Regulation of Skeletal Muscle Morphology in Type 2 Diabetic Subjects by Troglitazone and Metformin: Relationship to Glucose Disposal

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The goal of this work was to compare the effects of different antidiabetic therapies on the phenotype of skeletal muscle in type 2 diabetic subjects failing sulfonylurea therapy. Subjects were treated with a thiazolidinedione (troglitazone, TGZ) or a biguanide (metformin, MET) in addition to glyburide for 3 to 4 months. Insulin action was determined with a hyperinsulinemic (300 mU · m^{-2} · min^{-1}) euglycemic (5.0 to 5.5 mmol/L) clamp. Biopsies were obtained from the vastus lateralis muscle for morphological analysis. Despite similar glycemic control, relative increases in the insulin-stimulated glucose disposal rate (GDR) were greater after TGZ treatment (37 ± 8% increase, $P < .05$) than after MET (21 ± 11%, $P < .05$). Neither treatment had any effect on fiber type composition of the muscle. Capillary density was reduced in diabetic subjects compared to a nondiabetic group ($P < .01$) and was increased with TGZ treatment ($P < .05$), while MET was without significant effect. Diabetic muscle also displayed a lower mitochondrial volume density that was unaltered by either treatment. Both TGZ and MET therapy resulted in a reduction in the lipid content of muscle (percent fiber volume as lipid droplets); the relative decrease tended to be greater for TGZ (−33% *v* −23% for MET). The relative (%) improvement in GDR was correlated with the change in lipid content ($r = -0.756$, $P < .05$) after TGZ treatment; no such relationship was observed for MET. From these results we conclude that the higher potency of TGZ to increase capillary density and reduce the lipid content of muscle may contribute to its greater ability to improve glucose disposal in skeletal muscle of type 2 diabetic individuals.

DEFINING CHARACTERISTICS of type 2 diabetes include impaired utilization of glucose, resistance to the ability of insulin to stimulate glucose uptake and disposal in selective tissues, and inappropriate secretion of insulin to compensate for tissue insulin resistance.[1] The major site of insulin resistance in diabetes is skeletal muscle, where defects in glucose transport and both oxidative and nonoxidative glucose metabolism are common.[1] Besides biochemical and metabolic derangements, morphologic and structural differences are also observed between skeletal muscle from nondiabetic and type 2 diabetic individuals. One of these involves the fiber type composition of skeletal muscle. Many diabetic subjects have been reported to have a reduction in the content of type I oxidative fibers,[2,3] the most insulin-sensitive,[4] and an increase in type II fibers. A correlation has been observed between the content of type IIb glycolytic fibers and measures of insulin resistance.[5] Other investigators failed to find diabetes-related differences in muscle fiber type composition.[6] This discrepancy may be reflective of the heterogeneity of many features of diabetes.

Another common characteristic of insulin resistant states, including type 2 diabetes, is a reduction in the capillary density of skeletal muscle. Since one response of muscle to insulin is recruitment of capillaries to augment substrate delivery to muscle,[7] a lower capillary density could represent another contributor to lower insulin-stimulated glucose disposal. This defect may be an acquired one, as insulin-resistant first-degree relatives of type 2 diabetic subjects have a normal skeletal muscle capillary density.[3]

Considerable attention has been focused lately on the role of intramyocellular lipid content (IMCL) control of glucose metabolism and insulin action. Using a variety of procedures, a strong inverse correlation between muscle lipid content and insulin action has been established in both diabetic and nondiabetic subjects.[8-10] Increased skeletal muscle triglyceride accumulation may also be an early event in the development of insulin resistance in type 2 diabetic subjects, as nondiabetic offspring[11] or first-degree relatives of diabetics[12] display elevated intramyocellular lipids compared to weight-matched nondiabetic subjects with no family history of the disease.

A number of therapeutic approaches have proven useful in controlling hyperglycemia and improving insulin action in type 2 diabetic patients including biguanides such as metformin (MET)[13] and thiazolidinediones.[14,15] More recently, combination therapies have been employed with increasing frequency and effectiveness.[16] While the general mechanisms by which these various therapies exert their hypoglycemic effects are known, actions on the morphologic phenotype of skeletal muscle in type 2 diabetes are less well understood. In the current study, type 2 diabetic individuals failing control with sulfonylurea therapy had maximal doses of either MET or the thiazolidinedione troglitazone (TGZ) added to their treatment regimen. The goal was to match the extent of glycemic control and compare the impact of the therapies on previously reported skeletal muscle abnormalities in diabetes, as well as on whole body glucose disposal and insulin action.

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MATERIALS AND METHODS

Human Subjects and Treatment Protocol

Eighteen male and female type 2 diabetic subjects (age 30 to 70 years) who were poorly controlled (hemoglobin A_{1c} [HbA_{1c}] > 8.5% and fasting plasma glucose > 140 mg/dL) on at least half- maximal doses of any sulfonylurea agents were recruited. Except for diabetes, the subjects were healthy and on no other medications known to influence glucose metabolism.

After screening, their existing sulfonylurea medication was discontinued and all subjects uniformly started on glyburide 10 mg twice daily for at least 4 weeks. Baseline studies, including biopsies, were then performed and subjects randomized, in a blinded manner by using a randomization table with block design to either the TGZ or MET treatment group. Treatment involved TGZ titration of up to 600 mg/d or MET up to 2550 mg/d over 4 to 6 weeks as required to achieve glycemic goals. The targeted glycemic goal was a fasting plasma glucose of 80 to 120 mg/dL. Following 3 to 4 months of TGZ or MET treatment, patients were readmitted for repeat studies. Subjects were counseled to consume a fixed-calorie diet for the duration of the study. Insulin action was determined by a 3-hour hyperinsulinemic (300 mU · m^{-2} · min^{-1}) euglycemic (5.0 to 5.5 mmol/L) clamp; the glucose disposal rate (GDR) was measured during the last 30 minutes of the clamp.[17] Mean steady-state plasma insulin levels (7,734 ± 339 pmol/L) attained during the clamp did not differ between groups and were not altered by treatment. The insulin level attained provided maximal stimulation of whole-body glucose disposal. Glucose and insulin[18] levels were determined by standard techniques. These subjects were part of a larger study comparing the effects of TGZ and MET therapy on multiple clinical parameters.[19] A group of 5 nondiabetic subjects, without a family history of diabetes, were studied for comparison purposes. The experimental protocol was approved by the Committee on Human Investigation of the University of California, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol.

Muscle Biopsy

A percutaneous biopsy of vastus lateralis muscle was obtained before and after treatment with TGZ or MET using a 5-mm diameter biopsy needle (Bergstrom, Bignell Surgical Instruments, West Sussex, England) and negative pressure. Local anesthetic (1% xylocaine) was applied as a ring block of the tissue surrounding the biopsy, to avoid tissue edema and muscle fiber ultrastructural alterations induced by infiltration of anesthetic into the biopsy site.[20] All biopsies were taken at an approximate depth of 3 to 5 cm, 12 to 15 cm above the lateral knee joint space. The muscle samples from each biopsy were either immediately frozen in isopentane cooled to −140°C in liquid nitrogen and stored at −80°C for subsequent histochemical analysis, or immersion-fixed in glutaraldehyde fixative (6.25% glutaraldehyde solution in 0.1 mmol/L sodium cacodylate buffer; total osmolarity, 1,100 mOsm; pH 7.4) for processing for electron microscopy and morphometry.

Histochemistry

Eight-micron thick transverse sections were cut at −24°C on a cryostat (Jung-Reichert Cryocut 1800, Cambridge Instruments, Germany) and kept at −20°C until histochemical processing, which was done within 1 week of sectioning. After 5-minute fixation in a Guth and Samaha fixative at room temperature, the sections were incubated at 37°C for 1 hour in lead (Pb)-adenosine triphosphatase (ATPase) staining medium to simultaneously stain for fiber types I and II and capillaries.[21]

Tissue Preparation for Electron Microscopy

The glutaraldehyde-fixed samples were completely cut into thin longitudinal strips and processed for electron microscopy as previously described.[22] Four plastic-embedded blocks were cut from each biopsy into four transverse l-μm thick sections using an LKB Ultrotome III (LKB Instruments, Sweden) and stained with 0.1% toluidine blue aqueous solution. The sectioning angles used to provide sections transverse to the fiber axis in each block were determined as described previously.[22] Ultrathin transverse sections (50 to 70 nm) were cut from each block, contrasted with uranyl acetate and bismuth subnitrate[23] and electron micrographs for morphometry taken on 70 mm films with a Zeiss 10 electron microscope (Carl Zeiss, Inc, Germany).

Morphometric Analysis

The relative cross-sectional area and number of type I and type II fibers was estimated by point-counting using an eyepiece square grid test A100[24] on histochemical sections examined at a magnification of 250x with a light microscope. On average, 8 ± 1 fields, randomly selected by systematic random sampling, were measured, yielding 205 ± 19 fiber profiles in each sample. Capillary density (ie, capillary number per fiber cross-sectional area), capillary-to-fiber ratio (ie, capillary number per fiber number), and fiber cross-sectional area were measured by point counting on 1-μm thick plastic transverse sections. On average, 4.8 ± 0.2 fields, yielding 66 ± 4 fiber profiles in each sample, were measured with a light microscope using a 100-point square grid eyepiece at 400x magnification. The volume density of mitochondria, myofibrils, lipid droplets, and peroxisomes per volume of muscle fiber was estimated by standard point-counting procedure at a final magnification of 49,000x. Ten micrographs were obtained by systematic random sampling in one ultrathin transverse section from each block. Four randomly chosen blocks (total 80 micrographs) were

Table 1. Clinical Characteristics of Subjects

		TGZ		MET	
	Control	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
n (M/F)	5 (5/0)	10 (9/1)		9 (7/2)	
Age (yr)	47 ± 5	55 ± 2		55 ± 3	
BMI (kg/m^2)	27.9 ± 4.2	36.8 ± 2.4*	38.1 ± 2.6*†	33.6 ± 3.3	33.4 ± 3.2
Fasting plasma glucose (mmol/L)	4.9 ± 0.2	10.3 ± 0.5*	7.0 ± 0.6*†	11.7 ± 0.7*	7.7 ± 0.4*†
Fasting plasma insulin (pmol/L)	58 ± 5	228 ± 42*	192 ± 42*	244 ± 41*	227 ± 37*
HbA_{1c} (%)	5.9 ± 0.3	8.6 ± 0.4*	7.0 ± 0.5†	9.2 ± 0.5*	7.4 ± 0.3*†
GDR (mg · m^{-2} · min^{-1})	463 ± 53	266 ± 27*	355 ± 29*†	274 ± 18*	330 ± 34*

NOTE GDR is the glucose disposal rate determined from hyperinsulinemic (300 mU · m^{-2} · min^{-1}), euglycemic (5.0-5.5 mmol/L) clamp. Results are mean ± SEM.

*$P < .05$ *v* control.

†$P < .05$ *v* pre-Rx value for same individual.

Table 2. Influence of Diabetes and Treatment on Muscle Fiber Type Composition

		TGZ		MET	
	Control	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
Type I	44% ± 1%	32% ± 3%*	31% ± 7%	39% ± 4%	34% ± 3%
Type II	56% ± 1%	68% ± 3%*	69% ± 7%	61% ± 4%	66% ± 3%

*$P < .05$ *v* control subjects.

analyzed per sample. A microfilm reader (Documator DL2, Jenoptic, Jena, Germany) was used to project contact prints of the electron micrographs on a 144-point square grid. All morphometric measurements were performed blind, and the order of samples was randomized.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism program (Intuitive Software, San Diego, CA). Statistical significance was evaluated with Students's *t* test and repeated-measures analysis of variance (ANOVA). Paired analysis was used to determine the effect of treatment in the same subject. Data are presented as mean ± SEM. Significance was accepted at the $P < .05$ level. Because of limitations in tissue availability, not all analyses could be performed in all subjects. The number of subjects studied is given in the figure legends.

RESULTS

Experimental Subjects and In Vivo Effects of Treatment

After initial screening, type 2 diabetic subjects failing control on glyburide alone were randomized into 2 groups and fully characterized. Glucose intolerance and insulin resistance in the diabetic subjects was obvious from comparison to the nondiabetic group (Table 1). Subjects then had either MET or TGZ added to their treatment regimen and the studies repeated 3 to 4 months later. In the baseline (pre-Rx) state, subjects in the 2 treatment groups, even after randomization, were matched for age, obesity (body mass index [BMI]), and other clinical characteristics, including insulin action on whole-body glucose disposal (Table 1). After treatment, relative changes in fasting glucose, HbA_{1c}, and insulin levels were the same in both groups (Table 1), indicating that the extent of glycemic control was matched between groups. Insulin-stimulated whole-body glucose disposal improved with MET treatment by 21% ± 11% over the paired baseline value ($P < .05$). The improvement in insulin action was even greater after TGZ treatment: a 37% ± 8% increase ($P < .005$, $P < .05$ *v* MET response). The difference in the effect of treatment between groups was seen only for the relative (%) change; the absolute increments in GDR did not differ significantly.

Influence of Treatment on Muscle Morphology

Muscle fiber type composition was normal in the subjects in the MET treatment group. The subjects randomized to TGZ therapy, who tended to be more obese, displayed a reduced complement of type I fibers and a corresponding increase in type II fibers (Table 2). This pattern has been previously observed in type 2 diabetic subjects.[2,3] Neither treatment had any influence on fiber type composition.

Figure 1 shows light micrographs of portions of muscle bundles in muscle from control and pre-Rx diabetic subjects. Capillary density was significantly reduced in all the diabetic subjects studied (Fig 2). This was due to the combined effect of lower capillary number and greater fiber cross-sectional area in diabetics than controls, while each parameter alone did not differ from control values (Table 3). With MET treatment there was a tendency ($P < .20$) for capillary density to increase, while TGZ therapy resulted in a greater, and statistically significant ($P = .03$), increase in capillary density. This parameter was still lower than in nondiabetics after treatment.

Electron micrographs of portions of muscle fibers in muscle from control and pre-Rx diabetic are shown in Fig 3. Fiber mitochondrial volume density was also reduced in muscle from the diabetic subjects (Fig 4). Neither treatment had any effect on the mitochondrial volume density (Fig 4). Muscle content of peroxisomes was similar in all subjects and not significantly altered by treatment, though there was a tendency for the

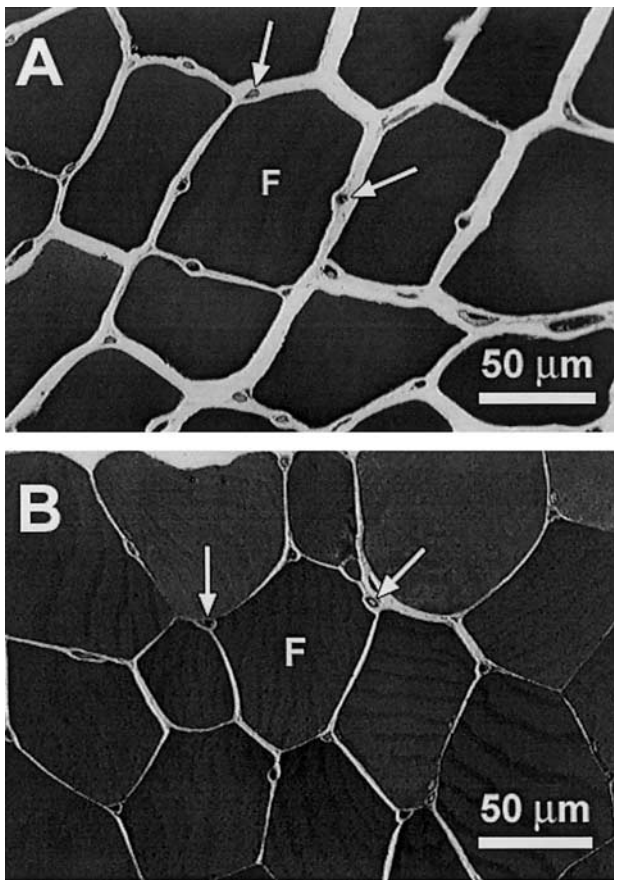

Fig 1. Representative light micrographs of portions of muscle bundles in transverse sections of m. vastus lateralis in control (A) and pre-Rx diabetic (B), showing muscle fibers (F) and capillaries (arrows).

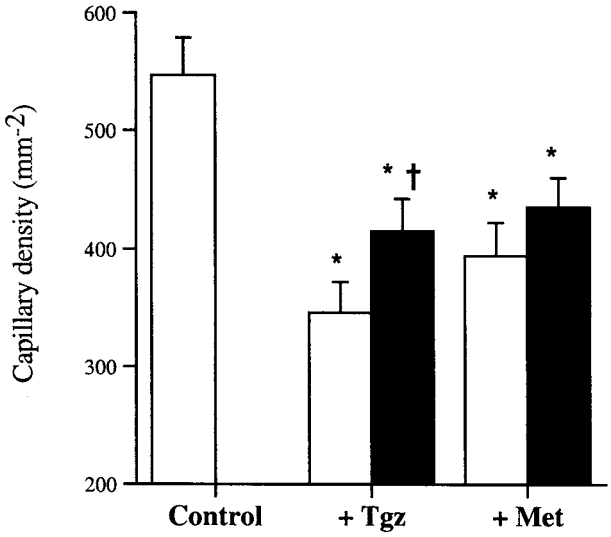

Fig 2. Influence of antidiabetic therapy on capillary density in skeletal muscle. After randomization into the different experimental groups, muscle biopsies were obtained before (□) and after (■) the indicated treatment (TGZ, n = 8; MET, n = 9). Capillary density of the muscle tissue was determined as described in Methods. Results are mean ± SEM. *$P < 0.05$ *v* control. †$P < 0.05$ *v* pre-Rx value for same individual.

volume density of peroxisomes to fall after either TGZ (from 0.34% to 0.07%, $P = .13$) or MET (0.22% to 0.06%, $P = .18$) treatment.

Influence of Treatment on Muscle Lipid Content

IMCL determined as the fraction of cell volume occupied by lipid droplets, was variable and in the baseline state did not differ between the groups (Fig 5). Both MET (23% ± 10% decrease, $P < .05$) and TGZ (33% ± 11% decrease, $P < .05$) treatment led to a reduction in muscle lipid content (Fig 5). Again, the tendency was for TGZ treatment to generate the greater effect, although this difference did not attain statistical significance. A further difference between the effects of the 2 agents is seen in the fact that there was a strong ($r = -0.756$, $P < .05$) correlation between reduction in muscle lipid content and relative improvement in whole-body GDR with TGZ treatment, but no such relationship for MET ($r = -0.237$, $P =$ not significant [NS]; Fig 6).

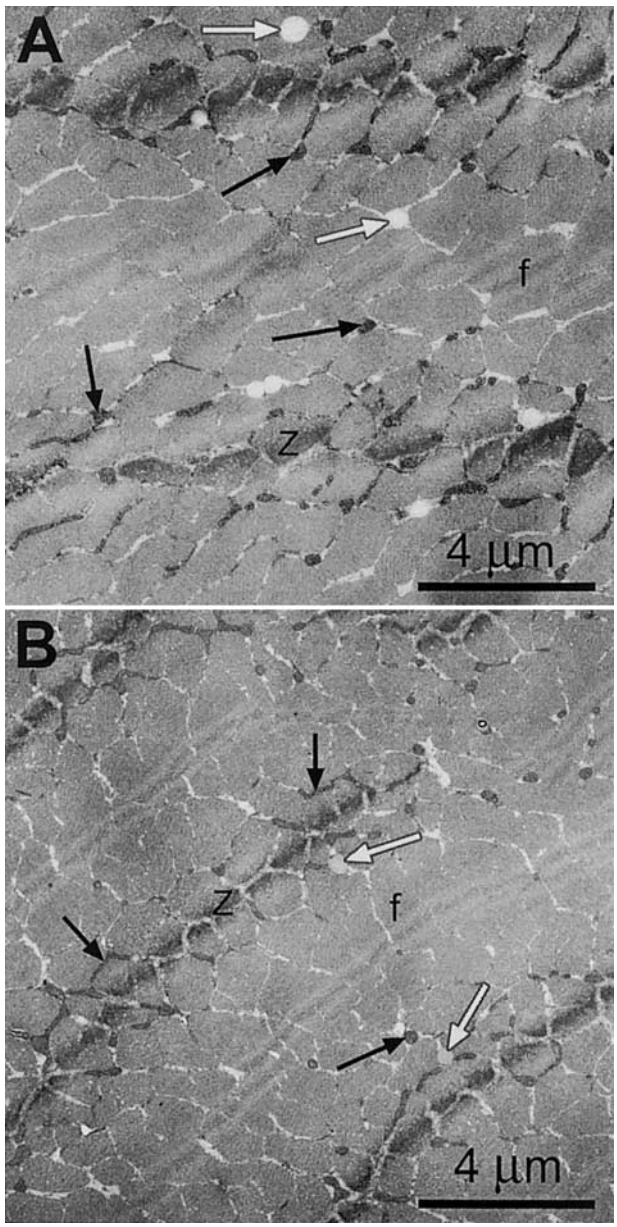

Fig 3. Representative electron micrographs of portions of muscle fiber in transverse sections of m. vastus lateralis in control (A) and pre-Rx diabetic (B), showing myofibrils (f), mitochondria (black arrows) and intracellular lipid droplets (white arrows).

DISCUSSION

Considerable heterogeneity has been noted for the influence of type 2 diabetes on skeletal muscle fiber type composition.[2,6] This variability was apparent in our diabetic study groups which, after randomization, displayed normal or slightly decreased complements of type I muscle fibers. Fiber type composition might not represent a defining characteristic of the

Table 3. Influence of Diabetes and Treatment on Muscle Capillarization and Fiber Size

		TGZ		MET	
	Control	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
Capillary-to-fiber ratio	1.64 ± 0.18	1.35 ± 0.18	1.54 ± 0.18	1.44 ± 0.08	1.57 ± 0.08
No. of capillaries around a fiber	3.98 ± 0.26	3.29 ± 0.33	3.68 ± 0.30	3.43 ± 0.21	3.83 ± 0.20
Fiber cross-sectional area (μm^2)	3,117 ± 459	3,899 ± 382	3,809 ± 511	3,765 ± 281	3,725 ± 310

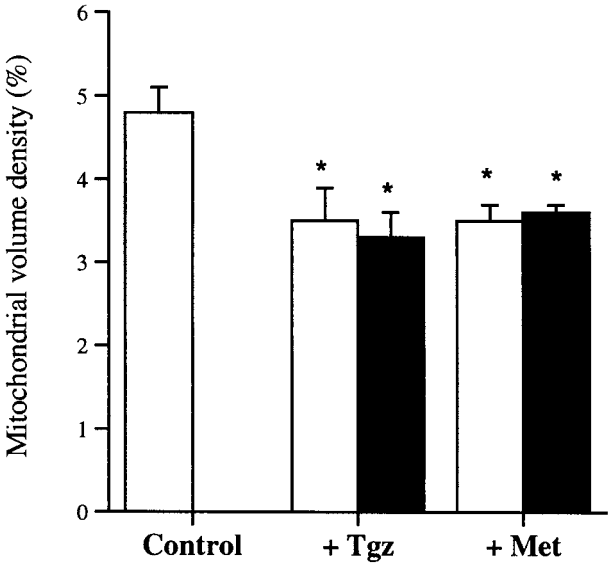

Fig 4. Influence of antidiabetic therapy on mitochondrial content of skeletal muscle. Muscle biopsies were obtained before (□) and after (■) the indicated treatment (TGZ, n = 7; MET, n = 9). Mitochondrial volume densities of the muscle fibers were determined as described in the Methods. Results are mean ± SEM. *$P < 0.05$ *v* control.

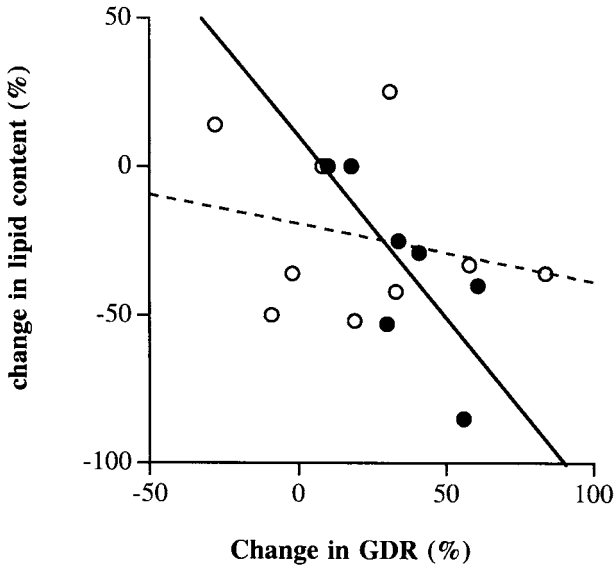

Fig 6. Correlational analysis of relationship between effect of TGZ (●) or MET (○) on changes in insulin-stimulated whole-body GDR and changes in skeletal muscle lipid content. Results presented as % change in the indicated parameter from the pre-Rx to the post-Rx condition.

disease, or at least not a reversible one, as insulin-resistant first-degree relatives of type 2 diabetic subjects also display an elevated complement of type II fibers.[3] There was no effect of antidiabetic therapy on muscle fiber type composition, even with improvements in glycemia and insulin levels. Thus, when present, altered fiber type composition may be an intrinsic property of diabetic muscle.

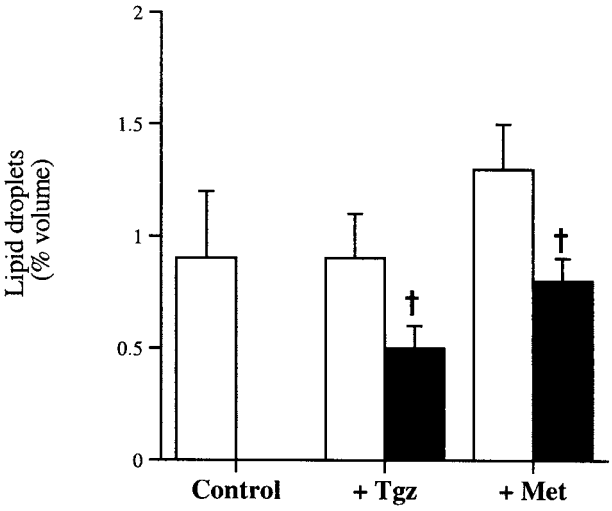

Fig 5. Influence of antidiabetic therapy on lipid content of skeletal muscle. Muscle biopsies were obtained before (□) and after (■) the indicated treatment (TGZ, n = 7; MET, n = 9). The fractional volume of muscle fiber occupied by lipid droplets was determined as described in Methods. Results are mean ± SEM. †$P < .05$ *v* pre-Rx value for same individual.

One determinant of substrate uptake into muscle is capillary density. One mechanism by which insulin increases glucose disposal is to recruit additional capillaries,[7] increasing the capacity to deliver substrate to the muscle. This insulin response is impaired in type 2 diabetes.[25] A reduced skeletal muscle capillary density is a common finding in type 2 diabetes[2] that, together with insulin resistance for capillary recruitment, could have a major impact on glucose disposal in muscle. The diabetes-related decrement in capillary density may be an acquired defect, as insulin-resistant, normoglycemic, first-degree relatives of type 2 diabetic subjects have normal capillary densities.[3] Prolonged exercise training has been shown to increase capillary density in skeletal muscle of obese type 2 diabetic subjects,[26] while increasing insulin action. The defect in capillary density was also partially reversible in our diabetic subjects, as both treatments increased this parameter, though only the response to TGZ attained statistical significance. The current data represent the first report of a pharmacologic intervention that can improve this diabetes-related defect in capillary density.

Disruptions in mitochondrial function and or structure, often due to mutations in the mitochondrial genome, are a common occurrence in certain syndromes of type 2 diabetes involving the pancreas, cardiac muscle, and skeletal muscle.[27] The current analysis does not provide information about mitochondrial function, but did reveal a diabetes-related decrement in the mitochondrial content of skeletal muscle that was not influenced by improvements in glycemia. That is in contrast to control of mitochondrial gene expression, which does appear to be an adaptive response to glucose flux in skeletal muscle.[28] While mitochondrial volume density was not altered by the treatments, a possible impact of either treatment on mitochon-

drial function cannot be ruled out. Indeed, MET has been shown to directly inhibit complex 1 of the respiratory chain in isolated mitochondria,[29] reducing gluconeogenesis, at least in the liver.

It is interesting to note that while TGZ has been identified as a ligand for the peroxisome proliferator-activated receptor gamma (PPARγ) class of nuclear receptors,[30] it did not cause peroxisomal proliferation in human skeletal muscle.

Several laboratories have established a strong relationship between IMCL and insulin resistance in both diabetic and nondiabetic subjects.[8,9,31] In the current report both treatments reduced IMCL along with improving insulin-mediated glucose disposal. Reduction in dietary lipid absorption due to biliopancreatic diversion also reduced IMCL, concomitant with an improvement in insulin-stimulated glucose disposal in morbidly obese individuals.[32] The reductions in IMCL in response to TGZ or MET treatment occurred in the absence of any significant change in circulating triglyceride or free fatty acid levels, suggesting that IMCL need not be directly related to circulating free fatty acid levels. TGZ and MET likely act through different mechanisms to regulate IMCL, as there was no statistical relationship between the magnitude of the changes in IMCL and GDR with MET therapy but a strong correlation for the effect of TGZ on both parameters. Of course, such an association is not proof of causation, but only suggestive of the possibility of an effect of TGZ on muscle lipid content. As one possible mechanism for this effect, we have recently shown, in complementary studies, that TGZ can directly upregulate free fatty acid oxidation in cultured human skeletal muscle cells.[33] Augmented free fatty acid oxidation in skeletal muscle could be one means to reduce IMCL. TGZ treatment of prediabetic Zucker Diabetic Fatty rats also reduced the triglyceride content of islets and reversed alterations in mitochondrial structure.[34] Alternatively, it has been postulated that thiazolidinediones such as TGZ may redirect fat from skeletal muscle to adipose tissue.[35] Whatever the mechanism, alleviation of lipotoxicity in tissues could be a general response to thiazolidinediones, contributing to their insulin-sensitizing actions.

It is interesting to note that, despite similar degrees of glycemic control, whenever effects on muscle morphology were observed (increase in capillary density and decrease in lipid content), the relative change was greater in response to TGZ compared to MET, just as was the result for insulin-stimulated glucose utilization. It is possible that these changes in muscle properties could contribute to insulin regulation of glucose disposal and that TGZ could be exerting a greater relative effect on skeletal muscle, while MET, as documented, would be acting primarily on the liver,[13] together with observed effects on other tissues.[36]

In summary, skeletal muscle from type 2 diabetic subjects displays multiple differences from the nondiabetic condition. These include reduced capillary density and fiber mitochondrial volume density and/or function. The current results present the first evidence that 2 different antidiabetic pharmacologic therapies can improve certain aspects of the phenotype of diabetic muscle, as well as lower muscle lipid content. Relatively speaking, the response of these parameters is greater to TGZ, as is the improvement of insulin-stimulated glucose disposal, which occurs primarily in muscle. Changes in muscle function with these treatments may have both direct and indirect components.

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