

# Troglitazone Decreases the Proportion of Small, Dense LDL and Increases the Resistance of LDL to Oxidation in Obese Subjects

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**OBJECTIVE** — Insulin resistance is associated with a predominance of small, atherogenic LDL particles that are more prone to oxidative modification. Treatment with the insulin-sensitizer troglitazone may improve LDL composition and resistance to oxidation.

**RESEARCH DESIGN AND METHODS** — In a randomized double-blind crossover design, 15 obese subjects were treated with either 400 mg troglitazone daily or placebo for 8 weeks. Insulin sensitivity (clamp), (apo)lipoproteins, LDL subclass pattern, plasma TBARS, and ex vivo LDL oxidation were determined.

**RESULTS** — Troglitazone treatment improved insulin sensitivity. LDL cholesterol increased from  $2.58 \pm 0.18$  to  $2.77 \pm 0.20$  mmol/l ( $P = 0.03$ ) because of an increase in large (buoyant) LDL<sub>1</sub> (from  $0.45 \pm 0.04$  to  $0.62 \pm 0.09$  mmol/l,  $P = 0.008$ ). Because small (dense) LDL<sub>3</sub> decreased, LDL<sub>1</sub>:LDL<sub>3</sub> ratio increased ( $P = 0.02$ ). Plasma TBARS concentration declined significantly, and the lag time of ex vivo LDL oxidation showed a small but significant increase.

**CONCLUSIONS** — In obese subjects, treatment with troglitazone improves insulin sensitivity, increases the ratio of large buoyant to small dense LDL, and appears to enhance the resistance of the LDL particle to oxidation. These qualitative changes in lipoproteins may have a beneficial effect on cardiovascular risk profile and compensate for a small increase in LDL cholesterol.

The increased risk for cardiovascular morbidity and mortality as found in patients with NIDDM may in part be explained by unfavorable changes in lipoproteins (1). In NIDDM patients, the major quantitative change is an elevation in triglyceride-rich lipoproteins, often accompanied by a decrease in HDL cholesterol concentration (2). Although the levels of LDL cholesterol are similar to those seen in the general population, differences in the composition of LDL may make these particles more atherogenic (3). Such qualitative changes include glycation of LDL, oxidation of LDL, and/or the presence in the

plasma of smaller and denser LDL. Dyslipidemia as observed in NIDDM may be related to hyperglycemia, as well as to the underlying insulin resistance (4).

Troglitazone is a recently developed compound that is capable of improving insulin sensitivity in human diabetes (5) and obesity (6). The exact mechanism of action is not yet known (7). Troglitazone contains, besides the thiazolidine-2-4-dione structure, an  $\alpha$ -tocopherol structure that was introduced to inhibit lipid peroxidation. As such, it may be expected that treatment with troglitazone may induce beneficial changes in lipid profiles, either by

improvement of insulin sensitivity or by an antioxidant effect. In vitro studies with troglitazone appear to confirm this possible action (8,9). Because a change in metabolic control (induced by whatever means) will induce quantitative and qualitative changes in lipoproteins in patients with diabetes (10), drug-induced changes in lipid concentrations may also be caused by the change in glucose metabolism in itself. To determine the direct effects of troglitazone on quantitative and especially qualitative lipoprotein parameters in vivo, we performed a randomized placebo-controlled double-blind crossover trial in obese individuals characterized by insulin resistance.

## RESEARCH DESIGN AND METHODS

### Subjects

The study group consisted of 15 obese, normotensive, healthy volunteers on no medication. Inclusion criteria were age between 25 and 50 years, nonsmoking, and a BMI between 27 and 36 kg/m<sup>2</sup>. All subjects had normal fasting glucose concentrations and a stable body weight. All gave written informed consent. The experimental protocol was approved by the hospital's ethical committee.

### Protocol

After inclusion, obese subjects received either two tablets of 200 mg of troglitazone once a day or placebo for 8 weeks in a randomized double-blind crossover design. Participants were strictly advised to maintain their weight and not to change their diet. At the end of the two treatment periods (time interval of ~10 weeks), 30 ml of blood was drawn after an overnight fast, with the subjects resting in supine position. The samples were collected in EDTA-containing tubes on melting ice. Blood was spun in a refrigerated centrifuge, and cepharose was added to the plasma. Samples were then frozen. Subsequently, a euglycemic-hyperinsulinemic clamp (insulin [Actrapid; Novo-Nordisk], infusion rate  $430 \text{ pmol} \times \text{m}^2 \times \text{min}^{-1}$  [60

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**Abbreviations:** apo, apolipoprotein; CV, coefficient of variation; NEFA, nonesterified fatty acid; TBARS, thiobarbituric acid reactive substance.

**Table 1—Effects of troglitazone on lipids and (apo)lipoproteins and oxidation parameters in 15 obese subjects**

	Placebo	Troglitazone	P value
Cholesterol (mmol/l)	4.91 ± 0.26	5.11 ± 0.27	0.22
Triglyceride (mmol/l)	2.11 ± 0.59	1.68 ± 0.35	0.34
VLDL cholesterol (mmol/l)	1.36 ± 0.30	1.34 ± 0.32	0.93
VLDL triglyceride (mmol/l)	1.80 ± 0.58	1.39 ± 0.33	0.34
HDL cholesterol (mmol/l)	0.98 ± 0.06	0.99 ± 0.07	0.67
LDL cholesterol (mmol/l)	2.58 ± 0.18	2.77 ± 0.20	0.03
LDL <sub>1</sub>	0.45 ± 0.04	0.62 ± 0.09	0.008
LDL <sub>2</sub>	0.96 ± 0.13	1.13 ± 0.09	0.087
LDL <sub>3</sub>	1.17 ± 0.20	1.02 ± 0.22	0.16
ApoA1 (mg/l)	1,211 ± 38	1,174 ± 42	0.15
ApoB (mg/l)	1,128 ± 70	1,156 ± 93	0.58
LDL oxidation			
Lag time (min)	97.4 ± 2.4	100.9 ± 2.2	0.012
Oxidation rate (mg · mmol <sup>-1</sup> · min <sup>-1</sup> )	14.1 ± 0.5	14.5 ± 0.6	0.53
TBARS (nmol/l)	1.06 ± 0.07	0.92 ± 0.04	0.04
Vitamin E (mg/g LDL protein)	2.90 ± 0.22	3.13 ± 0.21	0.29

Data are means ± SEM. LDL oxidation data refer to 10 subjects.

mU · dl<sup>-1</sup> · min<sup>-1</sup>) was performed, over 120 min. Body weight, waist and hip measurements, electrocardiogram, fat skinfold thickness, possible side effects, and serum safety chemical and hematological profiles were determined. Between each treatment periods, there was a 2 week washout period. Compliance, monitored by pill counts and diary records, was over 90%.

### Analytical methods

Plasma glucose was measured by the glucose oxidation method (Beckman Glucose Analyzer 2; Beckman Instruments, Fullerton, CA). Plasma insulin was measured with a double antibody radioimmunoassay (inter-assay coefficient of variation [CV] 6.2%). Plasma C-peptide was measured with a commercially available double antibody radioimmunoassay (Diagnostic, Los Angeles, CA; interassay CV 4.3%). Free fatty acids (nonesterified fatty acids [NEFAs]) were analyzed with an enzymatic method (ACS-ACOD, NEFA C-kit; Waco, Neuss, Germany). Total plasma cholesterol and triglyceride concentrations were determined by commercially available enzymatic reagents. VLDL was isolated from whole plasma by sequential ultracentrifugation at density 1.019 g/ml for 16 h at 36,000 rpm in a fixed angle rotor (TFT 45.6 rotor; Kontron, Zurich, Switzerland) in a Beckman L7-55 ultracentrifuge. HDL cholesterol was determined with the polyethylene glycol 6000 method (11). LDL cholesterol was cal-

culated by subtraction of VLDL and HDL cholesterol from total plasma cholesterol. Total plasma apolipoprotein (apo) A<sub>1</sub> and B was determined by immunonephelometry (12). To achieve accurate results in relation to the Centers for Disease Control Standardization Program, the obtained plasma apoB results were recalculated on the basis of exchange of sera with Dr. S. Marcovina (Northwest Lipid Research Laboratory, Seattle, WA). LDL subfractions were detected by single spin density-gradient ultracentrifugation according to a previously described method (13).

### Oxidation of LDL

Plasma isolation was immediately followed by LDL isolation by density-gradient ultracentrifugation (40,000 rpm for 18 h at 4°C) using an SW40 rotor (Beckman). The oxidation experiments were performed by a modification of the Esterbauer method (14). Each LDL preparation was oxidized twice in two separate oxidation runs on the same day. The interassay CVs for lag time and oxidation rate were 1.2 and 5%, respectively (*n* = 10). The content of thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde, in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid-malondialdehyde complex between 500 and 600 nm on a Shimadzu RFF-5000 recording spectrofluorometer. The vitamin E concentrations in LDL were determined by high-performance

liquid chromatography, as described previously (15).

### Statistical analysis

Coefficients of plasma glucose variation during the last 30 min of the clamp were 3.1 ± 0.4% (placebo) and 3.1 ± 0.3% (troglitazone). Whole-body glucose uptake was defined as the glucose infusion rate during the last 30 min of the clamp and expressed in μmol · kg<sup>-1</sup> · min<sup>-1</sup> (16). Differences in values for lipid, lipoproteins, whole-body glucose uptake, and fasting glucose and insulin concentrations during placebo and during troglitazone were tested by Student's *t* test. Pearson correlation coefficients were calculated to determine the association between changes in various parameters. All statistical analyses were performed using the SPSS personal computer software package (SPSS, Chicago). Results in tables and figures are expressed as means ± SE unless otherwise indicated. Significance was set at a *P* value of < 0.05.

**RESULTS** — The study population consisted of 15 subjects (M/W: 9/6), mean (± SD) age 37.4 ± 4.6 years, body weight 96.0 ± 9.0 kg, BMI 31.7 ± 2.9 kg/m<sup>2</sup>. Troglitazone was well tolerated by all subjects. Compared with normal values for insulin sensitivity in lean subjects in our laboratory, the obese subjects were clearly insulin resistant.

### Effect of troglitazone on metabolic parameters

Fasting plasma glucose did not change (placebo, 5.6 ± 0.2; troglitazone, 5.5 ± 0.1 mmol/l; NS). Fasting insulin concentration tended to decrease (placebo, 85.6 ± 8.2; troglitazone, 69.8 ± 6.8 pmol/l; *P* = 0.11). Body weight did not significantly change during either treatment period (placebo, +0.75 ± 0.46 kg; troglitazone, +0.41 ± 0.48 kg; NS). Blood glucose levels and steady-state plasma insulin concentrations were similar in both clamp procedures. Troglitazone increased whole-body glucose uptake from 26.8 ± 3.0 to 31.9 ± 3.3 μmol · kg<sup>-1</sup> · min<sup>-1</sup> (*P* = 0.028 vs. placebo), with a mean percentage increase of 23.1 ± 10.5% (*P* = 0.047).

### Effect of troglitazone on lipid concentrations and LDL subclass pattern

Table 1 shows the effects of troglitazone on plasma lipid levels and LDL subclass pattern. Troglitazone treatment was associated

with small decrements in NEFA (from  $0.61 \pm 0.07$  to  $0.47 \pm 0.05$  mmol/l,  $P = 0.06$ ), whereas total cholesterol, HDL cholesterol, and triglyceride concentrations did not change significantly. A small but significant increase in LDL cholesterol was observed. This increase was due to an increase in LDL<sub>1</sub> subfraction, whereas the LDL<sub>3</sub> subfraction tended to decrease. As a result, the ratio of LDL<sub>1</sub> to LDL<sub>3</sub> increased from  $0.65 \pm 0.15$  to  $1.15 \pm 0.26$  ( $P = 0.02$ ).

#### Effect of troglitazone on resistance to oxidation of LDL

During troglitazone treatment, plasma TBARS concentration decreased significantly (Table 1). Troglitazone appeared to have a small but significant beneficial effect on resistance of the LDL particle against oxidation: during treatment, oxidation lag time increased. The rate of oxidation did not change. The effect on lag phase was not due to changes in the concentration of vitamin E (Table 1).

#### Correlation between changes in insulin sensitivity and lipid levels

No correlations were found between troglitazone-induced changes in insulin sensitivity (change in whole-body glucose uptake during the clamp) and changes in lipid or lipoprotein levels. Correlations between the change in insulin sensitivity (glucose uptake during the clamp) and changes in LDL, LDL<sub>3</sub>, HDL, triglyceride, and lag time were 0.08, 0.14, 0.25, 0.11, and  $-0.08$  (NS for all).

**CONCLUSIONS** — The major observation of the present study is that troglitazone treatment improves the ratio of large buoyant to small dense LDL despite the absence of a clear change in plasma and VLDL triglycerides and HDL cholesterol. Furthermore, troglitazone appears to enhance the resistance of LDL particles to oxidation in vitro (increase in lag time) and to reduce lipid oxidation in vivo (decrease in TBARS). Because changes in lipoprotein profiles were unrelated to changes in insulin sensitivity, these effects of troglitazone appear to reflect a direct effect.

#### Effects of troglitazone on lipoprotein profiles

Recently, in a 48-week parallel randomized placebo control trial in patients with NIDDM, treatment with troglitazone induced an increase in total cholesterol, HDL cholesterol, and LDL cholesterol and

decreased triglyceride concentration compared with glyburide; the ratio of HDL to LDL was unchanged (17). In yet unpublished data, treatment with troglitazone was associated with dose-dependent alterations in serum lipid concentrations. With the highest dose used (600 mg), an increase in LDL cholesterol of 7–10% is reported (Parke-Davis: Data available at <http://www.warner-lambert.com/info/rezulin.html>). The ratio of HDL to LDL cholesterol did not change. ApoA concentration also remained unchanged. Because troglitazone significantly, albeit slightly, increases LDL cholesterol concentration, it is important to know what the qualitative properties of that LDL are. We demonstrate in this study that the increase in LDL cholesterol is due to an increase in large (buoyant) LDL, whereas the concentration of small dense LDL tends to decrease. As a result, the ratio of LDL<sub>1</sub> to LDL<sub>3</sub> increased. The quantitative increase in LDL is thus associated with a less atherogenic subclass pattern.

Earlier in vitro studies have reported that troglitazone increased resistance of the LDL particle to oxidation (9,10). We have demonstrated for the first time that small dense LDL is more susceptible to in vitro oxidation (15), as confirmed by several other groups (18). As such, it may be anticipated that a more favorable distribution of LDL size will be associated with an increased resistance to in vitro oxidation. This expectation is now confirmed in this study. However, our results refer only to 10 subjects and need confirmation. The observed significant decrease of TBARS during troglitazone treatment in this study supports an antioxidative effect.

#### Troglitazone and insulin resistance

The obese state represents “pure” insulin resistance, and thus changes in insulin sensitivity cannot be caused by changes in blood glucose concentrations (“glucose toxicity”) (19). Troglitazone improved total-body insulin-mediated glucose uptake in this group of subjects, consistent with earlier studies in humans (5,6), but the changes in this group of subjects under these strictly double-blind placebo-controlled conditions were modest. We found no relationship between the effects of troglitazone on lipoprotein profiles and the effect of insulin on insulin sensitivity. Therefore, the effects of troglitazone may reflect a direct action on lipid metabolism, possibly related to the specific biochemical (antioxidant) structure. In this study, we cannot provide

direct evidence for this hypothesis. Recently, an increase in lag time during in vitro oxidation was reported in normal volunteers after only 2 weeks’ use of troglitazone (20). Given the fact that the effect of troglitazone on insulin sensitivity appears to take at least 4 weeks to develop (17), this finding may support the concept that troglitazone exerts its effect on lipid oxidation by an antioxidant effect and not by a change in insulin sensitivity. It will be important to compare the possible direct antioxidant effect of troglitazone with administration of vitamin E in future trials. An alternative explanation for the effect of troglitazone on lipid composition is that troglitazone may have cross-reactivity with the receptors for which fibrates are the ligands (peroxisome proliferator-activated receptor- $\alpha$ ). Recent findings, however, appear to oppose this mechanism (21). Although the changes in lag time to in vitro oxidation appear small, it is currently not known to what extent changes in lipid oxidation correlate with clinical events.

In conclusion, we report that in obese, normotensive individuals, treatment with the insulin-action enhancer troglitazone is associated with a more beneficial ratio of large buoyant to small dense LDL. Troglitazone treatment appears to enhance the resistance to oxidation. These qualitative changes may compensate for the slight increases in total serum and LDL cholesterol and may improve the cardiovascular risk profile related to insulin resistance. The changes in lipoprotein parameters were not related to changes in insulin sensitivity, which suggests for a separate mode of action.

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