

Troglitazone Downregulates Δ -6 Desaturase Gene Expression in Human Skeletal Muscle Cell Cultures

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Δ -6 Desaturase, one of the rate-limiting enzymes, catalyzes the conversion of linoleic acid (C18:2 ω 6) into γ -linolenic acid (C18:3 ω 6), arachidonic acid (C20:4 ω 6), and further metabolites. Recently, it has been shown that human Δ -6 desaturase is expressed not only in liver but in a variety of human tissues, including muscle. Skeletal muscle is a major site of insulin action, and insulin sensitivity may be related to the fatty acid composition of muscle lipids. We examined the effects of troglitazone on the regulation of Δ -6 desaturase gene expression in human muscle cell cultures obtained from muscle biopsies ($n = 15$). Δ -6 Desaturase mRNA and peroxisome proliferator-activated receptor γ 2 (PPAR γ 2) mRNA were quantified by two-step RT-PCR, and the activity of the Δ -6 desaturase enzyme was estimated by gas chromatographic analysis of the ω 6-C18:3/C18:2 fatty acids ratio. In cells treated with 11.5 μ mol troglitazone for 4 days, PPAR γ 2 mRNA levels were significantly increased ($301.0 \pm 51.5\%$, $P < 0.05$) and Δ -6 desaturase mRNA levels were significantly decreased ($41.7 \pm 5.9\%$, $P < 0.0005$) compared with the untreated controls. In accordance with the decrease of Δ -6 desaturase mRNA, there was a significant decrease in the ω 6-C18:3/C18:2 ratio down to $47.4 \pm 7.5\%$ in cholesterol esters, $54.2 \pm 7.4\%$ in phospholipids, $56.7 \pm 6.5\%$ in nonesterified fatty acids, and $67.7 \pm 5.9\%$ in triglycerides. The troglitazone-induced decrease in Δ -6 desaturase mRNA is associated with a change in the unsaturated fatty acid composition of the muscle cells. These results add new aspects to the known thiazolidinedione effects on lipid metabolism. *Diabetes* 51: 1060–1065, 2002

Troglitazone, a thiazolidinedione, has been shown to enhance insulin sensitivity in patients with type 2 diabetes (1,2). One possible mechanism by which these effects are mediated is activation of the peroxisome proliferator-activated receptor γ (PPAR γ). PPAR γ exists in two isoforms, γ 1 and γ 2; PPAR γ 1 is the major form present in a variety of tissues (3), and PPAR γ 2 is expressed in pronounced amounts only in adipose tissue. In skeletal muscle, the presence of PPAR γ 2 was

shown in addition to the major PPAR γ 1 isoform (4). In a further study, it was demonstrated that troglitazone treatment increased PPAR γ 2 mRNA levels up to threefold in human muscle cell cultures (5). These results provide evidence for a direct PPAR γ -mediated response of muscle to these insulin sensitizers with otherwise primary effects on adipose tissue. The recent findings that the expression of lipoprotein lipase and fatty acid binding protein correlate with PPAR γ expression in human skeletal muscle support the hypothesis that PPAR γ activators may regulate fatty acid metabolism in skeletal muscle (6).

Different monounsaturated fatty acids, polyunsaturated fatty acids (PUFAs), and eicosanoids (e.g., 15-deoxy- Δ 12,14-prostaglandin J₂) have been shown to be natural PPAR γ ligands (7). Diets rich in these fatty acids have been reported to lower serum cholesterol and triglyceride levels in humans (8,9), suggesting that these effects are mediated through PPAR γ . In several studies (10,11), it has been shown that the fatty acid composition of serum lipids (12) and skeletal muscle phospholipids (12–14) is associated with insulin sensitivity. The serum cholesterol ester proportion of palmitic (C16:0), palmitoleic (C16:1 ω 7), and di-homo- γ -linolenic (C20:3 ω 6) acids correlates negatively, and that of linoleic acid (C18:2 ω 6) positively, to insulin sensitivity (13). Analysis of skeletal muscle phospholipids revealed decreased insulin sensitivity to be associated with decreased concentrations of PUFAs (14). Significant changes in the metabolism of essential fatty acids and their metabolites have also been found in patients with essential hypertension, coronary heart disease, and type 2 diabetes (15). With the exception of marine fat sources, the availability of these PUFAs for humans depend greatly on the uptake of dietary essential fatty acids and their elongation and desaturation. Δ -6 Desaturase, one of these rate-limiting enzymes, catalyzes the conversion of linoleic acid (C18:2 ω 6) into γ -linolenic acid (C18:3 ω 6), the precursor of arachidonic acid (C20:4 ω 6) and its metabolites. After cloning of human Δ -6 desaturase cDNA (16), Northern analysis revealed that it is expressed not only in liver but in a variety of human tissues including muscle (16,17).

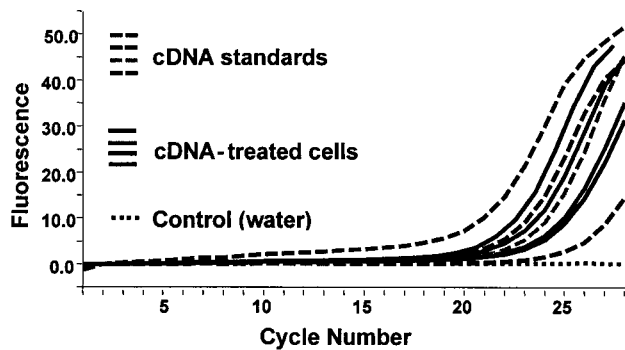
The objectives of the present investigation were to examine the effects of troglitazone on the regulation of Δ -6 desaturase gene expression in human muscle and subsequent alterations in fatty acid composition. We therefore quantified PPAR γ 2 and Δ -6 desaturase mRNA in treated and untreated human muscle cell cultures obtained from muscle biopsies. The activity of the Δ -6 desaturase enzyme

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EFA, essential fatty acid; MCR, metabolic clearance rate; PPAR γ , peroxisome proliferator-activated receptor γ ; PUFA, polyunsaturated fatty acid; TZD, thiazolidinedione.

Δ -6 desaturase

A Baseline adjustment

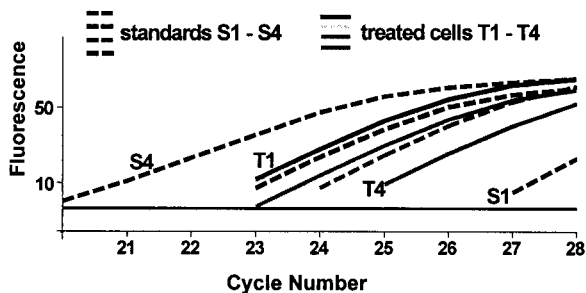
B Identifying the log-linear portion of the standard's amplification curve
S1-S4: increasing concentrations of cDNA (standards)
T1-T4: cDNA from cells treated with increasing concentrations of troglitazone

FIG. 1. Quantitative PCR of Δ -6 desaturase and PPAR γ 2 cDNA. Total RNA was isolated from myotubes treated with different concentrations of troglitazone (0, 2.875, 5.75, and 11.5 μ mol/l for 120 h) and reverse-transcribed. Quantitative PCR of cDNA was done using SYBR Green I Dye on a high-speed thermal cycler with an integrated microvolume fluorimeter (LightCycler; Roche Diagnostics). After removing background fluorescence (baseline adjustment, A) and identifying the log-linear portion of the standard's amplification curve (B), the crossing points were plotted versus the log of copy number. From the resulting crossing points (cycle numbers), a log-linear relationship could be established for the complete range of concentrations in all experiments, with mean squared errors between 0.0013 and 0.0035.

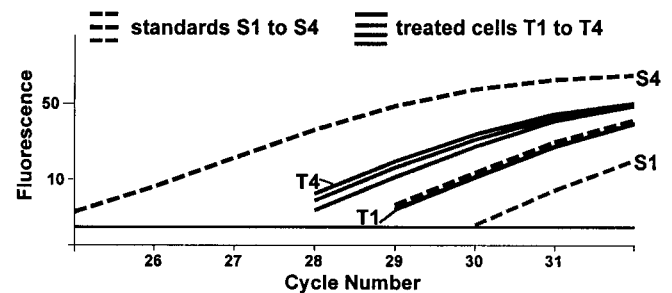
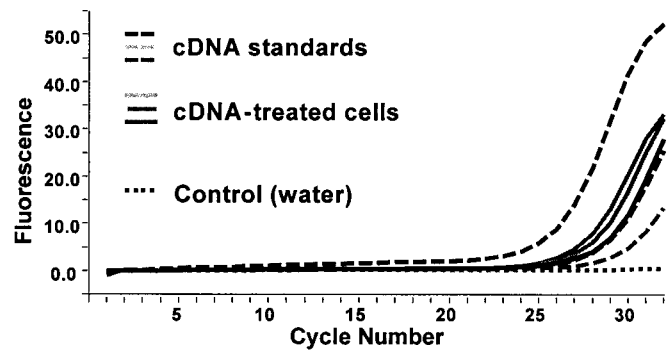
was quantified by measuring the ω 6-C18:3/C18:2 fatty acids ratio in the different lipids extracted from the cell cultures.

RESEARCH DESIGN AND METHODS

Subjects. Percutaneous biopsies of vastus lateralis muscle (18,19) were taken from 15 nondiabetic healthy volunteers (5 men and 10 women; age, 27.9 \pm 6.0 years; BMI, 22.2 \pm 2.2 kg/m²), and muscle tissue was processed for cell cultures or immediately snap-frozen in liquid nitrogen. Glucose tolerance of the volunteers was determined by standard oral glucose tolerance test. Insulin sensitivity was determined by euglycemic-hyperinsulinemic glucose clamp (20,21) and expressed as clamp-derived glucose metabolic clearance rate (MCR). All clinical chemistry parameters were analyzed in the central laboratory of the University of Tübingen according to standard protocols. The study protocol was approved by the ethics committee of the University of Tübingen. Informed written consent was obtained from all subjects.

Cell culture. Myoblasts were isolated from needle biopsies (vastus lateralis muscle) and cultured as previously described by Henry et al. (18), with minor modifications (22). At 80% confluence, cells were fused in α -minimal essential medium supplemented with 2% fetal bovine serum and 0.2% antibiotic-antimycotic solution. During the entire fusion period (120 h), cells were incubated with 11.5, 5.75, 2.875, or 0 μ mol/l troglitazone dissolved in DMSO (final concentration did not exceed 0.05%). The medium was changed every other day. By the end of the fusion period, cells were harvested in PBS to prepare total RNA for RT-PCR and extract total lipids for quantification of fatty acids.

Quantitation of Δ -6 desaturase and PPAR γ 2 mRNA by RT-PCR: RNA isolation and reverse transcription. Total RNA was isolated from myotubes treated with different concentrations of troglitazone (0, 2.875, 5.75, and 11.5 μ mol/l for 120 h) using the optimized phenol guanidinium isothiocyanate extraction method according to the manufacturer's instructions (peqGOLD TriFast; Peqlab Biotechnologie, Erlangen, Germany). Total RNA concentrations were determined by absorbance measurements at 260 nm. Subsequently,

PPAR γ 2

1 μ g total RNA was reverse-transcribed using AMV Reverse Transcriptase (1st Strand cDNA Synthesis Kit for RT-PCR; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Quantitation of cDNA. Quantitative PCR of cDNA was done using SYBR Green I Dye on a high-speed thermal cycler with an integrated microvolume fluorimeter (LightCycler; Roche Diagnostics). The principal method is described in detail elsewhere (23,24). Briefly, the glass capillaries used in the cycler for reaction vessels serve at the same time as optical elements for efficient illumination and fluorescence, allowing for continuous online and real-time monitoring of the reaction kinetics. Quantification with the LightCycler software includes removing background fluorescence, identifying the log-linear portion of the standard's amplification curve, and plotting crossing point (cycle number) versus the log of copy number (concentration). At the end of amplification, the PCR products can be melted, and a graph of the first negative derivative of the fluorescence ($-dF/dT$) versus temperature identifies specific products with different melting temperatures.

The following specific primers (5' to 3' orientation) were designed from Δ -6 desaturase sequence (Genbank accession no. AF126700) yielding a 460-bp fragment: cctacaatcaccagcagaa and ccgatgttgccacatagaga. The amplified product was sequenced (ABI PRISM, 310 Genetic Analyzer; Perkin Elmer, Rodgau-Jugesheim, Germany) to verify its identity. The PCR conditions were optimized using a gradient cycler (Eppendorf, Hamburg, Germany). Specific primers from the PPAR γ 2 sequence (Genbank accession no. AB005520) yielding a 267-bp fragment were designed as reported earlier (25). PCR amplification was performed using the LightCycler/DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instructions. Cycling parameters for Δ -6 desaturase cDNA were 95°C for 8 s, 64°C for 5 s, and 72°C for 12 s, with heating and cooling at a rate of 20°C/s. The temperatures for PPAR γ 2 were 95°C for 8 s, 60°C for 5 s, and 72°C for 11 s. The melting curves were acquired at a heating rate of 0.2°C/s, starting at 55°C for PPAR γ 2 and at 59°C for Δ -6 desaturase. For each set of experiments (cell cultures from one muscle biopsy, treated with 11.5, 5.75, 2.875, or 0 μ mol/l troglitazone), the Δ -6 desaturase and PPAR γ 2 cDNA from the control cells (no

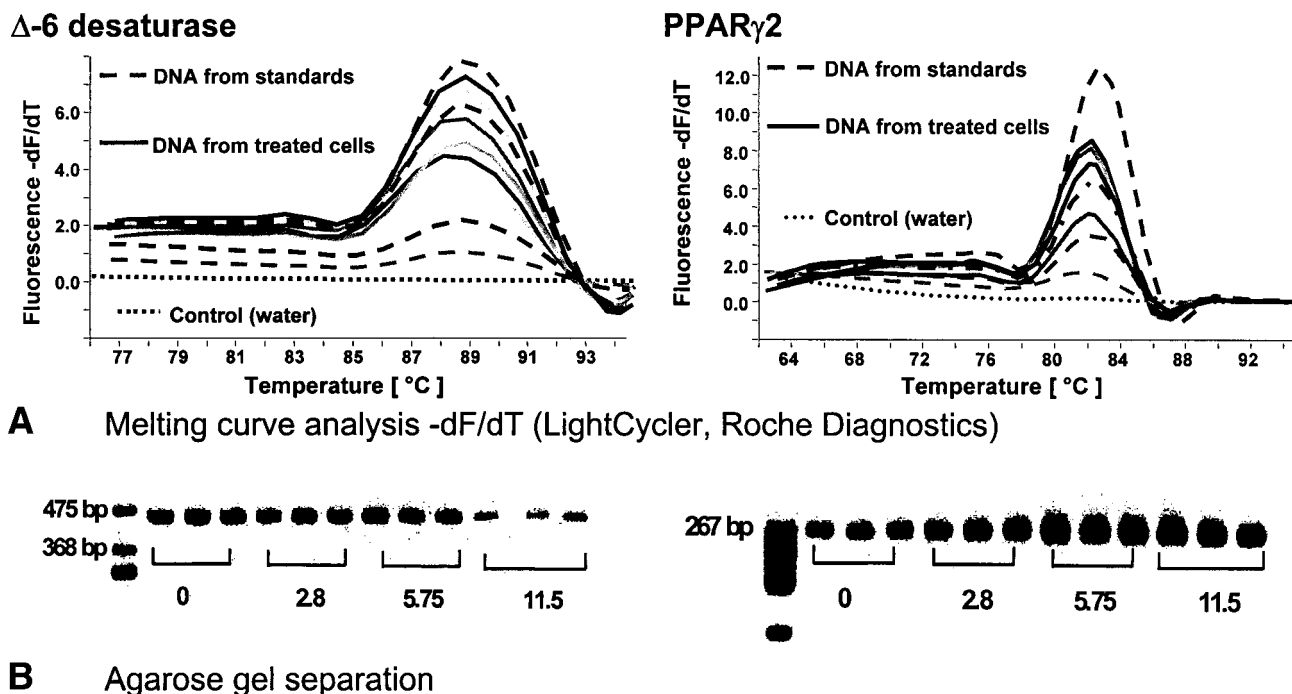


FIG. 2. Confirmation of amplification products (cells treated with 0, 2.8, 5.75, and 11.5 $\mu\text{mol/l}$ troglitazone). The specificity of amplification products is shown by the melting-curve analysis (A) and confirmed by agarose gel separation of the 460-bp fragment for Δ -6 desaturase cDNA and the 267-bp fragment for PPAR γ 2 cDNA (B).

troglitazone treatment) were diluted (1:1, 1:5, and 1:10) and used for the amplification standard curve. All analyses were done in triplicate and reported in percent increase or decrease relative to the untreated cells.

Fatty acid analysis. Quantitation of fatty acids was performed by capillary gas chromatography as previously reported (26), with minor modifications. Total lipids were extracted from the cells and separated by thin-layer chromatography into individual classes of phospholipids, nonesterified fatty acids (NEFAs), diglycerides, triglycerides, and cholesterol ester. The different lipids were scraped off the plate and transferred into screw-capped vials for direct transesterification (27–29). The fatty acid methyl esters of each class were separated by gas chromatography (HP 5890; Hewlett Packard, Waldbronn, Germany) with a flame ionization detector. Separation of the 20 most commonly found fatty acids, including linoleic acid (C18:2 ω 6) and γ -linolenic acid (C18:3 ω 6), was achieved with a fused silica column, 60 m \times 0.25 mm internal diameter, coated with a 0.2- μm film of Rtx-2330 (Restek, Bad

Homburg, Germany). Helium was used as carrier gas at a column head pressure of 16 psi. The oven temperature program was 130°C for 0.2 min, heated at a rate of 2°C/min, and held at 260°C for 10 min. Injection mode was 1 μl splitless with different times for the individual lipid classes. Standard curves were used for all 20 fatty acids, with *cis*-13,16,19-docosatrienoic acid as internal standard. Quantitation was done using HP Chemstation software. The results of linoleic acid (C18:2 ω 6) and γ -linolenic acid (C18:3 ω 6) were confirmed by gas chromatography mass spectrometry analysis.

Chemicals. All fatty acids, including the internal standards used for the calibration curve, were from Sigma (St. Louis, MO). All other reagents were of analytical grade. Troglitazone was a gift from Dr. H. Horikoshi, Sankyo Pharmaceuticals, Tokyo, Japan.

Statistical analysis. All results are presented as means \pm SE unless noted otherwise. Statistical analysis was done using Student's *t* test for paired

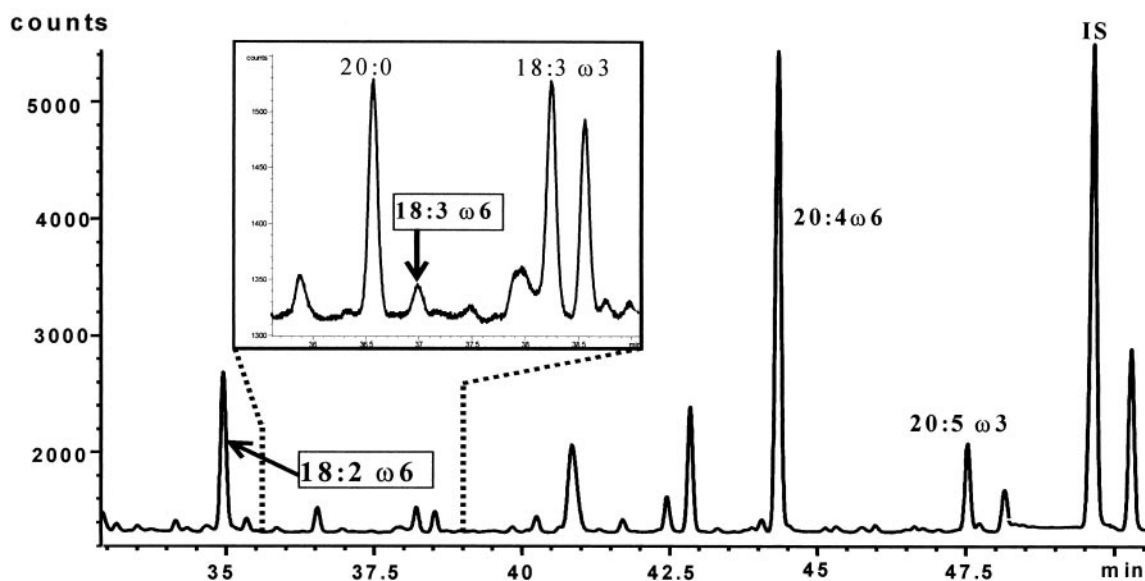


FIG. 3. Chromatogram of transmethylated fatty acids from cholesterol ester lipid fraction showing linoleic acid (C18:2 ω 6) and γ -linolenic acid (C18:3 ω 6). IS, internal standard.

comparison and multiple regression. A P value <0.05 was considered statistically significant. All analyses were done with SSP statistical package.

RESULTS

Subjects. Laboratory tests (means \pm SD) of fasting plasma glucose (4.8 ± 0.3 mmol/l), fasting insulin (5.3 ± 1.8 mU/l), triglycerides (0.89 ± 0.26 mmol/l), and NEFAs (218 ± 55 μ mol/l) were within reference intervals. All subjects had normal glucose tolerance tests according to American Diabetes Association criteria. Clamp-derived glucose MCRs were determined as 7.8 ± 2.2 ml \cdot kg $^{-1}$ \cdot min $^{-1}$.

Quantitation of Δ -6 desaturase and PPAR γ 2 mRNA by RT-PCR. Quantitation of Δ -6 desaturase mRNA and PPAR γ 2 mRNA from troglitazone-treated and untreated control cells was done with a two-step RT-PCR (LightCycler; Roche Diagnostics). Figure 1 shows the quantitation procedure for one set of experiments for both Δ -6 desaturase mRNA and PPAR γ 2 mRNA: after removing background fluorescence (baseline adjustment, Fig. 1A) and identifying the log-linear portion of the standard's amplification curve (Fig. 1B), the crossing points were plotted versus the log of copy number. From the resulting crossing points (cycle numbers), a log-linear relationship could be established for the complete range of concentrations in all experiments, with mean squared errors between 0.0013 and 0.0035. In addition to the specificity shown by the melting curve analysis (Fig. 2A), agarose gel separation was done to confirm the amplification products (Fig. 2B).

Fatty acid analysis. Quantitation of fatty acids was performed by capillary gas chromatography with flame ionization detection. Figure 3 shows a typical chromatogram of transmethylated fatty acids from the cholesterol ester lipid fraction (linoleic acid, γ -linolenic acid, and internal standard). The ω 6-C18:3/C18:2 ratio was used as activity index for Δ -6 desaturase.

Troglitazone effects on PPAR γ 2 and Δ -6 desaturase mRNA. Increasing levels of troglitazone to 11.5 μ mol/l caused a dose-dependent decrease in Δ -6 desaturase mRNA and an increase in PPAR γ 2 mRNA (Fig. 4A and B) in the human skeletal muscle cell cultures. In cells treated with 11.5 μ mol troglitazone for 4 days, PPAR γ 2 mRNA levels were significantly ($P < 0.05$, paired t test) increased to $301.0 \pm 51.5\%$ of the untreated controls (Fig. 5A), and Δ -6 desaturase mRNA levels were significantly ($P < 0.0005$, paired t test) decreased to $41.7 \pm 5.9\%$ of the untreated controls (Fig. 5B). The increase in PPAR γ 2 mRNA was associated with a decrease in Δ -6 desaturase mRNA in each individual, and there was a strong correlation ($P = 0.0012$, multiple regression) between the increase in PPAR γ 2 mRNA and the decrease in Δ -6 desaturase mRNA. There was no correlation between any of the in vitro troglitazone effects observed (increase in PPAR γ 2 mRNA, decrease in Δ -6 desaturase mRNA, or decrease in Δ -6 desaturase activity) and the in vivo determined MCR as insulin sensitivity marker.

The Δ -6 desaturase activity was estimated by quantifying the fatty acids in the different lipids extracted from the cells. A decrease in Δ -6 desaturase activity would cause a decrease in the ω 6-C18:3/C18:2 ratio. Paralleling the decrease of Δ -6 desaturase mRNA by troglitazone, there was a significant decrease of the ω 6-C18:3/C18:2 ratio ($P < 0.05$) in all lipid classes (Fig. 5C) under investigation:

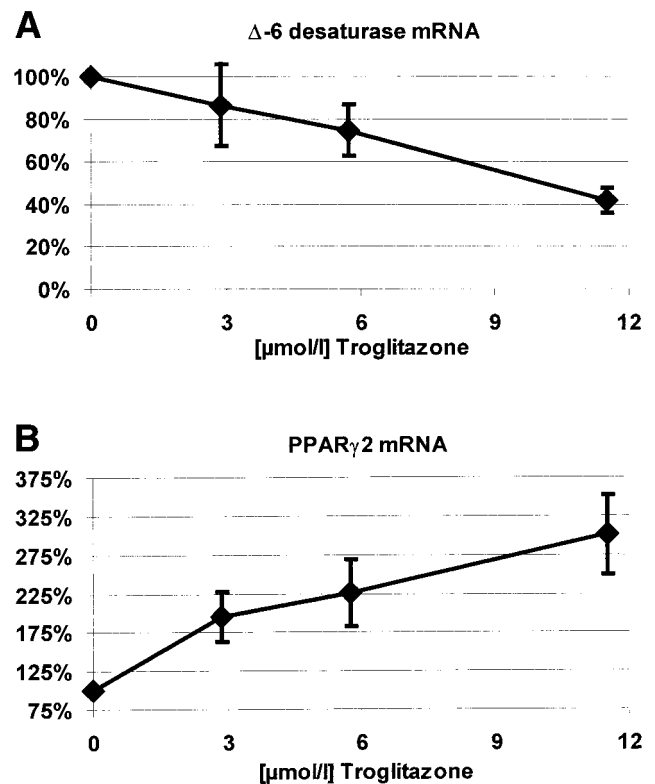


FIG. 4. Dose dependence. Increasing levels of troglitazone up to 11.5 μ mol/l caused a dose-dependent decrease (means \pm SE) in Δ -6 desaturase mRNA (A) and an increase in PPAR γ 2 mRNA (B) in human skeletal muscle cell cultures ($n = 15$).

relative to the untreated cell cultures, the ratio decreased to $47.4 \pm 7.5\%$ in cholesterol esters, $54.2 \pm 7.4\%$ in phospholipids, $56.7 \pm 6.5\%$ in NEFAs, and $67.7 \pm 5.9\%$ in triglycerides.

DISCUSSION

Our results, showing regulation of Δ -6 desaturase by troglitazone, are in good agreement with the downregulation of stearoyl-CoA desaturase 1 (SCD1) gene expression in 3T3-L1 adipocytes by thiazolidinediones (30). SCD1, a Δ -9 desaturase, catalyzes the conversion of stearic (C18:0) to oleic acid (C18:1 ω 9) and palmitic (C16:0) to palmitoleic acid (C16:1 ω 7). It was shown by mRNA differential display method that both troglitazone and pioglitazone downregulate SCD1 gene expression in 3T3-L1 adipocytes in a dose-dependent manner (30). SCD1 enzyme-catalyzed Δ 9-*cis* desaturation was subsequently inhibited as shown by the significantly lower content of C16:1 and C18:1 fatty acids (weight percentage) in the treated adipocytes.

Troglitazone and other thiazolidinediones (TZDs) mediate their effects by binding to and activating the transcription factor PPAR γ (31–33). Most studies on PPAR γ and TZDs have been performed in adipose tissue or tissue cultures. Improvements in skeletal muscle action and glucose metabolism may then be indirect, by lowering of lipid levels through the glucose-fatty acid cycle (34); however, with PPAR γ being present in skeletal muscle (4,35), there could also be direct effects. This might especially be true in type 2 diabetic and nondiabetic obese subjects, where PPAR γ mRNA expression in human skel-

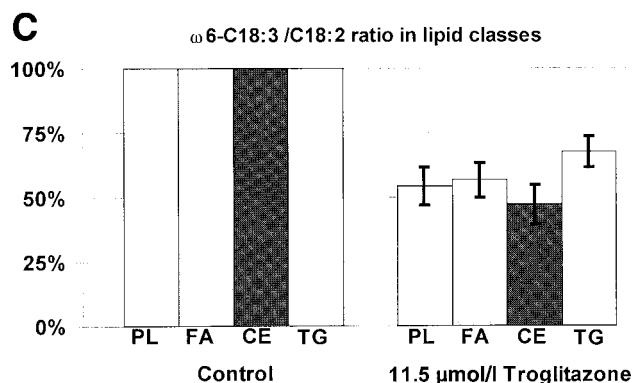
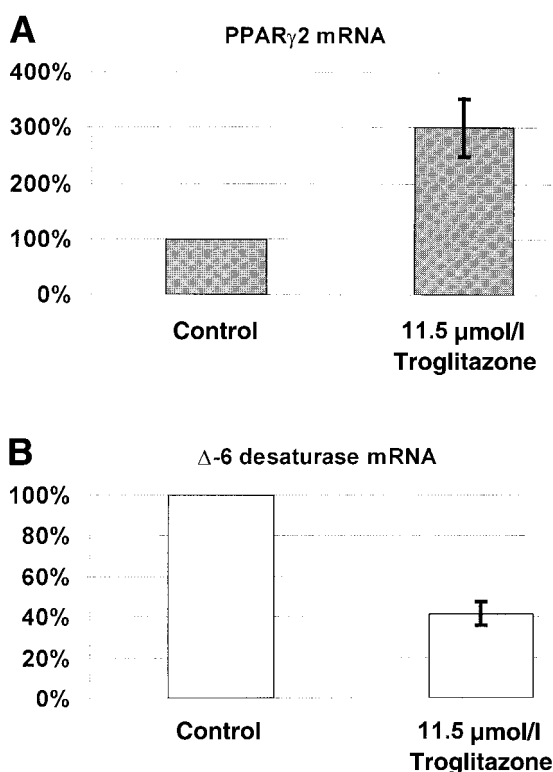


FIG. 5. Troglitazone effects. In cells ($n = 15$) treated with 11.5 μ mol troglitazone for 4 days, PPAR γ 2 mRNA levels were significantly ($P < 0.05$, paired t test) increased to $301.0 \pm 51.5\%$ of the untreated controls (Fig. 5A) and Δ -6 desaturase mRNA levels were significantly ($P < 0.0005$, paired t test) decreased to $41.7 \pm 5.9\%$ of the untreated controls (Fig. 5B). Paralleling the decrease of Δ -6 desaturase mRNA by troglitazone, there was a significant decrease of the ω 6-C18:3/C18:2 ratio (desaturase activity index) in all lipid classes under investigation ($P < 0.05$) (Fig. 5C). Relative to the untreated cell cultures, the ratio decreased to $47.4 \pm 7.5\%$ in cholesterol esters (CE), $54.2 \pm 7.4\%$ in phospholipids (PL), $56.7 \pm 6.5\%$ in NEFAs, and $67.7 \pm 5.9\%$ in triglycerides (TG). All results are presented as means \pm SE.

etal muscle is increased (35). Troglitazone treatment of human skeletal muscle culture (4 days, 11.5 μ mol/l) led to a 1.43 ± 0.25 -fold increase of PPAR γ 1 mRNA (90% of total PPAR γ) and a 2.5 ± 0.7 -fold increase of PPAR γ 2 mRNA (10% of total PPAR γ) compared with untreated controls (5). Next to the regulation of Δ -6 desaturase gene expression, we were therefore interested in the effects of troglitazone on the regulation of PPAR γ 2 mRNA in the same human muscle cell cultures obtained from muscle biopsies. Increasing the level of troglitazone up to 11.5 μ mol/l caused a dose-dependent increase in PPAR γ 2 mRNA levels in the human skeletal muscle cell cultures. In cells treated with 11.5 μ mol troglitazone for 4 days, PPAR γ 2 mRNA levels were significantly increased to $301.0 \pm 51.5\%$ of the untreated controls ($P < 0.05$, paired t test). There was a strong correlation ($P = 0.0012$, multiple regression) between the increase in PPAR γ 2 mRNA and the decrease in Δ -6 desaturase mRNA, suggesting that the regulation of Δ -6 desaturase gene expression is mediated by PPAR γ . In this regard, it will be of interest to see if a functional PPRE (PPAR response element) is found in the promoter region of the Δ -6 desaturase gene.

Regulation of desaturase gene expression will eventually lead to altered fatty acid composition of lipids. In the case of serum lipids, it has been shown that the serum cholesterol ester proportion of palmitic (C16:0), palmitoleic (C16:1 ω 7), and di-homo- γ -linolenic (C20:3 ω 6) acids correlated negatively, and that of linoleic acid (C18:2 ω 6) positively, to insulin sensitivity (12). Analysis of skeletal muscle phospholipids from healthy subjects revealed decreased insulin sensitivity to be associated with decreased concentrations of PUFAs (14). Although it is not statistically significant, there seems to be a noteworthy difference between Δ -6 and Δ -5 desaturase activity with regard to insulin sensitivity. The ratio of ω 6-C20:4/C20:3 as Δ -5

desaturase activity index correlated positively ($r = 0.84$, $P < 0.001$), whereas the ratio of ω 6-C18:3/C18:2 as Δ -6 desaturase activity index showed a negative coefficient ($r = -0.28$, $P > 0.05$) (14). These studies (12,14) suggest that PUFAs do not in general correlate with insulin sensitivity, but there seem to be differences between the individual PUFAs. To further elucidate the effect of troglitazone on muscle fatty acid composition and, in particular, on PUFAs, it would be important to investigate its effect on all the desaturases involved (Δ -9, Δ -6, and Δ -5) as well as the complete fatty acid profiles.

Δ -6 Desaturase enzymatic activity varies with hormonal and nutritional manipulation (16,36,37). In addition to being affected by insulin, fasting, and feeding, the activity is highly dependent on the composition of dietary fat (36–38). Fats rich in essential fatty acids (EFAs) cause a lower activity than those low in EFAs (37). In mice, a triolein-rich (EFA-deficient) diet caused a significantly higher Δ -6 desaturase enzymatic activity compared with a corn oil-rich (EFA-rich) diet (16). Both Δ -6 desaturase and Δ -5 desaturase in human Hep G2 cells are rate limiting to fatty acid interconversion and are upregulated under essential fatty acid-deficient conditions (39). Certain mono- and polyunsaturated fatty acids and their metabolites (7), as well as TZDs and fibrates, have been shown to interact directly with PPAR α and/or PPAR γ . Because PPARs appear to play a central role in the catabolism and storage of fatty acids, this could be the underlying molecular mechanism whereby dietary lipids affect such metabolic diseases as obesity, atherosclerosis, and type 2 diabetes.

In summary, we showed that increasing levels of troglitazone caused a dose-dependent increase in PPAR γ 2 mRNA levels and a decrease in Δ -6 desaturase mRNA in human skeletal muscle cell cultures. In cells treated with 11.5 μ mol/l troglitazone, PPAR γ 2 mRNA levels were sig-

nificantly increased and Δ -6 desaturase mRNA levels significantly decreased, with a corresponding decrease in Δ -6 desaturase enzyme activity. The decrease in Δ -6 desaturase mRNA levels led to a change in the muscle cells' unsaturated fatty acid composition; these results add new aspects to the known TZD effects on lipid metabolism in muscle.

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