Troglitazone Treatment Increases Protein Kinase B Phosphorylation in Skeletal Muscle of Normoglycemic Subjects at Risk for the Development of Type 2 Diabetes

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We investigated whether the effect of troglitazone on glucose disposal is associated with altered insulin signaling. Nondiabetic first-degree relatives of type 2 diabetic patients (age 30 ± 2 years, BMI 30 ± 1 kg/m^2; n = 20) were randomized in a double-blind manner to 3 months of troglitazone (200 mg/day) or placebo treatment. Before and after treatment, 3-h euglycemic-hyperinsulinemic glucose clamps (40 mU · m^{-2} · min^{-1}) were performed, and muscle biopsies were obtained immediately before and after the clamps. In the biopsies, insulin receptor kinase (IRK) activity, insulin receptor substrate (IRS)-1–associated phosphatidylinositol 3-kinase (PI3K) activity, Ser^{473} and Thr^{308} phosphorylation of protein kinase B (PKB), and protein expression of IRS-1, IRS-2, phosphoinositol-dependent kinase-1 (PDK-1), PKB, and GLUT-4 were determined. After troglitazone treatment, insulin-stimulated glucose disposal was increased compared with pretreatment and placebo (279 ± 37 vs. 211 ± 26 and 200 ± 25 mg · m^{-2} · min^{-1}; both $P < 0.05$). IRK and PI3K activities were not altered by troglitazone, but PKB Ser^{473} phosphorylation was enhanced compared with pretreatment and placebo at the clamp insulin level (138 ± 36 vs. 77 ± 16 and 55 ± 13 internal standard units; both $P < 0.05$) and with pretreatment at the basal level (31 ± 9 vs. 14 ± 4 internal standard units; $P < 0.05$). PKB Thr^{308} phosphorylation also tended to be higher, but this was not statistically significant. Troglitazone did not alter insulin receptor number or IRS-1, IRS-2, PKB, PDK-1, or GLUT-4 protein expression. We conclude that increased PKB phosphorylation may contribute to the insulin-sensitizing effects of thiazolidinediones in human skeletal muscle. *Diabetes* 51:2691–2697, 2002

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FDR, first-degree relative of type 2 diabetic patients; FFA, free fatty acid; IRK, insulin receptor kinase; IRS, insulin receptor substrate; PDK-1, phosphoinositol-dependent kinase-1; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PPAR, peroxisome proliferator–activated receptor.

Peroxisome proliferator–activated receptor (PPAR)γ agonists represent a new class of antidiabetic agents that improve glycemic control by increasing insulin sensitivity in target tissues such as muscle and adipose tissue (1–4). PPARγ is mainly expressed in adipose tissue and, to a lower extent, in muscle (5–7). The effects of PPARγ agonists on insulin sensitivity in skeletal muscle may therefore be direct (6,8–10) and/or involve adipose tissue PPARγ stimulation, which alters the secretion of factors by the adipocytes that modulate skeletal muscle insulin action (3,6,11–13).

One way in which PPARγ agonists could improve insulin-stimulated glucose disposal in skeletal muscle is by directly or indirectly enhancing insulin signaling. Insulin elicits its biological actions by an activation of the insulin receptor kinase (IRK), which leads to a tyrosine phosphorylation of several insulin receptor substrates (14). In skeletal muscle, insulin receptor substrate (IRS)-1 appears to be the most important of these substrates (15), and IRS-1 tyrosine phosphorylation promotes its association with the p85 regulatory subunit (p85) of phosphatidylinositol 3-kinase (PI3K) and activation of the kinase (14). PI3K activation has been shown to be a necessary, albeit insufficient, step for insulin-induced glucose transport (14,15). Insulin signaling downstream of PI3K appears to be mediated, at least in part, by the serine/threonine kinase protein kinase B (PKB), which is activated by phosphorylation at the Thr^{308} and Ser^{473} residues (16–21). Several lines of evidence suggest that PPARγ agonists enhance insulin signaling. In rat muscle (22), L6 myotubes (23), isolated human adipocytes (24), and cultured human muscle cells (10), PPARγ agonists have been shown to increase IRS-1 phosphorylation (22,23), PI3K activity (10), and/or PKB phosphorylation (10,22,24). Others have observed thiazolidinedione-induced increases of protein kinase C-ζ and -λ activation in rat adipocytes (25). As with PKB, protein kinase C-ζ and -λ are thought to be involved in the signaling downstream of PI3K. PPARγ agonist effects on insulin signaling and/or glucose disposal could be mediated, at least in part, by an altered expression of signaling proteins. This is supported by findings in isolated human adipocytes in which PPARγ agonists increased IRS-2 (26) or p85 expression (24). Moreover, in cultured cells and tissues from animals, the ability of various PPARγ agonists to augment glucose transport has often

TABLE 1
Subject characteristics

	Troglitazone group	Placebo group
n (F/M)	10 (5/5)	10 (7/3)
Age (years)	30.2 ± 2.4	30.8 ± 1.8
BMI (kg/m^2)	29.1 ± 0.8	31.1 ± 1.4
Fat-free mass (%)	67.1 ± 2.7	69.3 ± 3.0

Data are means ± SE.

(8,22,27), but not always (25,28), been associated with increases in GLUT-1 and/or GLUT-4 protein expression.

Despite these data in animals and cell systems, it remained unclear whether PPARγ agonist treatment of human subjects enhances insulin-stimulated glucose disposal, at least in part, by modulating known insulin signaling steps in skeletal muscle. If one addresses this question by comparing insulin signaling in diabetic subjects before and after PPARγ agonist treatment, one encounters the problem that any reduction of hyperglycemia, regardless of how it is achieved, could improve insulin signaling and action (29–31). In two recent studies with type 2 diabetic patients (4,32), the effects of the PPARγ agonist troglitazone on insulin signaling and action were therefore compared with the effects of metformin. Since both compounds led to comparable improvements in metabolic control but only troglitazone treatment enhanced PI3K and PKB activity in muscle (32) or PKB phosphorylation in adipocytes (4), the authors concluded that it was unlikely that a generalized reduction in glycemia was responsible for the observed troglitazone effects on insulin signaling.

The goal of our present study was to investigate the effects of a 3-month treatment with troglitazone on insulin signaling and action as independently as possible from treatment-induced changes of metabolic control. Therefore, the study was not performed with type 2 diabetic patients. Instead, first-degree relatives of type 2 diabetic patients (FDRs) who had normal glucose tolerance but were insulin resistant compared with nondiabetic subjects without a family history of diabetes (33) were recruited. The volunteers were randomized into two groups in a double-blind manner and treated for 3 months with troglitazone or placebo. Before and after these treatments, euglycemic-hyperinsulinemic glucose clamps were performed and skeletal muscle biopsies obtained at basal and steady-state clamp insulin levels. In these biopsies, the protein expression, insulin-stimulated phosphorylation, or activity of known insulin signaling intermediates were evaluated.

RESEARCH DESIGN AND METHODS

Subjects. A total of 20 FDRs (with at least 1 first-degree relative [parent] and 1 second-degree relative with type 2 diabetes) were studied (Table 1). All were Caucasians and had a sedentary lifestyle. The subjects were instructed not to engage in excessive physical activity for at least 2 days before each metabolic investigation. None had impaired glucose tolerance as determined by an oral glucose tolerance test or any other disease as evaluated by clinical investigation and standard laboratory assessments. Before entry into the study, the purpose and risks of the experimental protocol were carefully explained to all of the volunteers and informed written consent was obtained. The protocol was approved by the local ethics committee and was in accordance with the Helsinki II declaration.

Study protocol. Subjects were randomized into two groups in a double-blind manner and treated for 3 months with troglitazone (200 mg/day) or placebo (containing excipients similar to the troglitazone tablets). Before and after these treatment periods, 3-h euglycemic-hyperinsulinemic glucose clamps were performed. For the clamp studies, subjects were admitted to the clinical research center at 8:00 A.M. after an overnight fast. Intravenous catheters were inserted for infusion of insulin and glucose into the antecubital vein, and blood samples for plasma glucose analyses were drawn from a catheter of the dorsal wrist vein of the opposite arm. During the study, the subjects remained in a supine position with the hand designated for collection of blood samples placed and maintained in a heated box for arterialization of venous blood (34). After a 30-min relaxation period, a surface-adjusted primed-constant dose of [3-^{3}H]glucose was given (14 μCi Ci/m^2) (DuPont-New England Nuclear, Boston, MA). This was followed by a continuous infusion of [3-^{3}H]glucose (0.14 $\mu Ci \cdot min^{-1} \cdot m^{-2}$), and, after a 120-min basal tracer equilibration period, insulin (Actrapid; Novo-Nordisk, Bagsvaerd, Denmark) was infused at a rate of 40 $mU \cdot min^{-1} \cdot m^{-2}$ for 180 min. Euglycemia (5.9 ± 0.1 mmol/l) was maintained using a variable infusion of 18% glucose with added [3-^{3}H]glucose (34). Steady-state periods were defined as the last 30 min during basal and insulin-stimulated measurements, respectively, and the glucose disposal rate was calculated from the plasma concentrations of tritiated glucose and plasma glucose (34). Body composition was estimated with the bioimpedance method using the formula of Kushner and Schoeller (35). Plasma glucose, insulin, triglycerides, and free fatty acid (FFA) were analyzed as described (34).

Muscle biopsies. At the end of the basal and insulin-stimulated steady-state periods, percutaneous vastus lateralis muscle biopsies were obtained under local anesthesia (2% lidocaine without epinephrine) 20 cm above the knee (34). This was done at separate incision sites to avoid local effects of the prior biopsy and blood contamination. The biopsies were immediately frozen in liquid nitrogen. For the subsequent analytical procedures, ~50 mg frozen tissue was homogenized at 4°C in 500 μl solubilization buffer (8 mmol/l EDTA, 20 mmol/l HEPES, 1% IGEPAL, 530 mmol/l NaF, 30 mmol/l sodium pyrophosphate, 7 mmol/l sodium vanadate, 2 mmol/l dichloroacetic acid, 9 mmol/l phenylmethylsulfonyl fluoride, 21 mmol/l benzamidine, 5 μmol/l leupeptin, 4 μmol/l pepstatin, and 10 μg/ml aprotinin; pH 7.4). After 20 min, detergent insoluble material was removed by centrifugation (12,000*g* for 20 min at 4°C), and the protein concentration was measured using a protein-dye binding assay (BioRad, Munich).

IRK and binding capacities. These were measured as previously described (34,36,37). Briefly, 40 μl muscle sample lysates were added to microwells coated with anti-insulin receptor antibody for 16 h at 4°C. The wells were washed, and receptor-mediated ^{32}P incorporation into recombinant IRS-1 (17 nmol/l) (Upstate Biotechnology, New York, NY) was measured at 120 nmol/l ^{32}P-ATP. [^{125}I-Tyr-A^{14}]monoiodoinsulin (Amersham-Pharmacia, Freiburg, Germany) binding to immobilized insulin receptors was also measured in the wells (36). Insulin binding capacity was defined as the amount of specifically bound insulin at a concentration of 8.7 nmol/l (34,37).

PI3K activity. Solubilized muscle (500 μg protein) was added to protein G Sepharose beads (Pierce, Rockford, IL) to which anti–IRS-1 antibody (2 μg) (Upstate Biotechnology) had been bound. After 4 h at 4°C, the beads were washed three times with PBS containing 1% IGEPAL and 0.1 mmol/l sodium orthovanadate (pH 7.5), three times with 500 mmol/l LiCl, 100 mmol/l Tris, and 0.1 mmol/l sodium orthovanadate (pH 7.5), and twice in reaction buffer (10 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, and 0.1 mmol/l sodium orthovanadate; pH 7.5). The pellets were then suspended in 60 μl of reaction buffer supplemented with 15 mmol/l $MgCl_2$ and phosphatidylinositol (20 μg) (Sigma, Deisenhofen, Germany). The phosphorylation reaction was started by the addition of 10 μl of a solution that contained 720 μmol/l ATP (25 μCi/mmol) (NEN, Dreieich, Germany). After 20 min at room temperature, the reaction was stopped by the addition of 40 μl 25% HCl and 190 μl methanol/chloroform (1:1). The organic phase was extracted and applied to a silica gel thin-layer chromatography plate. Phosphatidylinositol-3 phosphorylation was determined using a phosphorimager.

Immunoblots. Tissue lysates (100 μg protein for the detection of IRS-1, IRS-2, phosphoinositol-dependent kinase-1 [PDK-1], PKB, PKB Ser^{473}, and PKB Thr^{308} phosphorylation and 50 μg protein for the detection of GLUT-4) were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Proteins were detected with antibodies against IRS-1 or -2, p85, PDK-1 (all from Upstate Biotechnology), PKB, Ser^{473}- or Thr^{308}-phosphorylated PKB (all from BioLabs, Beverly, MA) or GLUT-4 (Chemicon International, Temecula, CA), horseradish peroxidase–labeled secondary antibodies (Dako Diagnostics, Hamburg, Germany), and enhanced chemiluminescence (ECL; Amersham-Pharmacia, Freiburg, Germany). The antibodies against PKB and phosphorylated PKB were known to detect the α, β, and γ isoforms. Bands were quantified densitometrically with Molecular Analyst software (BioRad).

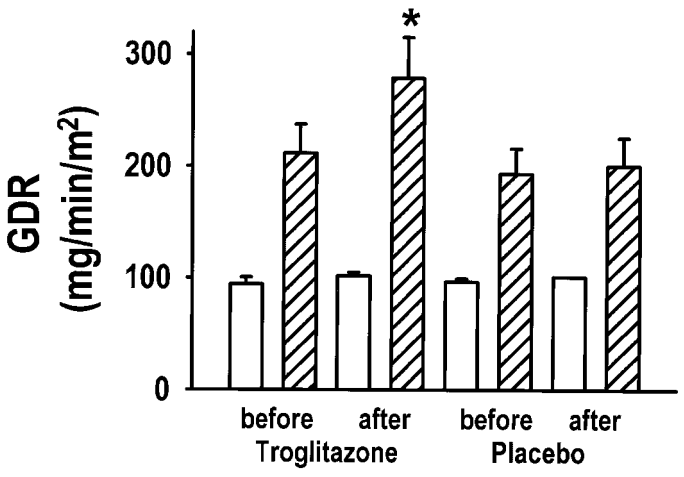

FIG. 1. Effects of troglitazone or placebo treatment on glucose disposal rate (GDR). Rates of total body glucose disposal at basal (□) or steady-state clamp insulin concentrations (▨) before and after 3 months of troglitazone or placebo treatment are shown. Data represent means ± SE. *P < 0.05 compared with respective pretreatment value.

Data analysis. To exclude distortion of the results stemming from interassay variation between PI3K assays or immunoblots, material from two troglitazone-treated and two placebo-treated subjects was analyzed in parallel per experiment (total of 16 samples: basal and insulin-stimulated biopsies from two clamps in four subjects). For most parameters, at least two internal standard samples (pooled human muscle samples from clamp biopsies) were also analyzed per experiment, and the densitometric (Western blots) or phosphorimager-derived value (PI3K activity) obtained for the individual samples was expressed as a percentage of the value obtained for these standards. For some parameters, internal standard data were not available and arbitrary units were calculated. This was done by dividing the densitometric- or phosphorimager-derived value obtained for the individual sample by the average value of all 16 samples that had been studied in parallel within the same experiment. Statistical comparisons were carried out by t tests for paired or unpaired data where appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

The subjects in the troglitazone and placebo group did not differ in BMI or percent fat-free mass (Table 1), and there were no significant changes in these parameters during the treatment period (data not shown). Basal glucose disposal was not different after treatment with troglitazone (Fig. 1); however, the plasma insulin concentration was decreased (Table 2), indicating increased insulin sensitivity. The insulin effect on glucose disposal was 50% higher after treatment with troglitazone, an effect not observed with placebo (Fig. 1). Troglitazone treatment also resulted in lower fasting plasma triglyceride levels (Table 2). Fasting FFA levels were not different after troglitazone treatment, although there was a significant difference of the suppressed FFA levels at the end of the clamp (Table 2).

The increase of plasma insulin concentration from basal to steady-state clamp levels resulted in two- to threefold increases of IRK and PI3K activities, and no significant effects of the troglitazone treatment were observed (Fig. 2*A* and *B*). PKB Ser^{473} phosphorylation was also increased three- to fourfold by the clamp insulin infusion, and, in contrast to IRK and PI3K activities, in which no troglitazone effects were found, insulin-stimulated PKB Ser^{473} phosphorylation was significantly higher after troglitazone treatment (94 or 152% compared with the respective pretreatment or placebo treatment level, respectively) (Fig. 2*C*). PKB Ser^{473} phosphorylation in the biopsies obtained at the basal insulin level was also significantly higher after troglitazone treatment compared with pretreatment. There was also a trend for an enhanced insulin action on the phosphorylation of the other activating site of PKB, Thr^{308}, after troglitazone treatment (Fig. 2*D*). However, most likely due to the higher variation of the results, there was no statistically significant difference.

There was a tendency to slightly higher protein expression of PDK-1 and PKB in the biopsies that were obtained after troglitazone treatment; however, statistically significant effects on protein expression were not observed for any of the investigated proteins (Table 3).

DISCUSSION

In the present placebo-controlled study, we report that the increase in insulin-stimulated glucose disposal after 3 months of troglitazone treatment was associated with an increased Ser^{473} phosphorylation of PKB at basal and clamp insulin concentrations. Because our study population consisted of insulin-resistant subjects with a genetic background for type 2 diabetes but normal glucose tolerance, the treatment did not alter the plasma glucose concentration. The observed increases of PKB phosphorylation and glucose disposal were therefore independent of a normalization of plasma glucose levels that by itself could have lead to a normalization of insulin signaling and action (29–31).

PKB has been proposed to be an intermediate in the signaling pathway by which insulin, among other things, controls glucose uptake (17,19,20). Although there remains some controversy with respect to the exact role of PKB in the insulin signaling toward glucose uptake (38,39), strong lines of evidence indicate that Thr^{308} and Ser^{473} phoshorylation and concomitant activation of the enzyme are important. Thus, overexpression of constitutively ac-

TABLE 2
Metabolic parameters before and after treatment with troglitazone or placebo (all in plasma)

	Troglitazone group		Placebo group	
	Before	After	Before	After
Fasting glucose (mmol/l)	6.0 ± 0.2	5.9 ± 0.2	5.9 ± 0.1	6.0 ± 0.1
Fasting triglycerides (mmol/l)	1.97 ± 0.38	1.39 ± 0.32*†	2.05 ± 0.48	2.36 ± 0.50
Fasting FFA (mmol/l)	0.49 ± 0.05	0.43 ± 0.07	0.44 ± 0.07	0.47 ± 0.07
Fasting insulin (pmol/l)	64.2 ± 7.6	46.4 ± 5.9*†	75.1 ± 10.7	74.5 ± 7.6
Clamp insulin (pmol/l)	392 ± 22	376 ± 12	437 ± 26	425 ± 26
Clamp FFA (mmol/l)	0.04 ± 0.01	0.02 ± 0.01*	0.07 ± 0.02	0.06 ± 0.01

Data are means ± SE. *$P < 0.05$ compared with pretreatment; †$P < 0.05$ compared with placebo treatment.

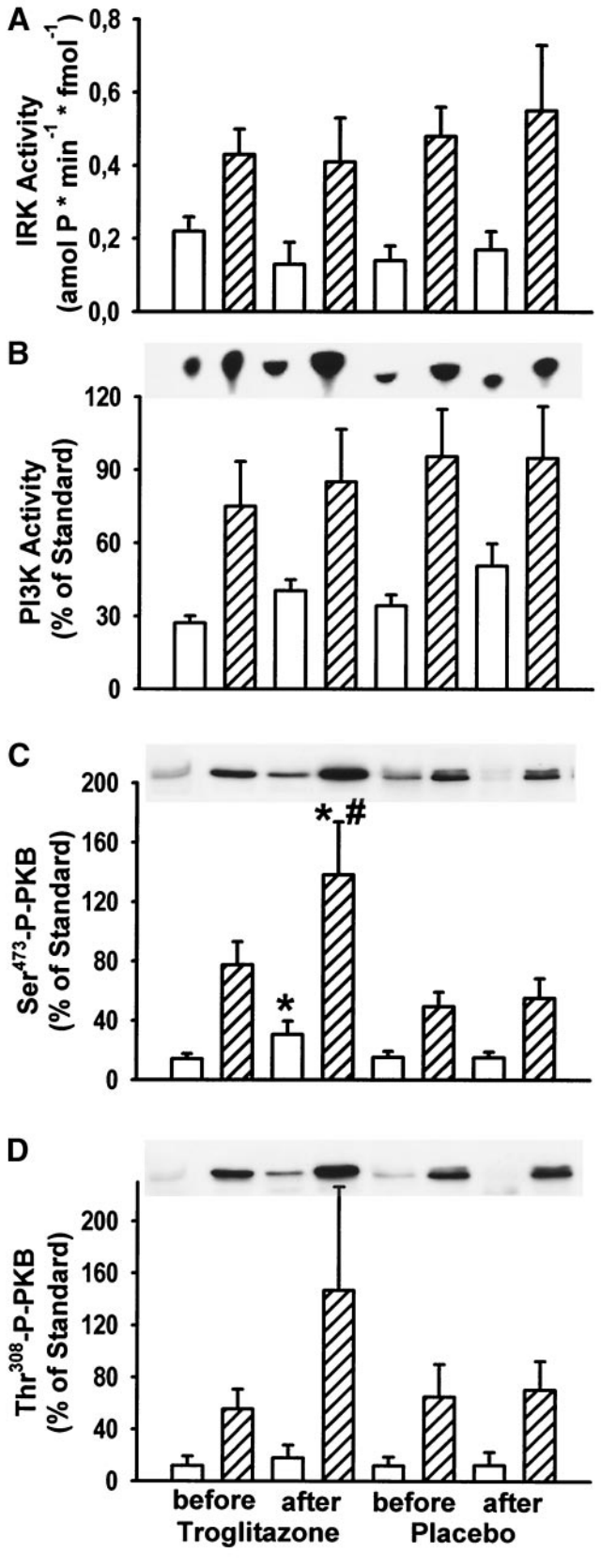

FIG. 2. Insulin signaling. Muscle biopsies obtained at basal (□) or steady-state clamp insulin concentrations (▨) before and after 3 months of troglitazone or placebo treatment were solubilized, and IRK activity (*A*), IRS-1–associated PI3K activity (*B*), and Ser^{473} (*C*) or Thr^{308} phosphorylation (*D*) of PKB were measured as described. Data represent means ± SE. **P* < 0.05 compared with respective pretreatment value; #*P* < 0.05 compared with placebo treatment.

tive forms of PKB induced glucose uptake, GLUT-4 translocation, and glycogen synthesis (16,40), and recently it was demonstrated that mice deficient in PKB-β, due to targeted disruption of the PKB-β locus, were insulin resistant in muscle and liver (21). Finally, in IRS-1–deficient mice, adenovirus-mediated gene therapy with an IRS-1 mutant lacking the binding site for p85 enhanced PKB activity and normalized insulin sensitivity without increasing PI3K activity, suggesting that PKB activation might be sufficient for reducing insulin resistance even without increasing PI3K activity (18). Therefore, the increased PKB Ser^{473} phosphorylation and the tendency to increased Thr^{308} phosphorylation after long-term troglitazone treatment observed in our study could be part of a mechanism that leads to the improved insulin sensitivity of glucose uptake and glucose storage (41) in human skeletal muscle.

The notion that the insulin-sensitizing effect of PPARγ agonists in skeletal muscle involves increased signaling at the level of PKB is consistent with previous reports. An increased insulin-stimulated PKB phosphorylation has also been observed in skeletal muscle of obese Zucker fatty rats that had been treated with troglitazone for 3 weeks (22), in the membrane fraction of cultured human skeletal muscle cells that had been treated with troglitazone for 5 days (10), and in isolated human adipocytes that had been treated for 12 h with the PPARγ agonist BRL 49563 (24). Moreover, increased insulin-stimulated adipocyte PKB serine phosphorylation (4) and enhanced insulin-stimulated skeletal muscle PI3K and PKB activity (32) have most recently been reported in type 2 diabetic subjects after troglitazone treatment for 3–4 months. Because these two latter studies, in contrast to our study, were performed with type 2 diabetic subjects, treatment-induced changes in plasma glucose levels were inevitable. Therefore, to demonstrate that the troglitazone effects on insulin signaling were not simply due to the changes in glycemia, a similar group of subjects was treated with metformin. Since both compounds led to comparable improvements in metabolic control, but effects on PI3K and PKB were only observed with troglitazone, the authors concluded that it was unlikely that a generalized reduction in glycemia was responsible for the observed troglitazone effects on insulin signaling (4,32).

In contrast to several previous reports, in which the troglitazone-induced increases in PKB phosphorylation or activity appeared to be related to an increased PI3K activity (10,32) or increased IRS-1 phosphorylation (22), our data suggest that the increase in PKB phosphorylation occurred in the absence of a change in IRS-1–associated PI3K activity. We cannot exclude that small differences in IRS-associated PI3K activity might have been missed in our study. On the other hand, it is certainly conceivable that, as suggested by our data, the troglitazone effect on PKB phosphorylation was independent of changes in PI3K activity. For example, it has been observed that hyperglycemia reduced insulin-stimulated PKB phosphorylation despite PI3K activity being unchanged (30) or even increased (31). The current model for insulin stimulation of PKB phosphorylation and activity involves the PI3K-mediated generation of phosphatidylinositol-3,4,5-tris and 3,4-bisphosphate in the plasma membrane. This induces the

TABLE 3
Insulin signaling protein expression before and after treatment with troglitazone or placebo

	Troglitazone group		Placebo group	
	Before	After	Before	After
IBC (fmol/mg)				
Basal	22.0 ± 2.6	24.6 ± 3.5	23.1 ± 3.1	21.2 ± 3.5
Clamp	23.2 ± 3.2	25.6 ± 3.9	25.7 ± 3.9	22.8 ± 3.0
IRS-1 (% of standard)				
Basal	138 ± 33	93 ± 11	80 ± 21	88 ± 17
Clamp	97 ± 16	96 ± 20	104 ± 23	104 ± 25
PKB (% of standard)				
Basal	143 ± 28	183 ± 51	122 ± 23	122 ± 25
Clamp	120 ± 18	188 ± 57	119 ± 24	99 ± 23
IRS-2 (AU)				
Basal	1.26 ± 0.31	1.20 ± 0.25	0.93 ± 0.43	0.73 ± 0.24
Clamp	1.22 ± 0.28	0.90 ± 0.22	0.76 ± 0.23	0.99 ± 0.28
PDK-1 (AU)				
Basal	0.88 ± 0.11	1.08 ± 0.15	0.83 ± 0.12	0.90 ± 0.10
Clamp	1.04 ± 0.18	1.20 ± 0.14	1.03 ± 0.12	1.04 ± 0.21
GLUT-4 (AU)				
Basal	0.81 ± 0.08	1.01 ± 0.20	0.84 ± 0.21	0.95 ± 0.13
Clamp	1.14 ± 0.18	0.98 ± 0.15	1.24 ± 0.26	1.08 ± 0.12

Data are means ± SE; $n = 8–10$. Insulin binding capacity (IBC) in the skeletal muscle lysates was determined as described in RESEARCH DESIGN AND METHODS and expressed per milligram solubilized muscle protein. The amounts of the other proteins were determined by the separation of muscle lysates with similar protein concentration by SDS-PAGE and subsequent immunoblots with the respective antibodies. AU, arbitrary units.

translocation of PKB from the cytosol to the plasma membrane and its phosphorylation by PDK-1 on the Thr^{308} site. PDK-1 appears to also be involved in the phosphorylation of the Ser^{473} site (17,19,42), and overexpression of PDK-1 has been shown to increase GLUT-4 translocation in adipocytes (43). Thus, there are multiple locations where the signal between PI3K and PKB could be modulated, including PDK-1 and the phosphoinositide phosphatases (44).

There was a tendency to slightly higher protein expression of PDK-1 and PKB in the biopsies obtained after troglitazone treatment (Table 3), and although this was not statistically significant, a contribution of such changes in protein expression to the troglitazone effects on PKB phosphorylation and/or insulin sensitivity cannot be excluded. Our finding that the insulin binding capacity and the protein expression of IRS-1, IRS-2, and GLUT-4 were not changed after therapy is in partial contrast to findings in human adipocytes, in which increased IRS-2 (26) or GLUT-4 (4) expression has been observed, and may indicate tissue-specific modulation of protein expression. The observation that GLUT-4 protein expression was not increased by troglitazone is also in contrast to a previous report in rat skeletal muscle (22) but consistent with most data in muscle cell culture systems (8,10,28).

Evidently, due to the limited amount of tissue available, only a small spectrum of the proteins that might contribute to the effects of PPARγ agonists on insulin signaling has been studied, and it is not clear whether, or to what degree, the effects on signaling observed in the present study contributed to the increase in insulin sensitivity. Other effects of PPARγ-agonists on insulin signaling that have been described include, for example, increased cbl-associated protein expression in 3T3L1-adipocytes (45) or increased protein kinase C-ζ and -λ activation in rat adipocytes (25). Nevertheless, our data demonstrate that a 3-month treatment of nondiabetic insulin-resistant human subjects enhances insulin action on PKB phosphorylation in skeletal muscle, the most important tissue for peripheral insulin-stimulated glucose disposal (46).

Whereas some studies suggest that skeletal muscle PKB activity or phosphorylation in response to insulin may be impaired in type 2 diabetes (47), other studies (48), including our own (33), indicate that this is not the case. Moreover, the FDRs in our study were insulin resistant compared with non-FDR control subjects, but insulin-stimulated PKB phosphorylation was similar (33). The troglitazone effect observed in the present study therefore does not appear to reflect a normalization of a diabetes-associated impairment of insulin-stimulated PKB phosphorylation or activity, but rather an enhancement above normal. This does not exclude, however, that the increased PKB phosphorylation contributes to the observed normalization of glucose disposal. It might, for example, compensate for impairments that exist in FDRs and type 2 diabetic subjects at other locations, e.g., downstream of the investigated signaling cascade or in alternative signaling pathways (33).

The advantage of an investigation of FDRs rather than type 2 diabetic subjects was that treatment-induced metabolic changes, which by themselves could have influenced insulin signaling, were much smaller. Thus, there were no detectable troglitazone effects on fasting plasma glucose or FFA concentrations, both of which are known to influence insulin signaling (30,31,49). FFA levels at the clamp insulin level were very low; therefore, it appears rather unlikely that the troglitazone-induced difference in plasma FFA levels observed under this condition affected insulin signaling. This does not, of course, exclude the possibility that the troglitazone effects on skeletal muscle PKB phosphorylation and/or glucose disposal were through secondary mechanisms. For example, altered

intramyocellular lipid levels (50), altered local metabolite concentrations, or altered adipocyte mediators of insulin resistance have contributed to the altered insulin signaling and/or action.

In summary, we have shown in normoglycemic FDRs that the increased glucose disposal after a 3-month treatment with troglitazone was associated with increased PKB phosphorylation in skeletal muscle. This increase in PKB phosphorylation may contribute to the insulin-sensitizing effects of PPARγ agonists.

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