

Effects of Metformin and Rosiglitazone Treatment on Insulin Signaling and Glucose Uptake in Patients With Newly Diagnosed Type 2 Diabetes

A Randomized Controlled Study

Håkan K.R. Karlsson,¹ Kirsti Hällsten,² Marie Björnholm,¹ Hiroki Tsuchida,¹ Alexander V. Chibalin,¹ Kirsi A. Virtanen,² Olli J. Heinonen,³ Fredrik Lönnqvist,^{1,4} Pirjo Nuutila,^{2,5} and Juleen R. Zierath¹

The effect of metformin or rosiglitazone monotherapy versus placebo on insulin signaling and gene expression in skeletal muscle of patients with newly diagnosed type 2 diabetes was determined. A euglycemic-hyperinsulinemic clamp, combined with skeletal muscle biopsies and glucose uptake measurements over rested and exercised muscle, was performed before and after 26 weeks of metformin ($n = 9$), rosiglitazone ($n = 10$), or placebo ($n = 11$) treatment. Insulin-mediated whole-body and leg muscle glucose uptake was enhanced 36 and 32%, respectively, after rosiglitazone ($P < 0.01$) but not after metformin or placebo treatment. Insulin increased insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation, IRS-1-associated phosphatidylinositol (PI) 3-kinase activity, and phosphorylation of Akt Ser⁴⁷³ and AS160, a newly described Akt substrate that plays a role in GLUT4 exocytosis, ~2.3 fold before treatment. These insulin signaling parameters were unaltered after metformin, rosiglitazone, or placebo treatment. Expression of selected genes involved in glucose and fatty acid metabolism in skeletal muscle was unchanged between the treatment groups. Low-intensity acute exercise increased insulin-mediated glucose uptake but was without effect on insulin signaling. In conclusion, the insulin-sensitizing effects of rosiglitazone are independent of enhanced signaling of IRS-1/PI 3-kinase/Akt/AS160 in patients with newly diagnosed type 2 diabetes. *Diabetes* 54:1459–1467, 2005

From the ¹Department of Surgical Sciences, Section of Integrative Physiology, Karolinska Institutet, Stockholm, Sweden; the ²Turku PET Centre, University of Turku, Turku, Finland; the ³Paavo Nurmi Centre, Sports and Exercise Medicine Unit, Department of Physiology, University of Turku, Turku, Finland; ⁴Biovitrum, Stockholm, Sweden; and the ⁵Department of Medicine, University of Turku, Turku, Finland.

Address correspondence and reprint requests to Juleen R. Zierath, PhD, Karolinska Institutet, Department of Surgical Sciences, Section of Integrative Physiology, S-171 77 Stockholm, Sweden. E-mail: juleen.zierath@fyfa.ki.se.

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DTT, dithiothreitol; FFA, free fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRS-1, insulin receptor substrate 1; LPL, lipoprotein lipase; PET, positron emission tomography; PI, phosphatidylinositol; PPAR- γ , peroxisome proliferator-activated receptor- γ ; SCD, stearoyl-CoA desaturase; TZD, thiazolidinedione; UCP, uncoupling protein.

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Impaired insulin-stimulated whole-body glucose uptake is a major characteristic of type 2 diabetes (1). As skeletal muscle quantitatively accounts for the major part of insulin-stimulated glucose disposal in humans (2), defects in this tissue are of particular importance for the development of insulin resistance. Impaired glucose uptake in skeletal muscle is due to defects in insulin action at the cellular level, presumably caused by defects in mechanisms regulating GLUT4 translocation to the plasma membrane (3,4). The functional disturbances in the insulin signaling pathway that account for impaired glucose uptake have not been fully elucidated. Type 2 diabetic patients have impaired insulin action on insulin receptor substrate 1 (IRS-1), phosphatidylinositol (PI) 3-kinase (5–8), and Akt (9,10). Defects in Akt signaling have only been noted at pharmacological insulin concentrations (9,10) and appear to be isoform specific (10). Because insulin signaling defects coincide with impaired glucose transport in skeletal muscle (5,8–10), therapeutic strategies that enhance insulin signaling may improve whole-body glucose uptake and glucose homeostasis in type 2 diabetic patients.

The glucose-lowering effects of metformin (dimethylbiguanide) in type 2 diabetes are well documented, both for monotherapy and for combination treatment (11–15). Despite the extensive clinical experience related to metformin use, the molecular mode of action has not been fully elucidated. Metformin primarily enhances hepatic insulin sensitivity and reduces hepatic glucose production (13). Thus, any improvement in peripheral insulin sensitivity achieved by metformin treatment may be secondary to the reduction in endogenous glucose production and the subsequent reduction in plasma glucose concentrations (13). Metformin also elicits direct effects on insulin action in skeletal muscle. Metformin exposure (2 h) of isolated skeletal muscle from insulin-resistant humans potentiated insulin-stimulated glucose transport (16) with no effect on basal glucose uptake. Furthermore, in cultured insulin-resistant C2C12 skeletal muscle cells, metformin restored insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity (17). In contrast to these in vitro data, metformin treatment of type 2 diabetic patients for 3–4 months improved whole-body

insulin-mediated glucose uptake, independent of enhanced insulin signaling (18). Thus, effects of metformin on insulin signaling are equivocal (17,18).

Rosiglitazone is a thiazolidinedione (TZD) and a peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist that has been increasingly used for the treatment of type 2 diabetes over the last few years. Although PPAR- γ is expressed at high levels in adipose tissue, this receptor is also expressed in the nucleus of skeletal muscle myocytes (19). Rosiglitazone improves both glucose control, primarily by enhancing peripheral insulin sensitivity, and fatty acid metabolism (20–25). Correlative changes in fatty acid metabolism and improvements in glucose homeostasis and insulin sensitivity may imply an indirect effect on skeletal muscle via adipose tissue (21). Treatment with troglitazone, another member of the TZD family, increases insulin-stimulated IRS-1-associated PI 3-kinase activity and Akt activity in skeletal muscle from type 2 diabetic patients (18) and enhances Akt phosphorylation in normal, glucose-tolerant, insulin-resistant, first-degree relatives of type 2 diabetic patients (26). However, because structurally different PPAR- γ ligands may have diverse effects, the effects of rosiglitazone treatment on insulin signaling in human skeletal muscle are difficult to predict, and responses may vary from those described after troglitazone treatment.

Studies in cultured cells and rodent studies provide evidence that TZDs achieve a therapeutic effect partly through changes in gene expression (27,28). In white adipose tissue from Zucker diabetic fatty rats, TZD treatment promotes a coordinated increase in the expression of a number of genes involved in glucose and fatty acid metabolism to increase the flux of fatty acids into adipose tissue (27). Conversely, in skeletal muscle, TZD treatment decreases the expression of genes regulating fatty acid transport and oxidation to facilitate an increase in glucose uptake and a decrease in fatty acid utilization in skeletal muscle. Although human studies indicate that TZD treatment leads to changes in gene expression in adipose tissue (29,30), little is known about the effects in skeletal muscle.

We determined the effects of metformin or rosiglitazone monotherapy on insulin signaling in skeletal muscle from patients with newly diagnosed type 2 diabetes. All patients were subjected to a euglycemic-hyperinsulinemic clamp, combined with a one-legged exercise, making a comparison between potential effects during resting and exercising conditions possible. This testing procedure was performed both before and after 26 weeks of treatment with metformin or rosiglitazone or placebo. Skeletal muscle biopsy samples were obtained on both occasions before and during insulin infusion, and signal transduction at the level of IRS-1/PI 3-kinase, Akt, and AS160, a newly described Akt substrate (31,32) that plays a role in GLUT4 exocytosis (33), was determined. A secondary aim was to determine the expression of genes involved in fatty acid and glucose metabolism and transcriptional responses in skeletal muscle among the treatment groups.

RESEARCH DESIGN AND METHODS

The biopsy samples were obtained from a subgroup of patients who participated in a recent clinical study (34). A complete set of biopsy samples to be used for this study of signal transduction was obtained from 9 patients in the metformin group, 10 patients in the rosiglitazone group, and 11 patients in the placebo group from the original cohort of patients. Mild type 2 diabetic

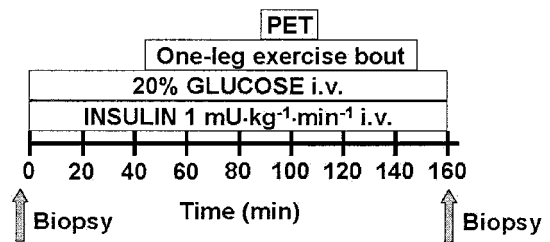


FIG. 1. Schematic representation of the study protocol. A euglycemic-hyperinsulinemic clamp procedure was performed before and after 26 weeks of treatment with metformin, rosiglitazone, or placebo. Euglycemic-hyperinsulinemic conditions consisted of a 160-min insulin infusion. Leg muscle glucose uptake was assessed using PET. The arrow represents the time at which muscle biopsies were obtained. One biopsy was obtained under basal conditions, and two biopsies were obtained after insulin infusion (from rested and exercised leg, respectively).

patients, as defined by new World Health Organization criteria (35), were randomly assigned to a protocol. The subjects were recruited by advertisement and from clients of the occupational health care service in Turku, Finland. Patients with cardiovascular disease, blood pressure $>160/100$ mmHg, any previous or present abnormal hepatic or renal function, or anemia and those taking antidiabetic medication or oral corticosteroid treatment were excluded. Written informed consent was obtained after the nature, purpose, and potential risks of the study were explained to the subjects. The subjects participated in a 4-week run-in period and obtained written diet instructions. Patients with a fasting plasma glucose value <6.1 or >11.0 mmol/l after this run-in period were excluded. Patients included in this double-blind study were randomly assigned into groups for participation in a 26-week double-blind trial with rosiglitazone (2 mg twice a day for 2 weeks, thereafter 4 mg twice a day), metformin (500 mg twice a day for 2 weeks, thereafter 1,000 mg twice a day) or placebo. The size of the pills was identical in all of the groups, consistent with the double-blind design of the study. The Ethical Committee of the Hospital District of Varsinais-Suomi approved the study protocol. The study was conducted according to principles of the Declaration of Helsinki. A schematic representation of the *in vivo* study protocol is depicted in Fig. 1.

Glucose uptake measurements. Whole-body glucose uptake was determined by the euglycemic-hyperinsulinemic clamp technique, and skeletal muscle glucose uptake was determined during the clamp procedure using ¹⁸F-labeled fluorodeoxyglucose and positron emission tomography (PET), as previously described (34). The euglycemic-hyperinsulinemic clamp consisted of a 160-min intravenous insulin infusion ($1 \text{ mU} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$) while normoglycemia was maintained by a variable infusion of 20% glucose. The PET measurement of skeletal muscle glucose uptake was performed between 90 and 110 min of the clamp. A one-legged intermittent isometric leg extension exercise was performed between 45 and 110 min of the clamp. The intensity of exercise was set to 10% of the maximal isometric force measured individually. Regions of interest were drawn in vastus lateralis muscle to quantify glucose uptake.

Muscle biopsy procedure. Local anesthesia (10 mg/ml lidocaine hydrochloride) was administered, and an incision was made in the skin and muscle fascia. Three vastus lateralis muscle biopsy samples were obtained from each subject on each clamp occasion using a Bergström needle with suction. Skeletal muscle biopsy samples were obtained before (basal) and 160 min after the onset of the insulin infusion in the rested (insulin-stimulated) and the exercised (insulin plus exercise) leg. When two biopsy samples were taken from the same leg, the second sample was taken 3 cm distal from the first incision site. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C . The samples were obtained after 160 min to coincide with the conclusion of the insulin clamp protocol. Although in rodent skeletal muscle, maximal insulin action signaling events are transient (36), evidence from human studies indicates that the time course for insulin signaling activity is sustained (8). Moreover, previous *in vivo* studies using a similar insulin infusion protocol in humans reported that mean values for insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity are stable throughout 100 min after the onset of insulin infusion (37). In a study of lean nondiabetic, obese nondiabetic, and obese subjects, *in vivo* insulin action on Akt 1/2 activity was similar between 15 min and 3 h (6). Consequently, insulin action on PI 3-kinase and Akt was assessed by Kim et al. (6) after a 3-h *in vivo* insulin infusion. Thus, we reasoned that the 160-min sampling time would allow for an appropriate measure of insulin signaling activity in human skeletal muscle under *in vivo* conditions.

Tissue processing. For protein measurement; muscle biopsies (40–50 mg) were freeze dried overnight; then dissected under a microscope to remove visible blood, fat, and connective tissue; subsequently homogenized in ice-cold buffer A (20 mmol/l Tris [pH 7.8], 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l MgCl₂, 1% Triton X-100, 10% [wt/vol] glycerol, 10 mmol/l NaF, 1 mmol/l EDTA, 5 mmol/l Na-pyrophosphate, 0.5 mmol/l Na₃VO₄, 1 μg/ml leupeptin, 0.2 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mmol/l dithiothreitol [DTT], 1 mmol/l benzamide, and 1 μmol/l microcystin); and then rotated for 30 min at 4°C. Samples were subjected to centrifugation (12,000g for 15 min at 4°C), and the protein concentration was determined in the supernatant using the Bradford method (Bio-Rad, Richmond, CA).

For the gene expression studies, a portion of the skeletal muscle biopsy (20–35 mg) that was obtained under the basal condition in the posttreatment period was homogenized in Tri Reagent (Sigma, St. Louis, MO), and total RNA was extracted according to the manufacturer's instructions. Extracted RNA was subjected to DNase I treatment using a DNA-free kit (Ambion, Austin, TX) according to manufacturer's instructions. cDNA was synthesized using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) using random hexamer primers according to the manufacturer's instructions. **IRS-1 tyrosine phosphorylation.** Aliquots of supernatant (800 μg protein) were immunoprecipitated with anti-IRS-1 antibody (M.G. Myers, Joslin Diabetes Center, Boston, MA) overnight at 4°C. Thereafter, protein A-Sepharose beads were added to the lysates and samples were incubated for 2 h at 4°C. The immunoprecipitates were washed three times with buffer A, two times with buffer B (0.1 mol/l Tris [pH 8.0] and 0.5 mol/l LiCl) and one time with buffer C (10 mmol/l Tris [pH 7.6], 0.15 mol/l NaCl, and 1 mmol/l EDTA). Pellets were resuspended in Laemmli buffer containing β-mercaptoethanol. Samples were heated at 95°C for 4 min and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked with Tris-buffered saline with 0.02% Tween containing 5% milk. Membranes were incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibodies (PY2005; Affinity, Exeter, U.K.) overnight at 4°C. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL) and quantified using densitometry and Molecular Analyst Software (Bio-Rad).

IRS-1-associated PI 3-kinase activity. A second aliquot of supernatant (1 mg protein) was immunoprecipitated with anti-IRS-1 antibody and washed, as described above. Immunoprecipitates were then washed one time with buffer D (20 mmol/l HEPES [pH 7.3], 1 mmol/l DTT, and 5 mmol/l MgCl₂) and thereafter resuspended in 20 μl of buffer E (20 mmol/l HEPES [pH 7.3], 20 mmol/l β-glycerophosphate [pH 7.2], 5 mmol/l Na₄P₂O₇, 30 mmol/l NaCl, and 1 mmol/l DTT). The kinase reaction was started by adding 30 μl of buffer F (buffer E containing 12.5 μmol/l ATP, 7.5 mmol/l MgCl₂, and 20 μg phosphatidyl inositol per reaction [Avanti Polar Lipids, Alabaster, AL] and 20 μCi [³²P]ATP per reaction) and carried out for 15 min at room temperature. The reaction was terminated by addition of 150 μl of 1% perchloric acid. Thereafter, a 2:1 mixture of methanol:chloroform was added followed by two washes with 1% perchloric acid, where the aqueous phase was removed between washes. The reaction product was applied onto a silica gel-coated thin-layer chromatography aluminum sheet (Silica Gel 60; Merck, Darmstadt, Germany) and was separated in a pre-equilibrated tank containing methanol:chloroform:ammonia:water (75:54:20:10) and quantified using a Phosphor-Imager (Image Reader BAS-1800 II; Fujifilm, Düsseldorf, Germany).

Western blot analysis. Protein expression of GLUT4 and IRS-1 and phosphorylation of Akt were determined. An aliquot of muscle lysate (40 μg protein) was mixed with Laemmli buffer containing β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked in fat-free milk for 2 h. Membranes were incubated with either anti-GLUT4 (G.D. Holman, University of Bath, Bath, U.K.), anti-IRS-1 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Akt (Ser⁴⁷³), or anti-phospho-(Ser/Thr) Akt substrate (PAS) antibodies (Cell Signaling Technology, Beverly, MA), washed in Tris-buffered saline with Tween and incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (Bio-Rad). Proteins were visualized and quantified as described above.

Gene expression analysis. Gene expression analysis was carried out utilizing a TaqMan-based Multi Fluidic Card (MFC) gene expression assay (Applied Biosystems, Foster City, CA). The MFC was specifically designed to assess the expression of 24 genes (5 endogenous controls and 19 target genes), where all genes were analyzed in duplicate. The following primer and probe sets from Applied Biosystems were lyophilized in the MFC well: MFC internal endogenous control 18S, Hs99999907_m1 (β₂-microglobulin), Hs99999905_m1 (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]), Hs99999909_m1 (hypoxanthine phosphoribosyltransferase 1), Hs99999910_m1 (TATA box binding protein), Hs00169627_m1 (fatty acid translocase [CD36]), Hs00192700_m1 (fatty acid transporter 4), Hs00167385_m1 (acetyl-CoA carboxylase α),

Hs00153715_m1 (acetyl-CoA carboxylase β), Hs00173425_m1 (lipoprotein lipase [LPL]), Hs00748952_s1 (stearoyl-CoA desaturase [SCD]), Hs00243297_m1 (uncoupling protein [UCP]3), Hs00177552_m1 (diacylglycerol kinase delta [DGK δ]), Hs00168966_m1 [GLUT4], Hs00606086_m1 (hexokinase 2), Hs00248750_m1 (Cbl associated protein [CAP]), Hs00360422_m1 (adiponectin receptor 1), Hs00234592_m1 (PPAR-γ), Hs00173304_m1 (PPAR-γ coactivator 1α [PGC1-α]), Hs00370186_m1 (PPAR-γ coactivator 1β [PGC1-β]), Hs00209379_m1 (PGC-1-related coactivator), Hs00602161_m1 (nuclear respiratory factor 1 [NRF-1]), Hs00231106_m1 (forkhead box O1A [FOXO1A]), and Hs00231674_m1 (sterol regulatory element-binding protein 1 [SREBP1]). Samples were applied into the MFC well. mRNA was determined according to technical documents supplied by the manufacturer.

Statistical analysis. Data are presented as means ± SE. Student's paired or unpaired *t* test was used to assess differences. Differences within and between groups were determined by ANOVA. Fisher's least significant difference post hoc analysis was used to identify significant differences. Pearson correlation analysis was applied to determine the existence of possible relationships between glucose uptake and insulin signaling. Differences were considered significant at *P* < 0.05.

RESULTS

The study participants in this investigation are part of a larger cohort included in a previous report (34). The clinical characteristics, including whole-body and skeletal muscle glucose uptake in response to metformin, rosiglitazone, or placebo treatment have previously been reported for the entire cohort (34). Clinical and metabolic characteristics of study participants in the present study are reported in Table 1. BMI and fasting plasma glucose were decreased after metformin treatment (*P* < 0.05) but not in rosiglitazone or placebo treatment. HbA_{1c} was improved after metformin (*P* < 0.001), but not after rosiglitazone (*P* = 0.08) or placebo treatment. Pretreatment fasting free fatty acid (FFA) levels were similar between the groups and unchanged after treatment. However, posttreatment serum FFA levels measured during the hyperinsulinemic clamp were decreased 30% (*P* < 0.05) and 48% (*P* < 0.01) after metformin and rosiglitazone treatment, respectively, and were unchanged in the placebo group.

Glucose uptake. Whole-body insulin-mediated glucose uptake was not altered by metformin or placebo treatment (Fig. 2A). In contrast, rosiglitazone treatment significantly increased whole-body insulin-mediated glucose uptake 36% (*P* < 0.01). Glucose uptake was also measured across a portion of vastus lateralis skeletal muscle using PET under insulin-stimulated conditions in rested and exercised legs (Fig. 2B and C, respectively). Insulin-stimulated leg muscle glucose uptake was not altered by either metformin or placebo treatment in either the rested or exercised leg. In contrast, rosiglitazone treatment increased insulin-stimulated leg muscle glucose uptake 32% in the rested (*P* > 0.05) and 70% (*P* < 0.01) in the exercised leg. The effects of metformin and rosiglitazone treatment on whole-body and leg glucose uptake are compatible with our previous results in the larger cohort (34). The improvement in leg glucose uptake was not due to increased total GLUT4 content. GLUT4 protein expression was not altered by any treatment regimen (data not shown).

IRS-1 tyrosine phosphorylation. Insulin action on IRS-1 and PI 3-kinase was determined before and after treatment with metformin, rosiglitazone, or placebo. Before treatment, insulin infusion led to a 1.7-fold increase in IRS-1 tyrosine phosphorylation in vastus lateralis skeletal muscle (*P* < 0.01), with similar effects in both rested and exercised legs. Insulin-stimulated IRS-1 tyrosine phosphorylation was reassessed 26 weeks after metformin,

TABLE 1
Clinical and metabolic characteristics of the patients

	Metformin		Rosiglitazone		Placebo	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment	Pretreatment	Posttreatment
<i>n</i> (M/F)	9 (6/3)		10 (8/2)		11 (9/2)	
Age (years)	57.7 ± 2.9	—	58.2 ± 2.1	—	58.6 ± 2.5	—
BMI (kg/m ²)	28.8 ± 1.3	28.0 ± 1.1*	28.2 ± 1.0	28.2 ± 1.2	29.4 ± 1.3	29.5 ± 1.3
HbA _{1c} (%)	7.1 ± 0.3	6.4 ± 0.3‡	6.8 ± 0.3	6.4 ± 0.2	6.3 ± 0.1	6.2 ± 0.1
Glucose (mmol/l)	8.3 ± 0.6	7.1 ± 0.4*	7.0 ± 0.3	6.8 ± 0.3	7.2 ± 0.3	7.5 ± 0.3
Insulin (mU/l)	10.8 ± 2.4	8.4 ± 1.3	6.8 ± 0.5	6.4 ± 0.4	10.2 ± 1.8	9.8 ± 1.2
C-peptide (nmol/l)	0.89 ± 0.12	0.61 ± 0.07*	0.72 ± 0.06	0.57 ± 0.05*	0.83 ± 0.09	0.71 ± 0.05*
Fasting FFA (μmol/l)	458 ± 71	496 ± 63	596 ± 59	552 ± 86	598 ± 70	511 ± 58
Clamp FFA (μmol/l)	145 ± 26	102 ± 16*	110 ± 14	57 ± 5†	100 ± 20	93 ± 19
Total cholesterol (mmol/l)	4.2 ± 0.2	4.4 ± 0.2	4.9 ± 0.3	5.5 ± 0.4*	4.5 ± 0.3	4.4 ± 0.3
HDL cholesterol (mmol/l)	1.0 ± 0.1	1.1 ± 0.1†	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
LDL cholesterol (mmol/l)	2.6 ± 0.2	2.5 ± 0.2	2.9 ± 0.3	3.7 ± 0.3†	2.7 ± 0.3	2.7 ± 0.3
Triglycerides (mmol/l)	1.4 ± 0.1	1.5 ± 0.2	1.8 ± 0.3	1.5 ± 0.2	1.1 ± 0.1	1.0 ± 0.1
Plasma lactate (mmol/l)	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1

Data are means ± SE. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. pretreatment.

rosiglitazone, or placebo treatment. Insulin-stimulated IRS-1 tyrosine phosphorylation in skeletal muscle was not altered by metformin, rosiglitazone, or placebo treatment under either rested or exercised conditions. Furthermore, protein expression of IRS-1 was not altered after any of the treatment protocols (data not shown).

IRS-1-associated PI 3-kinase activity. Insulin infusion increased IRS-1-associated PI 3-kinase activity twofold in skeletal muscle before treatment (Fig. 3). Neither metformin (Fig. 3A) nor rosiglitazone (Fig. 3B) treatment enhanced insulin-stimulated IRS-1-associated PI 3-kinase activity. However, insulin action on IRS-1-associated PI 3-kinase activity was increased ($P < 0.05$) in the placebo group (Fig. 3C). This may be a coincidental finding or a consequence of slightly lower PI 3-kinase activity at baseline in the placebo group. Acute exercise did not alter insulin-stimulated IRS-1-associated PI 3-kinase activity in any of the treatment groups.

Akt phosphorylation. Insulin infusion increased Akt Ser⁴⁷³ phosphorylation ~2.3 fold (Fig. 4). Insulin-stimulated Akt phosphorylation was not altered after treatment with metformin (Fig. 4A), rosiglitazone (Fig. 4B), or placebo treatment (Fig. 4C). Furthermore, acute exercise did not alter insulin action on Akt phosphorylation in any of the groups. Furthermore, protein expression of Akt was not altered after any of the treatment protocols (data not shown).

AS160 phosphorylation. Insulin elicits phosphorylation of a 160-kDa Akt substrate, as detected by immunoblot experiments using the PAS antibody (38). AS160 was identified by immunoprecipitation experiments using an antibody against the COOH-terminal 12 amino acids of mouse AS160 (PTNDKAKAGNKP) (31). Insulin infusion increased AS160 phosphorylation approximately twofold before treatment (Fig. 5). Insulin-stimulated AS160 phosphorylation was unaltered after treatment with metformin (Fig. 5A), rosiglitazone (Fig. 5B), or placebo (Fig. 5C). Furthermore, acute exercise did not alter insulin action on AS160 phosphorylation in any of the groups.

Gene expression analysis. Five endogenous control genes were analyzed and compared. GAPDH was the most stable endogenous control gene in this assay and was used

to normalize data by dividing the expression of each target gene by the expression of GAPDH. We were unable to perform the gene expression analysis under pretreatment conditions owing to insufficient availability of samples. Thus, we compared expression of the target genes in skeletal muscle biopsies obtained posttreatment among the groups. Expression of the 19 target genes was similar for metformin, rosiglitazone, and placebo treatment (Table 2). However, a tendency for an increase in LPL and SCD mRNA and a reduction in UCP3 mRNA expression was noted in rosiglitazone- versus metformin- or placebo-treated subjects.

Correlation analysis. In an effort to resolve whether leg muscle glucose uptake reflects whole-body glucose uptake, insulin action on whole-body and leg muscle glucose uptake in the type 2 diabetic subjects was compared before they underwent pharmacological treatment (Fig. 6A). Insulin action on whole-body glucose uptake was positively correlated with leg glucose uptake ($r = 0.88$; $P < 0.001$). Similar data are reported for the entire study cohort (34), and results are presented for this subgroup for comparative purposes. In addition, a correlation analysis was performed to resolve whether a linear relationship exists between insulin action signal transduction and leg muscle glucose uptake. Insulin-stimulated IRS-1-associated PI 3-kinase activity and leg muscle glucose uptake were positively correlated (Fig. 6B; $r = 0.57$; $P < 0.01$). Similarly, a positive correlation was noted between Akt Ser⁴⁷³ phosphorylation and leg muscle glucose uptake (Fig. 6C; $r = 0.51$; $P < 0.05$).

DISCUSSION

We and others have observed insulin signaling defects at the level of IRS-1 and PI 3-kinase in skeletal muscle from type 2 diabetic patients (5–8), concomitant with impaired insulin-stimulated glucose uptake (5,8). Thus, strategies to enhance insulin signaling may be efficacious in the treatment of skeletal muscle insulin resistance. A previous report provides evidence that treatment of patients with poorly controlled type 2 diabetes with metformin for 3–4 months enhanced insulin-mediated glucose uptake independent of improved insulin signaling (18). However,

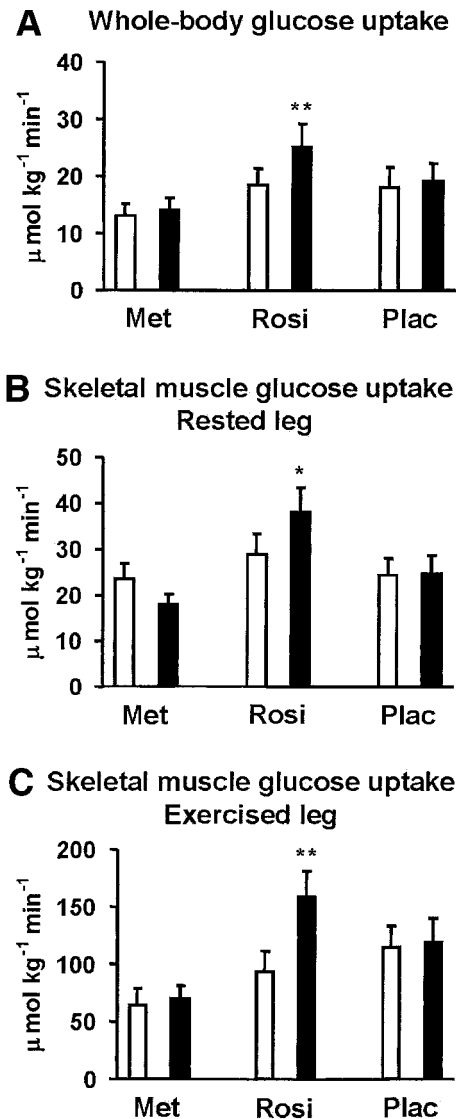


FIG. 2. Whole-body and leg muscle glucose uptake rates. Whole-body glucose uptake (A) and leg muscle glucose uptake over the rested (B) and exercised (C) legs were determined during the euglycemic-hyperinsulinemic clamp procedure before (\square) and after (\blacksquare) treatment. Leg glucose uptake was assessed across a portion of the vastus lateralis skeletal muscle using PET. Data are means \pm SE for 9–11 subjects per group. * $P < 0.05$, ** $P < 0.01$ for post- vs. pretreatment value. Met, metformin; Rosi, rosiglitazone; Plac, placebo.

treatment with troglitazone for 3–4 months improved both insulin-mediated whole-body glucose uptake and insulin action on PI 3-kinase and Akt (18). Furthermore, treatment of obese type 2 diabetic subjects for 1 month with rosiglitazone also enhanced insulin-mediated whole-body glucose uptake, coincident with increased IRS-1-associated PI 3-kinase and atypical protein kinase C activity (24). These studies imply that the insulin-sensitizing effect of troglitazone in obese type 2 diabetic subjects is partly caused by enhanced insulin signaling in skeletal muscle. One common feature between these reports (18,24) is that obese type 2 diabetic subjects with poorly controlled diabetes were studied. However, there is no evidence as to whether similar effects on insulin signaling occur in moderately obese type 2 diabetic subjects or even subjects with newly diagnosed type 2 diabetes, in whom the metabolic derangements are presumably less severe. Thus, we

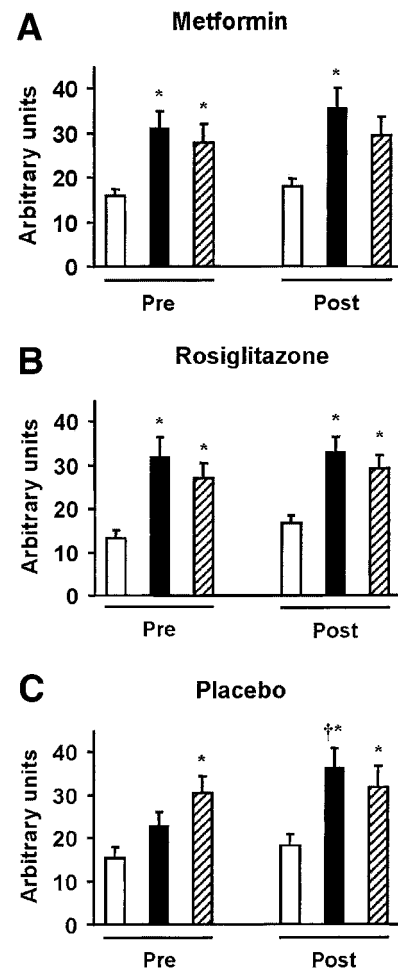


FIG. 3. IRS-1-associated PI 3-kinase activity in skeletal muscle. Muscle biopsies were obtained under basal (\square) or insulin-stimulated conditions in rested (\blacksquare) or exercised (\hatched) legs before and after treatment with metformin (A), rosiglitazone (B), or placebo (C). PI 3-kinase activity was measured in IRS-1 immunoprecipitates. Results were quantitated using a PhosphorImager. Data are means \pm SE arbitrary units for six to nine subjects per group. * $P < 0.05$ vs. basal; † $P < 0.05$ for post- vs. pretreatment for the insulin-stimulated condition.

evaluated whether the insulin-sensitizing effects of metformin and rosiglitazone involve enhanced insulin signaling and changes in gene expression in skeletal muscle from patients with newly diagnosed type 2 diabetes.

Patients with newly diagnosed type 2 diabetes received metformin, rosiglitazone, or placebo for 26 weeks. Consistent with previous reports in obese patients with poorly controlled type 2 diabetes, metformin treatment improved HbA_{1c} and fasting blood glucose levels but was without effect on insulin-mediated glucose uptake (12,18,39) and signal transduction (18). Because improvements in glucose homeostasis after metformin treatment are primarily due to an insulin-sensitizing effect on the liver leading to decreased hepatic glucose production (12,39,40), our results are not surprising. In contrast, rosiglitazone treatment enhanced insulin-mediated whole-body and leg muscle glucose uptake but was without effect on HbA_{1c} and fasting blood glucose. The improvement in insulin-mediated glucose uptake reported here and in our larger study cohort (34) is consistent with previous studies in which either troglitazone (18,41) or rosiglitazone (22,24, 25) was administered, further supporting an insulin-sensi-

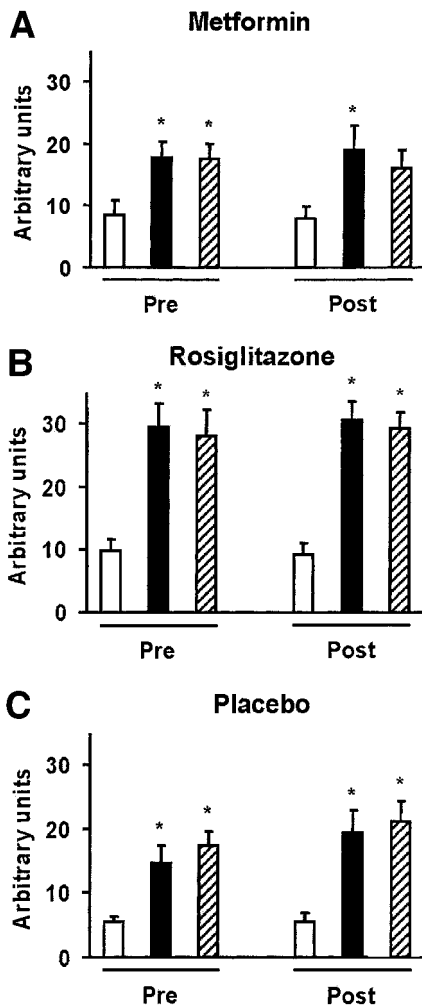


FIG. 4. Phosphorylation of Akt in skeletal muscle. Muscle biopsies were obtained under basal (□) or insulin-stimulated conditions in rested (■) or exercised (▨) legs before and after treatment with metformin (A), rosiglitazone (B), or placebo (C). Phosphorylation of Akt kinase was measured by immunoblot analysis using an anti-phospho-Ser⁴⁷³ Akt antibody. Results were quantitated using densitometry. Data are means \pm SE arbitrary units for 7–11 subjects per group. * $P < 0.05$ vs. basal value.

tizing effect on skeletal muscle. However, the lack of an improvement in HbA_{1c} and fasting blood glucose is an inconsistent finding, because both improved (18,21,22,25) and unchanged (23,42) glycemic control has been observed in type 2 diabetic patients undergoing TZD treatment. Moreover, insulin signaling was not enhanced, despite the marked improvement in glucose uptake. This implies that there is not a simple relationship between insulin signaling and glucose uptake, as evidenced by our correlative study (Fig. 6), revealing a weak positive relationship between insulin action on glucose uptake and signal transduction at the level of PI 3-kinase or Akt. We speculate that defects in GLUT4 translocation, rather than decreased phosphorylation of signaling intermediates, may contribute more to the reduced glucose uptake in skeletal muscle from patients with newly diagnosed type 2 diabetes, because improved insulin signaling was not required for improved glucose uptake. Moreover, changes in glucose homeostasis in response to rosiglitazone treatment may be partly related to increased insulin sensitivity in adipose tissue, because serum FFA levels were mark-

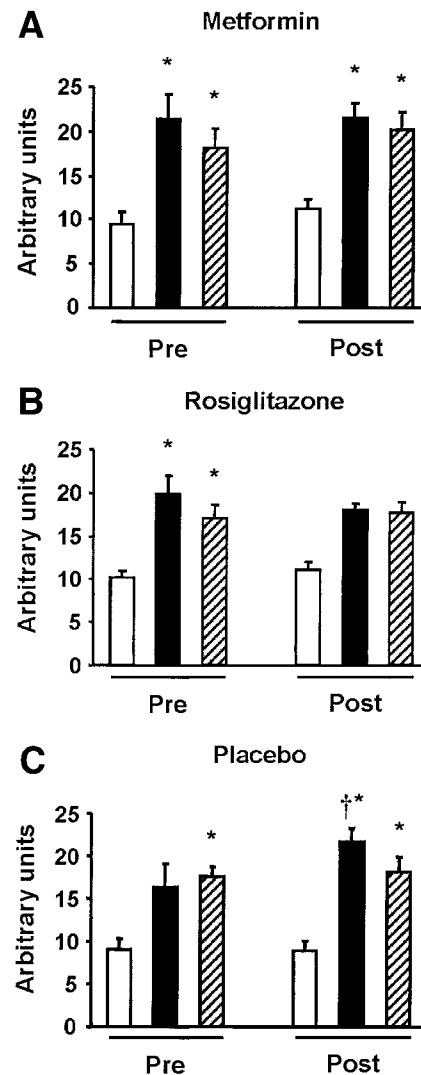


FIG. 5. Phosphorylation of AS160 in skeletal muscle. Muscle biopsies were obtained under basal (□) or insulin-stimulated conditions in rested (■) or exercised (▨) legs before and after treatment with metformin (A), rosiglitazone (B), or placebo (C). Phosphorylation of AS160 was measured by immunoblot analysis using an anti-phospho-Akt substrate antibody. Results were quantitated using densitometry. Data are means \pm SE arbitrary units for seven to nine subjects per group. * $P < 0.05$ vs. basal, † $P < 0.05$ for post- vs. pretreatment for the insulin-stimulated condition.

edly reduced under insulin-stimulated conditions. This is further supported by the clinical observation in the larger study cohort, in whom rosiglitazone significantly increased glucose uptake in adipose tissue (43).

Emerging evidence suggests that treatment of type 2 diabetic patients or nondiabetic first-degree relatives of type 2 diabetic patients with either troglitazone (18) or rosiglitazone (24–26) improves insulin signal transduction. However, our results in moderately obese patients with newly diagnosed type 2 diabetes indicated that this is not a universal observation, because we failed to observe improvements in insulin signaling. Previous studies performed in obese patients with poorly controlled type 2 diabetes provide evidence that insulin-stimulated IRS-1-associated PI 3-kinase activity in skeletal muscle obtained during a euglycemic-hyperinsulinemic clamp was enhanced after 1–4 months of TZD treatment (18,24,25). In contrast, in patients with newly diagnosed type 2 diabetes,

TABLE 2
mRNA expression in skeletal muscle posttreatment

	Metformin	Rosiglitazone	Placebo	<i>P</i> value
<i>n</i>	9	10	10	
Lipid transport and metabolism				
CD36	1060 ± 186	1027 ± 133	1000 ± 161	NS
FAT4	10.5 ± 2.3	10.5 ± 2.3	12.3 ± 4.4	NS
ACC-α	2.7 ± 0.6	3.3 ± 0.6	3.4 ± 0.9	NS
ACC-β	61 ± 13	65 ± 13	68 ± 12	NS
LPL	104 ± 25	253 ± 88	144 ± 28	NS
SCD	0.7 ± 0.3	1.4 ± 0.4	0.9 ± 0.2	NS
UCP3	420 ± 87	285 ± 46	480 ± 114	NS
DGK δ	36 ± 10	30 ± 4	38 ± 14	NS
Glucose transport and metabolism				
GLUT4	145 ± 40	151 ± 35	193 ± 47	NS
Hexokinase 2	25 ± 11	28 ± 9	22 ± 8	NS
CAP	265 ± 40	281 ± 39	224 ± 33	NS
Adiponectin receptor 1	322 ± 49	277 ± 42	244 ± 40	NS
Transcription factors				
PPAR-γ	3.4 ± 0.8	4.2 ± 0.6	5.1 ± 2.0	NS
PGC1-α	148 ± 16	169 ± 29	126 ± 15	NS
PGC1-β	11.8 ± 2.2	14.4 ± 2.9	17.6 ± 5.6	NS
PRC	7.3 ± 1.6	7.3 ± 1.6	7.6 ± 1.9	NS
NRF-1	52 ± 14	47 ± 8	70 ± 33	NS
FOXO1A	30 ± 5	24 ± 3	28 ± 3	NS
SREBP1	33.6 ± 7.7	27.7 ± 4.2	32.3 ± 8.8	NS

Data are mean ± SE arbitrary units normalized to GAPDH.

in whom metabolic derangements are less severe, insulin action on IRS-1 tyrosine phosphorylation and IRS-1-associated PI 3-kinase activity was unchanged after 26 weeks of rosiglitazone treatment. Although our findings contrast with those of studies in which improved insulin signaling has been observed in patients with poorly controlled type 2 diabetes (18,24,25), our results are consistent with a lack of improvement in IRS-1-associated PI 3-kinase activity in nondiabetic normoglycemic first-degree relatives of type 2 diabetic patients after 3 months of TZD treatment (26).

The effects of TZD treatment on insulin-mediated Akt phosphorylation in patients with poorly controlled type 2 diabetes are equivocal. Evidence for (18) and against (24) improvements in insulin action on Akt have been observed in patients with poorly controlled type 2 diabetes. However, insulin action on Akt phosphorylation was unchanged in skeletal muscle from this cohort of patients with newly diagnosed type 2 diabetes after rosiglitazone treatment. Akt has been linked to glucose transport through the identification of AS160, a protein containing a GTPase-activating domain for Rabs, which are small G-proteins required for membrane trafficking (31,32). Phosphorylation of AS160 is required for the insulin-induced translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes (33). AS160 phosphorylation is impaired in skeletal muscle from type 2 diabetic patients (38). Insulin-stimulated phosphorylation of AS160 was unchanged after rosiglitazone treatment. This was consistent with our results for upstream insulin signal transducers, because insulin action on IRS-1 tyrosine phosphorylation, IRS-1-associated PI 3-kinase activity, and Akt phosphorylation was not improved under these conditions. Moreover, enhanced IRS-1 signaling may not be a prerequisite for improvements in insulin action on Akt, because TZD treatment of nondiabetic normal glycemic first-degree

relatives of type 2 diabetic patients is associated with an increase in Akt phosphorylation, independent of any positive change in upstream signaling (26). Despite these inconsistent results among various human studies designed to assess the effects of TZD treatment on insulin signaling (18,24–26), all of these studies provide evidence for a profound improvement in insulin action on peripheral glucose uptake. Thus, changes in insulin signaling cannot fully account for the enhanced skeletal muscle glucose uptake in response to TZD treatment.

Several lines of evidence suggest that TZDs achieve a therapeutic effect partly through changes in gene expression (27,28). In diabetic KK/Ta mice, pioglitazone treatment (2 weeks) increased UCP2 and decreased UCP3 mRNA expression (44), providing evidence that glitazone treatment promotes changes in gene expression in skeletal muscle. In Zucker diabetic fatty rats, rosiglitazone treatment (2 weeks) increases mRNA expression of SCD and FAT/CD36 in skeletal muscle (45). However, data regarding the effects of TZD treatment on gene expression in human skeletal muscle are lacking. Interestingly, in this cohort, a tendency for an increase in LPL and SCD mRNA and a reduction in UCP3 mRNA expression were noted in rosiglitazone- versus metformin- or placebo-treated subjects. Furthermore, in cultured skeletal myotubes from type 2 diabetic patients, rosiglitazone treatment (4 days) increases mRNA expression of FAT/CD36 (46). Thus, improvements in glucose and lipid metabolism after TZD treatment may occur in concert with changes in gene expression. However, in this cohort, mRNA expression of genes involved in glucose and lipid metabolism and transcription (Table 2) was similar among subjects with newly diagnosed type 2 diabetes treated with metformin, rosiglitazone, or placebo. Moreover, protein expression of IRS-1, Akt, and GLUT4 was unchanged between the groups.

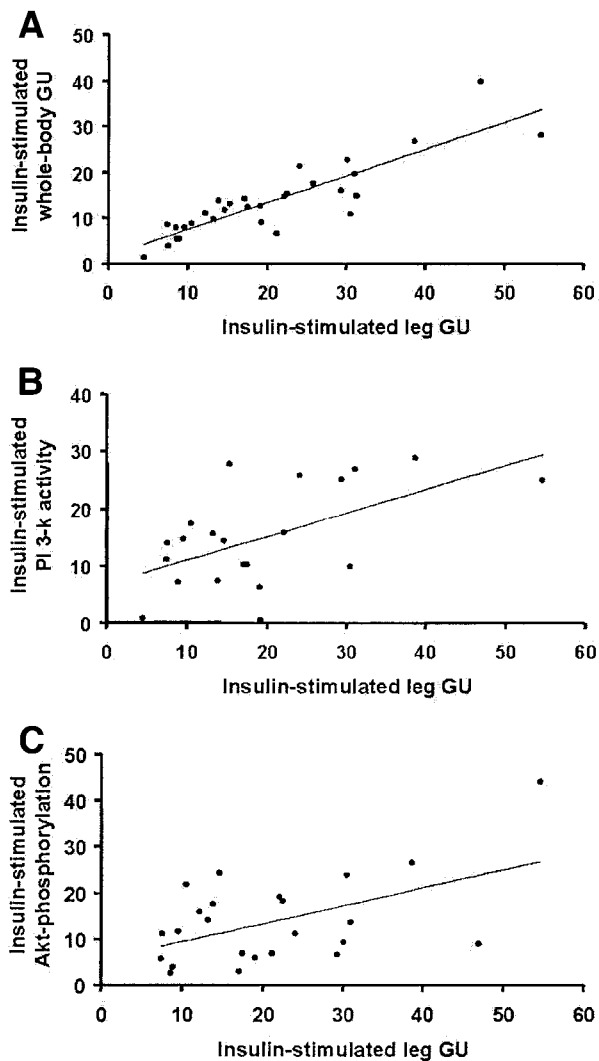


FIG. 6. Correlation analysis. **A:** Correlation between insulin-stimulated whole-body glucose uptake (GU) and insulin-stimulated leg muscle glucose uptake in the rested leg in the entire study cohort before pharmacological intervention ($r = 0.88$, $P < 0.001$, $n = 30$). **B:** Correlation between insulin-stimulated IRS-1-associated PI 3-kinase activity and insulin-stimulated leg muscle glucose uptake in the rested leg before pharmacological intervention ($r = 0.57$, $P < 0.01$, $n = 21$). **C:** Correlation between insulin-stimulated Akt phosphorylation and insulin-stimulated leg muscle glucose uptake in the rested leg before pharmacological intervention ($r = 0.51$, $P < 0.05$, $n = 24$).

Thus, changes in expression in these candidate genes or selected insulin signaling proteins in skeletal muscle does not appear to account for improvements in glucose homeostasis. Clearly more extensive studies using an unbiased gene array approach may reveal gene expression signatures in skeletal muscle to account for effects of these pharmacological treatments on glucose homeostasis.

Insulin-dependent and -independent pathways mediate glucose uptake in skeletal muscle. Muscle contraction through exercise is associated with an insulin-independent increase in glucose uptake (47). Furthermore, insulin and exercise have additive effects on glucose uptake that can be readily observed in humans in vivo during the euglycemic-hyperinsulinemic clamp (37). Thus, we determined whether metformin or rosiglitazone treatment would alter exercise effects on insulin-stimulated leg glucose uptake. We hypothesized that this approach would allow us to

ascertain whether metformin or rosiglitazone would improve glucose uptake through an insulin-independent mechanism. Before any pharmacological treatment, exercise had an additive effect on insulin-stimulated leg muscle glucose uptake in subjects with newly diagnosed type 2 diabetes. Metformin treatment did not alter the exercise-induced increment in insulin-stimulated leg muscle glucose uptake, consistent with the observation that metformin primarily targets liver and decreases hepatic glucose production. In contrast, rosiglitazone treatment markedly increased the exercise-induced increment in insulin-stimulated leg muscle glucose uptake. This effect was not associated with parallel increased insulin signaling in the exercised muscle, consistent with a previous report in young healthy volunteers (37). We attempted to determine phosphorylation of 5'-AMP-activated protein kinase, because it has been implicated as a mediator of insulin-independent glucose uptake (48), as well as a target for both rosiglitazone and metformin in vitro in cultured myotubes (49). However, due to the relatively low intensity of the exercise protocol and the delay in the timing of the muscle biopsy (10 min after exercise cessation), we did not observe any measurable changes in 5'-AMP-activated protein kinase phosphorylation after exercise (data not shown). Nevertheless, our in vivo measurements provide evidence that rosiglitazone treatment enhanced both insulin-dependent and -independent glucose uptake.

In conclusion, in moderately obese subjects with newly diagnosed type 2 diabetes, rosiglitazone treatment improves skeletal muscle glucose uptake through a mechanism(s) independent of insulin signaling at the level of IRS-1/PI 3-kinase/Akt/AS160 or changes in expression of selective candidate genes involved in glucose or lipid metabolism.

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