± 1.0</td>
<td>5.8 ± 0.6</td>
<td>7.2 ± 0.8</td>
<td>7.7 ± 0.8</td>
</tr>
</tbody>
</table>

Date are means ± SD.

been treated with sulfonylureas and 7 had been treated with diet alone. The same dose of sulfonylureas had been continued during troglitazone administration. Another 30 randomly recruited patients with type 2 diabetes, who were treated with diet alone (diet group; n = 10), sulfonylurea (SU) alone (SU group; n = 10), or insulin alone (insulin group; n = 10) served as control subjects. Patients with cancer, myocardial infarction, cerebral infarction, and arteriosclerosis obliterans were excluded from this study, because these diseases are known to raise plasma VEGF levels (25,26). We obtained informed consent from all patients, and this study was approved by Yamaguchi University School of Medicine Review Board.

**Measurement of plasma VEGF.** Venous blood (5 ml) was collected into tubes containing di-sodium EDTA. The blood samples were centrifuged at 4°C. Two volumes of 1 ml of plasma were immediately stored at −30°C and maintained at this temperature until analysis. Plasma VEGF was measured with a highly sensitive VEGF enzyme-linked immunosorbent assay system (Amersham, Paisley, UK) using human recombinant VEGF165 as a standard and the specific monoclonal antibody. There was no significant cross-reactivity or interference with other cytokines and growth factors.

**Effects of troglitazone and rosiglitazone on VEGF mRNA levels in 3T3-L1 adipocytes.** 3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% donor calf serum in an atmosphere of 10% CO₂ at 37°C. Differentiation into adipocytes was induced as described previously (27,28). More than 90% of the cells expressed the adipocyte phenotype. For Northern blotting experiments, 3T3-L1 adipocytes were placed in a culture dish and pretreated with serum-free DMEM for 12 h and then washed twice with cold phosphate-buffered saline, and total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). In another set of experiments, cells were treated with 0.1 or 1 μmol/l rosiglitazone for 2 h, and total RNA was similarly isolated.

As a probe, a VEGF cDNA fragment (449 bp), including the coding region of mouse VEGF165 (GenBank accession no. M95200), was cloned from 3T3-L1 adipocytes. Total RNA (7 μg) was electrophoresed in 1% agarose gel, transferred to a nylon membrane, and hybridized with 32P-labeled VEGF cDNA fragments using standard methods (29). After a stringent wash with 0.1 × sodium chloride–sodium citrate (1.5 mmol/l NaCl, 15 mmol/l Na₃citrate, pH 7.0) and 0.1% SDS at 42°C, autoradiographs were digitally scanned and quantitated using BAS2000 (Fujifilm, Tokyo).

**Statistical evaluation.** Multiple comparisons among four groups were performed using the Kruskal-Wallis rank test (post hoc: Dunn’s procedure). Correlation analysis was performed with Spearman’s test and trends with Friedman’s test. One-way analysis of variance (post hoc: Tukey) was used to determine multiple comparisons of VEGF mRNA levels in 3T3-L1 adipocytes. Results are given as means ± SD. Values of P < 0.05 were considered statistically significant.

**RESULTS**

**Effect of troglitazone on plasma VEGF concentration in diabetic patients.** Baseline characteristics of the 60 Japanese patients with type 2 diabetes (27 men and 33 women, mean age 59.0 ± 10.5 years) enrolled are shown in Table 1. These four groups (diet, SU, insulin, and troglitazone) were matched for age, duration of diabetes, and BMI. The only exception was slightly lower HbA1c levels in the SU group (P = 0.004).

As shown in Fig. 1, the plasma VEGF concentrations in the troglitazone group (120 ± 135 pg/ml, n = 30) were significantly higher than those in the diet (29.2 ± 36.1 pg/ml, n = 10), SU (25.8 ± 22.2 pg/ml, n = 10), and insulin (24.6 ± 19.0 pg/ml, n = 10) groups (P < 0.001). In addition, weight gain during the 3- to 6-month troglitazone treatment period showed a weak correlation with plasma VEGF levels in female patients (r = 0.49, n = 18, P = 0.0498; data not shown). No significant differences in plasma VEGF values were observed between male (147.0 ± 191.1 pg/ml, n = 12) and female patients (101.6 ± 80.8 pg/ml, n = 18) treated with troglitazone. Plasma VEGF levels exceeded the normal upper limit of 38.3 pg/ml, reported for Japanese subjects (30), in 8 of 12 male patients and 15 of 18 female patients. The ratios did not differ significantly between men and women.

To further study the possible causal relationship between troglitazone and plasma VEGF elevation, plasma VEGF was measured before and after troglitazone treat-

![FIG. 1. Plasma VEGF concentrations in diabetic subjects. Plasma VEGF levels of individual patients (*) are indicated. *P < 0.001 vs. other groups. Horizontal lines represent mean values.](image-url)
ment in five other type 2 diabetic patients (two men and three women, all treated with SU: glibenclamide). The same dose of glibenclamide was maintained throughout the study. Troglitazone treatment had increased plasma VEGF levels from 20.2 ± 7.0 pg/ml before to 83.6 ± 65.9 pg/ml 3 months after the start of administration. Although no hepatic dysfunction was observed in any of these five patients, troglitazone administration was terminated based on the report of possibly fatal hepatocellular injury (11,12). Plasma VEGF had returned to the basal level (28.0 ± 11.6 pg/ml) 3 months after discontinuation (Fig. 2).

**Effect of troglitazone and rosiglitazone on VEGF mRNA levels in 3T3-L1 adipocytes.** We next tested whether troglitazone affects VEGF expression in vitro using cultured adipocytes. Northern blot analysis demonstrated that incubation with 1 and 10 μmol/l troglitazone for 2 h significantly (P < 0.05) increased VEGF mRNA levels in 3T3-L1 adipocytes (Fig. 3A and B). The increase in VEGF mRNA was barely detectable with 0.1 μmol/l troglitazone but was marked with 1 μmol/l troglitazone, which is within the concentrations observed clinically. VEGF mRNA levels peaked with 10 μmol/l troglitazone (2.55 ± 0.38-fold increase above basal, n = 4). When cells were incubated with 2 μmol/l troglitazone, a concentration exerting a submaximal effect, the increase in the VEGF mRNA level was not detected at 30 min but was clearly detected at 1 h. The VEGF mRNA level peaked within 2 h and was maintained at this level for at least 6 h (data not shown). The 1 μmol/l concentration of troglitazone seemed to be as potent as hypoxia caused by N2 flush. We also studied the effects of rosiglitazone, another TZD, on VEGF expression in 3T3-L1 adipocytes. We found that 2-h incubation with 0.1 or 1 μmol/l rosiglitazone dose-dependently increased VEGF mRNA levels, with a statistical significance (Fig. 4A and B).

**DISCUSSION**

Cross-sectional as well as longitudinal measurements of plasma VEGF levels, in diabetic patients, demonstrated that troglitazone administration is involved in an increase in plasma VEGF levels. Furthermore, we have demonstrated that troglitazone as well as rosiglitazone, at concentrations observed clinically, dose-dependently increases VEGF mRNA levels in 3T3-L1 adipocytes.

VEGF was initially identified based on its ability to stimulate vascular permeability and was subsequently shown to be an endothelial cell-specific mitogenic and angiogenic factor (15,19). This growth factor has been demonstrated to be involved in normal and pathological processes, including tumor progression, collateral vessel formation in ischemic tissues, and inflammation. Concerning diabetes, it is of particular importance that VEGF may be involved in the development of diabetic retinopathy, including retinal edema, hemorrhage, and neovascularization (31,32). Therefore, our findings indicate potential adverse effects of troglitazone on this major diabetic complication. Although it is also possible that increased VEGF levels may instead exert beneficial effects on revascularization (24), for example in diabetic subjects with arteriosclerosis obliterans, vascular complications (especially retinopathy) must be followed with great care in troglitazone-treated subjects. It might also be possible that an increase in VEGF levels, if it occurs in TZD-treated subjects.
animals, plays a role in the induction and proliferation of colon polyps in these animals (12,13).

An increase in VEGF levels may be associated with troglitazone-induced edema, because VEGF is a potent vascular permeability factor. An association of edema with increased VEGF levels has been demonstrated in many pathological conditions, including Crow-Fukase syndrome (33), ovarian hyperstimulation syndrome, (34) and pre-eclampsia (35). It is also possible that increased VEGF levels contribute to weight gain, by causing fluid retention in the third space, in troglitazone-treated patients, even in those without apparent edema. We observed a weak correlation between weight gain and plasma VEGF levels in female diabetic patients treated with troglitazone.

VEGF mRNA and protein secretion are reportedly induced by adipocyte differentiation (36), and TZDs such as troglitazone induce adipocyte differentiation through binding to PPAR-γ (37). Therefore, it is not unreasonable to speculate that troglitazone increases plasma VEGF levels. However, adipocyte differentiation is not likely to be responsible for the increased VEGF mRNA in 3T3-L1 adipocytes observed herein, because the adipocytes we used were fully differentiated and no apparent change in adipocyte number or shape was noted with 2-h troglitazone-treatment. Troglitazone is believed to act predominantly via an increase in transcription, exerting through PPAR-responsive elements. The consensus PPAR-responsive element sequence is AGGTCA-NAGGTCA (DR-1), but no such sequence is present in the 1.2-kb 5′-upstream region of the VEGF gene (38). However, a recent study showed that troglitazone stimulates the interaction between PPAR-γ and cAMP response element (CREB)-binding protein (CBP) (39). CBP is a large nuclear molecule that coordinates a variety of transcriptional pathways with chromatin remodeling. CBP has many nuclear factor responsible elements that interact with PPAR-γ, estrogen receptor, thyroid-hormone receptor, and retinoid X receptor (40,41). Furthermore, a CBP binding site is present in the VEGF gene. Therefore, troglitazone may regulate VEGF gene expression through modulating the interaction between CBP and PPAR-γ. Obviously, further studies are needed for testing this possibility. Because incubation with 0.1 or 1 μmol/l rosiglitazone for 2 h also dose-dependently increased VEGF mRNA levels in 3T3-L1 adipocytes, it seems that our findings are not limited to troglitazone but can rather be generalized to all glitazones. This important issue also merits further investigation.

In summary, we demonstrated that 1) troglitazone treatment raised plasma VEGF levels in diabetic patients and 2) troglitazone and rosiglitazone increased VEGF mRNA levels in 3T3-L1 adipocytes. Vascular complications, most notably diabetic retinopathy, must be followed carefully in those whose treatment regimens include TZDs.

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