Rapid Publications

Troglitazone Reduces LDL Oxidation and Lowers Plasma E-selectin Concentration in NIDDM Patients

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Troglitazone, an oral antidiabetic agent with antioxidant properties, has previously been shown to increase the resistance of LDL to oxidation in vitro and in vivo in healthy volunteers. In a randomized, placebo-controlled, parallel-group study in 29 patients with NIDDM, we tested the effect of troglitazone (200 mg once daily) on the resistance of LDL to oxidation and on circulating levels of preformed lipid hydroperoxides and the adhesion molecule E-selectin. Resistance of LDL to oxidation was assessed by measuring *1*) fluorescence development induced by copper treatment (lag phase), and *2*) amount of thiobarbituric acid–reactive substances (TBARS) generated by incubation with umbilical vein endothelial cells. At 8 weeks, the lag phase was increased by 23% ($P < 0.01$ by analysis of covariance [ANCOVA]) in the patients receiving troglitazone ($n = 18$) compared with the group receiving placebo ($n = 11$). At the same time, TBARS were 3.63 ± 0.10 nmol/l (vs. 5.32 ± 0.10 nmol/l in the placebo group, $P = 0.009$), LDL hydroperoxide concentration was reduced from 1.48 ± 0.03 to 1.19 ± 0.03 ng/mg (no change in the placebo group, $P < 0.01$), and plasma E-selectin levels decreased from 56.5 ± 2.33 to 43.7 ± 1.77 μg/l (no change in the placebo group, $P < 0.01$). In NIDDM, troglitazone may slow down the development of atherosclerosis by modifying LDL-related atherogenic events. *Diabetes* 47:130–133, 1998

Troglitazone is an oral agent currently under clinical development for the treatment of NIDDM. In pre-clinical studies, troglitazone has been shown to improve insulin sensitivity, to increase the conversion of glucose to glycogen, and to reduce hepatic gluconeogenic enzyme activity (1). In clinical studies, within the therapeutic range of 200–600 mg once daily, troglitazone improves glycemic control and insulin sensitivity (2). Furthermore, troglitazone appears to have beneficial effects on the dyslipidemia of NIDDM, i.e., the combination of high triglyceride with low HDL cholesterol levels (2).

Early studies showed that troglitazone, which has structural similarities to α-tocopherol, reduces lipid peroxidation (3). Recently, we have shown that troglitazone increases the resistance of LDL to oxidation both in vitro (4) and in vivo in healthy volunteers (5). Oxidative modification confers potentially atherogenic properties to LDL particles (6). Furthermore, enhanced susceptibility of LDL to oxidation has recently been reported in diabetic patients (7). Through nonenzymatic protein glycation, hyperglycemia increases oxidative stress via the generation of oxygen free radicals (8). Increased plasma hydroperoxides, another product of oxidative stress, have also been observed in NIDDM patients (9,10).

The expression of some adhesion molecules on endothelial cells is modulated by oxidized LDL (11,12). Increased levels of serum adhesion molecules have been detected in patients with, or at risk of developing, atherosclerosis (13), as well as in those with IDDM or NIDDM (14–16).

Following up on our previous observations, in the present placebo-controlled study, we assessed the effects of troglitazone on the oxidative susceptibility of LDL in NIDDM patients (4,5). We used an in vitro model to measure the susceptibility of LDL to copper-induced and endothelial cell–mediated oxidation, specifically analyzing the influence of variables such as α-tocopherol content in LDL and hydroperoxide concentration. In addition, we determined the effect of troglitazone on plasma E-selectin concentrations.

RESEARCH DESIGN AND METHODS

Subjects. Twenty-nine NIDDM patients, 18 men and 11 women (age, 45–55 years; BMI, 25–35 kg/m^2; fasting plasma glucose [FPG], 7–15 mmol/l; HbA_{1c}, 3.8–9.1%), participated in the study. All patients were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal, hematological, and psychiatric disease as determined by history and clinical workup. Subjects who had received vitamin supplementation during the previous 3 months and probucol during the previous 12 months were excluded. In hypertensive patients taking antihypertensive agents, drug type and dosage were kept constant throughout the study. With regard to antidiabetic therapy, patients either were untreated or had been treated with diet and/or oral sulfonylureas or biguanides. Antidiabetic oral agents were withdrawn at least 3 weeks before patients entered into the study. During the entire study period, subjects were placed on a weight-maintenance diet (the habitual composition of the Italian diet is 15% protein, 50% carbohydrate, and 35%

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ANCOVA, analysis of covariance; ANOVA, analysis of variance; FPG, fasting plasma glucose; 15-HETE, 15-hydroxyeicosatetraenoic acid; HUVEC, human umbilical vein endothelial cell; TBARS, thiobarbituric acid–reactive substances.

fat). We also recruited 61 age-sex matched healthy subjects (36 men and 25 women) from the hospital staff to serve as the control group. Their mean age was 48.3 ± 3 years, and their mean BMI was 25.2 ± 0.6 kg/m^2. None of the controls were taking vitamin supplements. The study protocol was approved by the Institutional Review Board of the Region of Tuscany as well as the Ethics Committee of the CNR Institute of Clinical Physiology. All subjects gave informed, written consent to participate in the study.

Design. This was a double-blind, randomized, placebo-controlled parallel-group study in which 18 patients (age, 55 ± 1 year [mean ± SE]; BMI, 28.2 ± 0.5 kg/m^2; diabetes duration, 9 ± 1 year; FPG, 12.6 ± 0.7 mmol/l; HbA_{1c}, 7.4 ± 0.2%) received troglitazone (200 mg once daily, orally) and 11 patients (age, 55 ± 1 year; BMI, 28.7 ± 0.8 kg/m^2; diabetes duration, 10 ± 2 years; FPG, 11.8 ± 1.0 mmol/l; HbA_{1c}, 7.0 ± 0.5%) received placebo. In each patient, the following variables were measured on day 0 (before treatment) and on day 56 of treatment: total plasma cholesterol and triglycerides; HDL, LDL, and VLDL cholesterol; susceptibility of LDL to in vitro copper-induced and cell-mediated oxidative modification; α-tocopherol content in LDL; LDL hydroperoxides (as 15-hydroxyeicosatetraenoic acid [15-HETE]); and plasma E-selectin concentration.

Methods. For lipoprotein separation, whole blood was collected into Vacutainer tubes (Becton Dickinson, Meylan, France) containing EDTA (1 mg/ml) and immediately centrifuged at 2,000 rpm for 20 min at 4°C. Plasma was stored at 4°C and processed for lipoprotein separation within 1 day. Lipoproteins were isolated by sequential ultracentrifugation in NaBr solutions (17) containing EDTA (1 mg/ml) and stored at 4°C. To minimize LDL oxidation during isolation, all solutions used in this process were deoxygenated by argon bubbling. LDL was stored under nitrogen at 4°C in a sterile, dark environment and used within 1 day.

Susceptibility of LDL to oxidation by Cu^{2+} was evaluated by measuring the length of the lag phase based on the development of fluorescence during copper-catalyzed LDL oxidative modification as described (18).

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins as described (12) and used at passages 2–4. LDL oxidation was prepared by adding 1.5 ml of serum-free F-12 medium containing 200 μg/ml protein to each well of HUVECs and incubating for 24 h at 37°C, as previously described (12). The extent of LDL oxidation was determined by measuring the concentration of thiobarbituric acid–reactive substances (TBARS) as described (9). Aliquots of the incubation mixture containing 200 μg of LDL were removed and added to tubes containing 0.05 ml of 2% butylated hydroxytoluene, 2 ml of 0.67% thiobarbituric acid, and 10% trichloroacetic acid (2:1). The tubes were heated at 100°C for 10 min and then cooled and centrifuged at 2,500 rpm for 10 min. The absorbance of the supernatant fraction was read at 532 nm, and the quantitation was achieved by comparison with a standard curve of malonyldialdehyde equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane, as previously described (9).

High-performance liquid chromatography was used to measure α-tocopherol, as previously described (4). LDL hydroperoxides were measured as 15-HETE by a radioimmunoassay method using the 15-HETE [^{3}H] RIA Kit (PerSeptive Diagnostics, Cambridge, MA). Before measurement, 15-HETE was extracted as previously described (12). The sensitivity of the method was ~2.5 pg/0.1 ml. The intra-assay variation was <4%. Plasma E-selectin was determined using the Soluble E-Selectin kit (British Bio-technology Products, Abingdon, U.K.), as previously described (16). The assay, which is standardized against purified soluble forms of recombinant E-selectin, has an intra-assay coefficient of variation of <4%.

Cholesterol and triglyceride levels in plasma and lipoprotein fractions were determined by Technicon Autoanalyzer II (Technicon, Tarrytown, NY) methodology. Protein was measured by the Pierce BCA protein assay reagent (Rockford, IL). Plasma glucose was measured by the glucose oxidase method on the Beckman Glucose Analyzer (Beckman Instruments, Palo Alto, CA) (19). HbA_{1c} in plasma was measured by a minicolumn chromatographic procedure (20).

Statistical analysis. Between-group comparison after 8 weeks of treatment was performed by means of analysis of covariance (ANCOVA), with pretreatment values as covariates. Parameters were log-transformed before analysis in order to improve the assumptions of homogeneity of variance and normality of distribution. For each parameter, an estimate of the ratio of the adjusted geometric means for troglitazone relative to placebo was obtained, together with a 95% CI, and was tested for significance. Statistical significance of differences among group means was tested with analysis of variance (ANOVA). Values were expressed as means ± SE.

RESULTS

Troglitazone was very well tolerated by all patients, and none had any significant subjective or biochemical side effects. No patient dropped out of the study. Body weight decreased by 1.0 ± 0.5 kg in the treatment group and by 2.0 ± 0.7 kg in the placebo group (NS). Data on the effects of troglitazone in patients with NIDDM are shown in Table 1. Over the 8 weeks of treatment, mean FPG rose slightly in the placebo group whereas it decreased by ~10% in the troglitazone group, a difference of borderline statistical significance. A similar trend was evident for serum HbA_{1c} levels, which, however, did not reach statistical significance. No significant change in the serum levels of LDL cholesterol or triglycerides was observed in either group. In contrast, resistance of LDL to oxidation by copper (expressed as the lag phase of the corresponding in vitro reaction) was significantly improved, and the generation of TBARS on challenging LDL particles with HUVECs was significantly reduced in the patients receiving troglitazone as compared with those receiving placebo. The TBARS value at day 0 was inversely related to the length of the lag phase ($r = 0.58$, $P < 0.01$), and the treatment-induced changes in the two variables were closely related to one another in the troglitazone group ($r = 0.61$, $P = 0.007$).

While α-tocopherol content in LDL was unchanged after either placebo or troglitazone, both LDL hydroperoxide levels and E-selectin concentrations in plasma were significantly reduced by troglitazone treatment compared with placebo. LDL hydroperoxide levels at day 0 were reciprocally related to the lag phase ($r = 0.91$, $P < 0.001$), and the troglitazone-induced changes in these two variables were directly correlated ($r = 0.58$, $P < 0.01$). Finally, troglitazone-induced changes in plasma E-selectin were positively related with the corresponding changes in both length of the lag phase ($r = 0.44$, $P < 0.05$) and TBARS values ($r = 0.47$, $P < 0.03$).

Data on the LDL lag phase and on plasma concentrations of LDL hydroperoxide and E-selectin for control subjects and NIDDM patients before and after troglitazone treatment are shown in Table 2. Before treatment, the lag phase was significantly shorter, and plasma concentrations of LDL hydroperoxide and E-selectin were higher, in the NIDDM patients than in the controls. Troglitazone treatment increased the LDL lag phase and reduced the LDL hydroperoxide and E-selectin in plasma to normal concentrations.

DISCUSSION

In the present trial, 8 weeks of administration of troglitazone (200 mg once daily) to NIDDM patients increased resistance of LDL to oxidation in vitro by 23%. This finding is consistent with our previous results in healthy volunteers, in whom significant increments in the resistance of LDL to oxidation were seen at doses of 400 mg twice daily after 1 and 2 weeks of treatment (27 and 24%, respectively) (5). In vitro incorporation of troglitazone into the LDL particle resulted in a comparable lengthening of the lag phase (4). The in vitro technique for measuring susceptibility of LDL to oxidation that we have used has been related to predisposition to atherosclerosis in various models (21–23). The antioxidant probucol, which has been shown to counteract the progression of atherosclerotic lesions in rabbits (24), has also reduced the susceptibility of LDL to in vitro oxidation (21). Supplementation with α-tocopherol has also reduced oxidative modification in a similar model (25). Two recent large-scale studies have provided evidence that supplementation with α-tocopherol significantly decreases the risk of coronary artery disease (26,27).

The pattern of LDL oxidation seen in the current study is consistent with the classic lipid peroxidation model (28). Under circumstances in which other variables are largely unaffected, the amount of LDL antioxidants is critical in

TABLE 1
Effects of troglitazone in patients with NIDDM

	Day 0		Day 56		
	Placebo	TGZ*	Placebo	TGZ*	*P*
FPG (mmol/l)	11.8 ± 1.0	12.6 ± 0.7	12.8 ± 1.5	11.5 ± 0.8	0.098
HbA_{1c} (%)	7.0 ± 0.5	7.4 ± 0.2	7.2 ± 0.7	7.1 ± 0.4	NS
Serum triglycerides (mmol/l)	1.81 ± 0.30	2.85 ± 0.85	1.83 ± 0.34	3.55 ± 1.57	NS
Serum total cholesterol (mmol/l)	5.32 ± 0.33	5.88 ± 0.28	5.41 ± 0.26	6.28 ± 0.44	NS
Serum LDL cholesterol (mmol/l)	3.26 ± 0.20	3.31 ± 0.18	3.37 ± 0.17	3.24 ± 0.18	NS
Serum HDL cholesterol (mmol/l)	1.14 ± 0.04	1.25 ± 0.07	1.13 ± 0.06	1.23 ± 0.08	NS
LDL α-tocopherol (μg/mg)	6.46 ± 0.23	6.09 ± 0.18	6.46 ± 0.14	6.31 ± 0.14	NS
Lag phase (min)	73.3 ± 1.65	71.0 ± 1.26	73.4 ± 1.48	87.3 ± 2.10	<0.01
TBARS (nmol/l)	5.51 ± 0.13	5.66 ± 0.11	5.32 ± 0.10	3.63 ± 0.10	0.009
LDL hydroperoxide (ng/mg)	1.44 ± 0.04	1.48 ± 0.03	1.46 ± 0.04	1.19 ± 0.03	<0.01
Plasma E-selectin (μg/l)	53.2 ± 2.37	56.5 ± 2.33	55.9 ± 2.38	43.7 ± 1.77	<0.01

Data are means ± SE; *P* values are for the difference between troglitazone and placebo at day 56 after adjustment for day 0 values by ANCOVA. *200 mg/day. TGZ, troglitazone.

determining the potential of scavenging radicals and, therefore, the initiation rate of the lipid peroxidation cascade. It is significant that α-tocopherol concentrations in LDL did not differ between the patients receiving troglitazone and those receiving placebo. In copper-induced LDL oxidation, the initiation rate depends on the amount of preformed lipid hydroperoxides (29). Therefore, troglitazone is likely to increase the resistance of LDL to oxidation by reducing preformed lipid hydroperoxides. In both this study and the healthy volunteer study, length of the LDL lag phase and lipid hydroperoxide concentration were correlated in all of the subjects investigated.

The troglitazone-related decrease in oxidative modification of LDL by HUVECs in NIDDM patients is an important finding. The process of LDL modification induced by the endothelial cell in vitro closely resembles the corresponding process in vivo. It may therefore play a crucial role in the sequence of events leading to the formation of foam cells and atherosclerotic plaques. We have previously suggested that although the mechanism by which cells initiate LDL oxidation remains to be established, it is possible that troglitazone, as a scavenger of free radicals involved in perpetuating the process, delays initiation of the lipid peroxidation cascade (4).

The presence of circulating E-selectin demonstrates endothelial cell activation (13). Furthermore, E-selectin levels are elevated in patients with IDDM and NIDDM (14–16). It seems that rather than being directly related to glycemic control, oxidized LDL particles modulate the expression of adhesion molecules on endothelial cells (11,12). This modulation is thought to provide a signal for monocytes to accumulate at the subendothelium, and to trigger a succession of events providing the earliest morphological evidence of atherosclerotic disease (30). Troglitazone's ability to significantly decrease plasma E-selectin levels and its correlation with oxidative modification of LDL suggest that this compound has the potential to delay certain atherogenic activities.

In this series of NIDDM patients, the effect of troglitazone on FPG levels was small and did not reach full statistical significance. In larger randomized, placebo-controlled studies, the same dosage has shown statistically significant reductions in FPG as early as week 2 and in HbA_{1c} at week 8 (2). However, it has been recognized that a full antihyperglycemic effect requires doses of 400–800 mg/day, and that patients with milder degrees of fasting hyperglycemia respond better than more hyperglycemic NIDDM patients (31). It is nevertheless remarkable that a dose of troglitazone that had a small effect on plasma glucose and lipid levels was already fully efficacious in enhancing LDL resistance to oxidation and reducing plasma concentrations of LDL hydroperoxide and E-selectin. The fact that these parameters reverted to normal levels after troglitazone treatment further supports this conclusion. This novel antidiabetic agent thus represents a promising adjuvant in the management of the overall burden of NIDDM, of which atherosclerotic cardiovascular disease is the major component.

TABLE 2
LDL lag phase and LDL hydroperoxide and E-selectin plasma concentrations for control group and NIDDM patients before and after troglitazone treatment

	Control group	NIDDM before TGZ	NIDDM after TGZ
n	61	29	18
LDL lag phase (min)	92.2 ± 1.7	71.9 ± 1.42*	87.3 ± 2.10
LDL hydroperoxide (ng/mg)	1.10 ± 0.04	1.46 ± 0.03*	1.19 ± 0.03
Plasma E-selectin (μg/l)	39.9 ± 3.4	55.2 ± 2.21*	43.7 ± 1.77

Data are means ± SE. *Value differs from the control group ($P < 0.01$). TGZ, troglitazone.

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