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Effects of troglitazone on in vitro oxidation of LDL and HDL induced by copper ions and endothelial cells

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Summary Troglitazone is a new oral antidiabetic agent able to reduce lipid peroxidation. In this study we evaluated its effect on the susceptibility of LDL and HDL to in vitro oxidation induced by copper ions and endothelial cells. In Cu^{++}-induced LDL modification, different amounts of troglitazone were added to aliquots of the same pool of plasma with subsequent ultracentrifuge separation of LDL and HDL. Differences in LDL and HDL susceptibility to in vitro oxidation with Cu^{++} were studied by measuring the changes in fluorescence intensity (expressed as lag phase). LDL derived from plasma incubated with different amounts of troglitazone were also incubated with umbilical vein endothelial cells (HUVEC), the modification being monitored by LDL relative electrophoretic mobility and fluorescence. During Cu^{++}- and HUVEC-induced LDL oxidation, the decay rate of vitamin E, and the potency of troglitazone as a radical scavenger in comparison with vitamin E were also studied. Troglitazone determined a significant, dose-dependent decrease in Cu^{++}-induced LDL and HDL oxidation. Incubation with HUVEC was also followed by a progressive, significant decrease of LDL relative electrophoretic mobility and fluorescence intensity. During Cu^{++}- and HUVEC-induced-LDL modification, troglitazone significantly reduced the rate of vitamin E decay. In this study we also demonstrated that under the same oxidative stress, troglitazone was much more potent as a radical scavenger than vitamin E. In conclusion, the results demonstrate that troglitazone can reduce LDL and HDL in vitro oxidation and that, during this process, it can protect vitamin E. In addition to ensuring blood glucose control, the drug may therefore be useful in inhibiting lipoprotein peroxidation. [Diabetologia (1997) 40: 165–172]

Keywords Cu^{++}, endothelial cells, LDL oxidation, oxidized LDL, troglitazone, vitamin E, alpha tocopherol, diabetes mellitus, atherosclerosis.

Low density lipoprotein (LDL) oxidation is believed to play an important role in the development of atherosclerotic plaques [1–5]. This view is supported by LDL with characteristics similar to those of oxidatively modified LDL being present in atheromatous lesions [6]. Studies in cultured cells have demonstrated that oxidized LDL is chemotactic for monocytes and stimulates monocyte endothelial cell interaction [7, 8]. It can also cause initial endothelial cell damage and increase uptake by scavenger receptors on macrophages, leading to cholesterol accumulation and foam cell formation [7, 9].

Oxidative modification of LDL is not likely to occur in circulation [3]. In view of the difficulties in identifying oxidized lipoprotein in plasma, many workers have attempted to analyse the oxidation susceptibility of isolated LDL particles in vitro [10, 11]. If LDL can penetrate macrophages only after its modification [3], the ability of LDL to resist oxidation could be an

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Abbreviations: BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; LDL, low density lipoprotein; REM, relative electrophoretic mobility.

important factor in atherogenesis. The progression of atherosclerotic lesions is counteracted by antioxidants such as probucol [12, 13] and the LDL isolated from antioxidant-treated animals shows a reduced susceptibility to in vitro oxidation [13].

Many studies report increased susceptibility of LDL to in vitro oxidation in patients with, or at risk of developing, atherosclerosis. This enhanced susceptibility has been observed in patients with familial hypercholesterolaemia [14, 15], coronary artery disease [16, 17], renal failure [18, 19], hypertension [20] and diabetes mellitus [21].

Troglitazone is a new oral antidiabetic agent reported to enhance insulin sensitivity [22], increase the conversion of glucose to glycogen in rat HepG2 cells [22], reduce hepatic gluconeogenic enzyme activity in rats and mice [23] and to improve metabolic control in non-insulin-dependent diabetic patients [24–26]. Troglitazone has also been demonstrated to reduce lipid peroxidation [27].

Atherosclerosis is the major complication of diabetes, accounting for more than 70 % of total mortality in all forms of the disease [28]. The extent and progression of atherosclerosis in non-human primates are related to resistance of LDL to oxidation [13]. The present study analyses the effect of troglitazone on the susceptibility of LDL to oxidation induced by Cu^{++} and endothelial cells in vitro. Since oxidative modification of high density lipoprotein (HDL) has been shown to cancel its ability to stimulate efflux of cholesterol from foam cells [29], this study also investigates whether troglitazone can affect HDL oxidation in vitro.

Materials and methods

Addition of troglitazone to plasma. Blood samples were collected from healthy volunteers into vacutainer tubes containing EDTA (1 mg/ml), and immediately centrifuged at 2000 rev/min for 20 min at 4 °C. Plasma was pooled and different amounts of troglitazone (from 0 to 25.0 μg/ml plasma) were added to aliquots of the same plasma pool for 12 h at room temperature. The aliquots were then stored at 4 °C and processed for separation of LDL and HDL within 3 days.

Subjects received a full verbal and written explanation of the nature and purposes of the study and each gave written informed consent prior to participation in the study. The study was conducted in accordance with the Declaration of Helsinki.

Lipoprotein separation. LDL and HDL were isolated by sequential ultracentrifugation in NaBr solutions [30] containing 1 mg/ml EDTA and stored at 4 °C. In order to minimize LDL and HDL oxidation during isolation, all solutions used in this process were deoxygenated by bubbling with argon. LDL and HDL were stored under nitrogen at 4 °C in a sterile, dark environment and used within 3 days. Immediately before oxidation tests LDL and HDL were separated from EDTA and from diffusible low molecular mass compounds, by gel filtration on PD-10 Sephadex G-25 M gel (Pharmacia, Uppsala, Sweden) in 0.01 mol/l phosphate-buffered saline (136.9 mmol/l NaCl, 2.68 mmol/l KCl, 4 mmol/l Na_2HPO4, 1.76 mmol/l KH_2PO4) pH 7.4.

LDL and HDL oxidation by Cu^{++}. The method for LDL and HDL oxidation with Cu^{++} and for the evaluation of its susceptibility to oxidation, i. e. evaluation of the length of the lag phase, was based on the development of fluorescence during copper-catalysed LDL oxidative modification. The method, described elsewhere [11], was slightly modified for automation. Briefly, after gel-filtration to remove EDTA, the fractions eluting at the void volume were pooled, adjusted with the same buffer (10 mmol/l phosphate-buffered saline, pH 7.4) to a protein concentration of 0.2 mg/ml and used immediately in the presence of 1 μmol/l Cu^{++}. The HPLC for the automatic determination of the LDL and HDL oxidation curve was a Gold system (Beckman Instruments, inc., Palo Alto, Calif., USA), including one pump, an autosampler (Gilson, model 232 Bio; Biolabo Instruments, Milano, Italy) with a stirrer and temperature control, a PS2/50 IBM computer with the Gold acquisition and processing data software, and a fluorescence detector (Spectrophotofluorimeter Shimadzu RF-5000, Kyoto Japan). A mixing joint and a retarding stainless-steel coil, instead of the column, were connected between the sample injection valve and the detector. The size of the retarding coil was selected to ensure a delay in reaction time, as measured from the peak retention, of about 6 s at a flow rate of 1 ml/min. Fluorescence was checked every 20 min, from time 0 to 260 min.

Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins according to the method of Jaffe [31], and used at passage 2–4 as previously described [32]. The cells were grown in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) filled with 10 ml of Ham's F-12 medium (Sigma, St. Louis, Mo., USA) containing 10 % fetal calf serum (FCS) (Seromed, Berlin, Germany), 2 mmol/l glutamine (Seromed), 30 μg/ml endothelial cell growth supplement (Sigma), 100 μg/ml heparin (Sigma), 100 U/ml penicillin-streptomycin (Sigma), 100 μg/ml streptomycin (Sigma) and 2.5 μg/ml amphotericin (Sigma). The flasks were incubated at 37 °C, 100 % humidity and 5 % CO_2. The medium was refreshed every 2 days. At the beginning of each experiment the cells were detached by 0.01 % trypsin/EDTA (Sigma). The trypsin was inactivated by dilution, and the cells were washed and counted. Cells were plated at a concentration of 20 000 cells/cm^2 on a multiwell plate (9.6 cm^2/well) (Falcon), grown for 3 days and then used for the incubations. At that time the cells were non-confluent ($0.26–0.40 \times 10^5$ cells/cm^2).

HUVECs were harvested and characterized in terms of acetylated LDL binding and factor VIII expression, according to previously described techniques [33].

LDL oxidation by HUVEC. Endothelial cell-modified LDL was prepared by adding 1.5 ml of serum-free F-12 medium containing 200 μg/ml LDL protein to each 35 mm well of endothelial cells, and incubating for 3, 5, 8, 11, 15 and 22 h at 37 °C in triplicate. LDL (200 μg protein/ml) was also oxidized in the absence of cells as a control. Oxidation was arrested by the addition of 200 μmol/l EDTA and refrigeration. LDLs corresponding to the different times of incubation were re-isolated by ultracentrifugation at density = 1.15. The degree of LDL oxidation was evaluated by monitoring LDL fluorescence at 430 nm, with excitation at 360 nm (Spectrophotofluorimeter Shimadzu RF-5000), as previously described [11].

Cytotoxicity evaluation. To assess cell survival, hexosaminidase, a stable cytosolic enzyme released by cells when they

undergo lysis, was measured according to the method of Landegren [34]. Briefly, the substrate for the enzyme hexosaminidase, p-nitrophenol-N-acetyl-b-D-glucoseaminide (Sigma), was dissolved at 7.5 mmol/l in 0.1 mol/l citrate buffer, pH 5. The solution was then mixed with an equal volume of 0.5 % Triton X-100 (Sigma) in water and added to cells in volumes of 60 μl, for 60 min at 37 °C. The reaction was blocked by addition of 50 mmol/l glycine (Sigma) buffer, pH 10.4. Absorbance was measured in a Bio-Rad microplate reader (Model 450; Bio-Rad Laboratories S.r.L., Milan, Italy) at 405 nm. Results, expressed as percentage toxicity, were calculated as (E-S) × 100/M-S, where E represents the average absorbance readings from the experimental wells, S the average spontaneous release and M the maximum release after cell lysis.

LDL vitamin E time course and decay rate during LDL oxidation. The time course of LDL vitamin E and its initial decay rate was measured during the Cu^{++}- and HUVEC-catalysed LDL oxidation. LDL alpha-tocopherol was determined by a previously described HPLC method [35]. The initial rate of alpha-tocopherol decay was determined by fitting the points with a single exponential model, using a non-linear regression program based on the Marquard algorithm (Enzfitter, Biosoft, London, UK).

To assess the potency of troglitazone as a radical scavenger in comparison to vitamin E under the same oxidative stress, the data on vitamin E initial decay rates were also analysed in a previously described competitive kinetic model [36].

Other methods. Protein was measured by the Pierce BCA protein assay reagent [37].

The extent of LDL lipid peroxidation and LDL apoprotein B modification induced by Cu^{++} and HUVEC was also monitored by evaluating the relative electrophoretic mobility (REM) of LDL. LDL REM, evaluated on agarose gel at the end of Cu^{++} and HUVEC incubation, was expressed in terms of REM as compared to LDL stored at 4 °C for not more than 1 week.

Troglitazone concentration in LDL was measured by an HPLC system (Gold system; Beckman Instruments) with ultra-violet detection.

Statistical analysis

Differences in lag phase, REM and vitamin E levels and decay rate were analysed by one-way analysis of variance, followed by a multiple comparison test.

To compare mean alpha-tocopherol and fluorescence values obtained from LDL containing different concentrations of troglitazone after incubations with Cu^{++} and HUVEC at different times, a three-way analysis of covariance with repeated measures was performed. The "V" BMPD program and statistical software manual was used (University of California Press, Berkeley, Calif., USA). When significant results were obtained with the analysis of covariance, multiple comparison between the three different concentrations was performed by the Tukey test [38]. For each variable, the Dunnett test [39] was used to compare the mean value at different times of incubation with the mean at time 0.

Results

If otherwise not specifically indicated the results are expressed as mean ± SD.

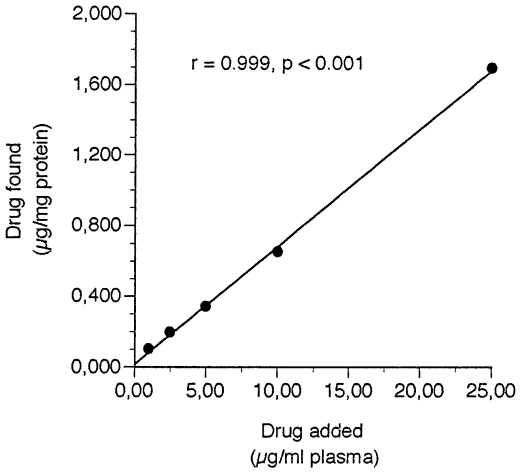

Fig. 1. Correlation between the concentration of troglitazone added to plasma and that found in LDL. Different amounts of troglitazone (from 0 to 25.0 μg/ml plasma) were added to aliquots of the same plasma pool for 12 h at room temperature. Samples were then processed by ultracentrifugation to separate LDL and HDL as indicated in Materials and methods. Troglitazone in LDL was assayed as indicated in Materials and methods. Data are mean of six different assays

Under our experimental conditions, the exposure of plasma to different amounts of troglitazone resulted in increasing LDL drug concentrations. Figure 1 shows the correlation between the concentration of troglitazone added to plasma and that found in LDL. The increase was a linear function of the amount added, up to 25 μg/ml plasma. The yield of incorporation was 6.1 ± 1.5 %.

After incubation of plasma with increasing amounts of troglitazone, LDL and HDL were separated by ultracentrifugation. Susceptibility to oxidation (lag phase) was measured after incubation with Cu^{++}. A typical oxidation curve of LDL with, and without troglitazone (at a concentration of 10 μg/ml plasma), is shown in Figure 2. Results on LDL lag phases at the different troglitazone concentrations and their absolute variations are indicated in Table 1. When compared to control conditions, a significant increase in the LDL lag phase (+ 13.2 ± 2.1 min) was observed at a drug concentration of 0.50 μg/ml plasma ($p < 0.01$). Starting from this concentration, the exposure of plasma to increasing amounts of troglitazone determined a dose-dependent, significant increase in LDL lag phase ($p < 0.01$).

The results of this study also show that the susceptibility of HDL to oxidation was lower, and the length of the lag phase accordingly longer in the presence of troglitazone. Figure 3 shows the HDL lag phase at different concentrations of troglitazone. At 10 and 25 μg/ml plasma of troglitazone, the HDL lag phase increased from 75 ± 2.1 to 108 ± 1.9 min and 137 ± 2.4 min, respectively ($p < 0.01$).

After incubation with HUVEC, the REM of control LDL was 1.79 ± 0.14 cm; the presence of

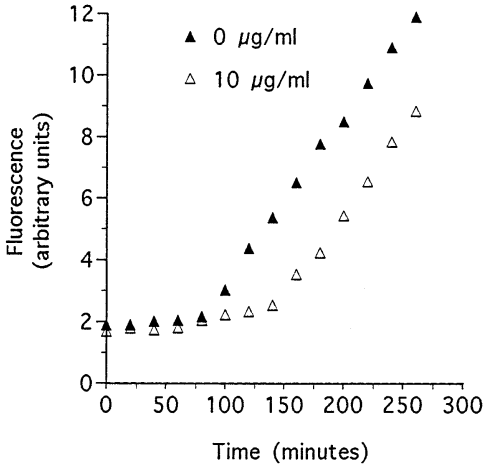

Fig. 2. Typical oxidation curve of LDL induced by Cu^{++}, without or with troglitazone (10 μg/ml plasma). Troglitazone (10 mg/ml plasma) was added to aliquots of the same plasma for 12 h at room temperature. Samples were then processed by ultracentrifugation to separate LDL as indicated in Materials and methods. After gel-filtration for removing EDTA, the fractions eluting at the void volume were pooled, adjusted to a protein concentration of 0.2 mg/ml and oxidized immediately with 1 μmol/l Cu^{++}

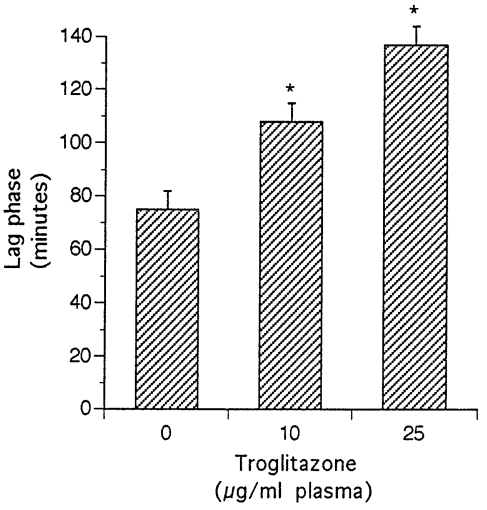

Fig. 3. Data (± SD) on HDL lag phase at different concentrations of troglitazone. Troglitazone (10–25 μg/ml plasma) was added to aliquots of the same plasma for 12 h at room temperature. Samples were then processed by ultracentrifugation to separate HDL as indicated in Materials and methods. After gel-filtration for removing EDTA, the fractions eluting at the void volume were pooled, adjusted to a protein concentration of 0.2 mg/ml and oxidized immediately with 1 μmol/l Cu^{++}. Data represent the mean of six different HDL oxidations. *$p < 0.01$ compared to control

Table 1. Effect of different concentrations of troglitazone added to plasma before LDL separation on LDL lag phase expressed in min and on its absolute variation in min (Δ min)

Drug added (μg/ml)	Drug found (μg/mg protein)	Drug found (mol/mol)	Lag-phase (min)	Δ (min)
0	–	–	94.0 ± 1.1	
0.05	ND	–	95.6 ± 1.2	1.6 ± 1.2
0.10	ND	–	95.6 ± 1.1	1.6 ± 1.1
0.50	ND	–	107.2 ± 1.4	13.2 ± 2.1
1.00	0.101	0.100	115.3 ± 0.9[a]	21.3 ± 1.8
2.50	0.193	0.232	121.7 ± 1.6[a]	27.7 ± 1.7
5.00	0.339	0.411	129.1 ± 0.9[a]	35.1 ± 1.9
10.0	0.651	0.789	135.4 ± 1.2[a]	41.4 ± 1.8
25.0	2.691	2.002	143.8 ± 1.5[a]	49.8 ± 1.8

Values of drug are mean of 6 different assays
Values of lag phase are mean ± SD of 6 different LDL oxidations
[a] Means of lag phases that were significantly higher ($p < 0.01$) by analysis of variance and multiple comparison test

troglitazone reduced the LDL REM to 1.25 ± 0.10 cm ($p < 0.01$), to 1.04 ± 0.09 cm ($p < 0.01$) and to 1.00 ± 0.06 cm ($p < 0.01$) at 2.5, 5 and 10 μg/ml plasma, respectively.

The degree of HUVEC-induced LDL oxidation was also evaluated by monitoring LDL fluorescence at 430 nm with excitation at 360 nm at the indicated times. This was done after re-isolation of LDL from the medium by ultracentrifugation. Data on LDL fluorescence are indicated in Figure 4. The analysis of covariance showed significant differences in LDL fluorescence values between the four different troglitazone concentrations as well as between times for all four. At all concentrations, p values ranged from < 0.005 to < 0.001.

The extent of endothelial cell cytotoxicity (expressed as percent cytotoxicity), induced by incubation with LDL (± troglitazone) was always less than 3 %.

LDL vitamin E values after incubation of LDL with Cu^{++} and HUVEC provided a good fit with a single exponential decay model (r ranging from 0.90 to 0.98, $p < 0.001$). The exponential decay of LDL vitamin E after incubation with HUVEC is shown in Figure 5.

From the monoexponential decay equations, the LDL vitamin E initial decay rate was calculated. Data on LDL vitamin E initial decay rates are indicated in Table 2. The LDL containing increasing amounts of troglitazone showed significantly lower decay rates than control LDL ($p < 0.001$).

Starting from the assumptions that vitamin E decay is due to its reaction with free radicals and that under the same oxidative stress troglitazone competes with vitamin E in the same reaction, the effect of troglitazone on vitamin E decay is assumed to be proportional to its reactivity on free radicals. Consequently the extent of vitamin E protection against the decomposition induced by free radicals can be considered a measure of the potency of a given radical scavenger. On the basis of these assumptions the potency of troglitazone as a radical scavenger was assessed in comparison to vitamin E in the following competitive kinetic model:

$V_0/V = 1 + (K_{tr}/K_e) \cdot ([Tr_0]/[E_0])$ (1) where:

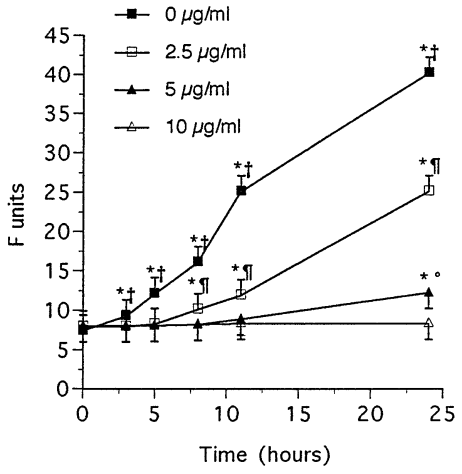

Fig. 4. Data (± SD) on the time course of LDL fluorescence formation during incubation with umbilical vein endothelial cells (HUVEC) of LDL containing different concentrations of troglitazone. HUVEC-modified LDL was prepared by adding 1.5 ml of serum-free F-12 medium, containing 200 μg/ml LDL protein, to each 35 mm well of endothelial cells, and incubating for 3, 5, 8, 11, 15 and 22 h at 37 °C. Oxidation was arrested by the addition of 200 μmol/l EDTA and refrigeration. LDLs corresponding to the different times of incubation were re-isolated by ultracentrifugation at density = 1.15. The degree of LDL oxidation was evaluated by monitoring LDL fluorescence, as described in Materials and methods. Data represent the mean of six different LDL oxidations. *different from time 0, $p < 0.001$; † higher than at a plasma troglitazone concentration of 2.5, 5 and 10 μg/ml, $p < 0.005$; ¶ higher than at a plasma troglitazone concentration of 5 and 10 μg/ml, $p < 0.001$; ° higher than at a plasma troglitazone concentration of 10 μg/ml, $p < 0.001$

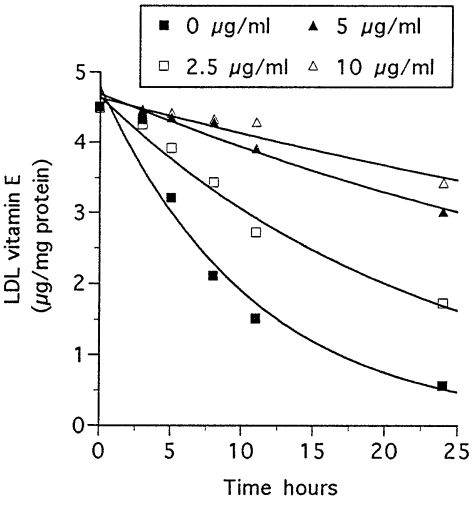

Fig. 5. Exponential decay of LDL vitamin E, obtained after incubation of LDL with HUVEC at different troglitazone concentrations. Different amounts of troglitazone (from 0 to 25.0 μg/ml LDL) were added to aliquots of the same plasma pool for 12 h at room temperature. LDL was then oxidized by HUVEC, as indicated in Materials and methods. At the indicated times, LDL vitamin E was measured as described in Materials and methods. The points (mean of 6 different LDL oxidations) were fitted with a single exponential model by a non-linear regression program, based on the Marquard algorithm (see Materials and methods)

V_0 = initial decay rate of LDL vitamin E without troglitazone;
V = initial decay rate of LDL vitamin E with troglitazone;
K_{tr} = rate constant for the reaction between radicals and troglitazone
K_e = rate constant for the reaction between radicals and vitamin E
$[Tr_0]$ = initial LDL troglitazone concentration;
$[E_0]$ = initial LDL vitamin E concentration.

In Figure 6, the scatter plot is shown for $[Tr_0]/[E_0]$ against V_0/V after incubation of LDL with Cu^{++} and HUVEC. These parameters proved to be associated (correlation coefficient = 0.99, $p < 0.001$ for both Cu^{++} and HUVEC). From the equation 1, the slope of the line, i. e. the ratio between the reactivity of troglitazone compared to vitamin E (K_{tr}/K_e ratio), was calculated. The K_{tr}/K_e ratio was (mean ± SEM) 5.94 ± 0.66 after incubation with Cu^{++}, and 57.97 ± 6.7 after incubation of LDL with HUVEC.

Discussion

There is growing evidence of a relationship between susceptibility of LDL to in vitro oxidation and atherosclerotic risk [13, 16, 17]. A novel strategy in the prevention of atherosclerosis could therefore be to prevent the deleterious effect of oxidized LDL by reducing the oxidation rate.

The results of this study show that troglitazone, a new oral antidiabetic agent with antioxidant capacity, decreased the susceptibility of LDL to oxidation by Cu^{++} and HUVEC in a dose-dependent manner. At least for the lowest concentrations, the troglitazone concentrations used in these experiments compare with the concentrations observed at therapeutic doses in human subjects. The reported median fasting plasma concentration of troglitazone at 400 mg two times per day in healthy volunteers was 0.42 μg/ml, while at 2 h post-dose it was 1.98 μg/ml [40].

The data for LDL oxidation induced by Cu^{++} are consistent with the classic kinetic model proposed by Niki for lipid peroxidation [41], where the length of the lag phase is directly related to the amount of antioxidant and inversely related to the initiation rate. The amount of LDL antioxidant, under circumstances in which other variables were probably largely unaffected, is therefore critical in increasing the potential of scavenging radicals and thus delaying the initiation of the lipid peroxidation chain. Our data are also consistent with studies in which an increase of

Table 2. Basal LDL troglitazone, LDL vitamin E and LDL vitamin E decay rates obtained after incubation of LDL containing different concentrations of troglitazone with Cu^{++} and HUVEC

	Troglitazone (μg/mg protein)	Vitamin E (μg/mg protein)	Decay rate (min^{-1})
Cu^{++}	0.000	4.95	1.13E-01 ± 0.291E-01
	0.651	4.61	6.97E-02 ± 0.290E-02[a]
	1.690	4.51	3.48E-02 ± 0.161E-02[a]
HUVEC	0.000	4.86	1.53E-03 ± 0.120E-03
	0.193	4.65	6.99E-04 ± 0.41E-04[a]
	0.396	4.65	2.68E-04 ± 02.6E-04[a]
	0.599	4.61	1.83E-04 ± 0.25E-04[a]

Values of troglitazone and vitamin E are mean of 6 different assays
Decay rate are mean ± SEM of 6 different LDL oxidations.
[a] $p < 0.001$ (vs troglitazone 0 μg/mg protein)

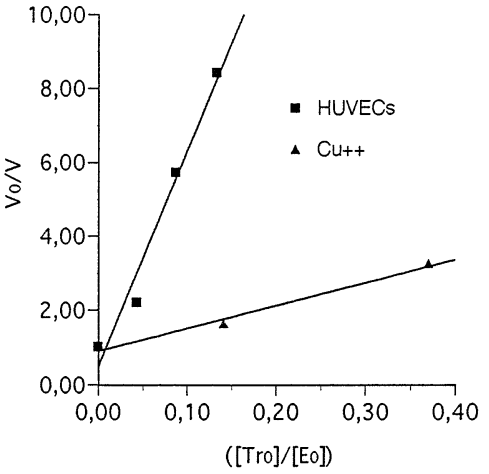

Fig. 6. Scatter plot for $[Tr_0]/[E_0]$ against V_0/V after incubation of LDL with Cu^{++} ($r = 0.99$, $p < 0.001$) and HUVEC ($r = 0.99$, $p < 0.001$). The data on vitamin E initial decay rates were analysed in the following competitive kinetic model: $V_0/V = 1 + (K_{tr}/K_e) \cdot ([Tr_0]/[E_0])$ where: V_0 = initial decay rate of LDL vitamin E at 0 μg/ml troglitazone; V = initial decay rate of LDL vitamin E with troglitazone; K_{tr} = rate constant for the reaction between radicals and troglitazone; K_e = rate constant for the reaction between radicals and vitamin E; $[Tr_0]$ = initial LDL troglitazone concentration; $[E_0]$ = initial LDL vitamin E concentration

LDL antioxidants, by incubation or oral supplementation, always resulted in a strict proportional increase in the length of the lag phase [42–44].

Troglitazone also determined a dose-dependent decrease in modification of LDL by HUVEC. Although cell-mediated oxidative modification of LDL has been the subject of several studies, the mechanism by which cells initiate LDL oxidation remains unclear. Both extracellular superoxide radical [45] and lipoxygenase activity [46, 47] have been proposed. Whichever mechanism of initiation may be involved, this first led to the hypothesis that the action of the modifying cells is to accelerate formation of lipid peroxides within the LDL particle. Recently, it has also been proposed that the modifying cells could directly provide peroxidative products to the LDL [32, 48]. Troglitazone could increase the potential of scavenging lipid peroxyl radicals, but also modify the potential of the modifying cells to produce peroxidative products and consequently delay the start of the lipid peroxidation chain.

In this study we also demonstrated that the susceptibility to in vitro oxidation of HDL derived from plasma supplemented with troglitazone was significantly lower than that of control samples. Recently, Nagano et al. [29] reported that oxidative modification occurred in HDL, as in LDL. This modification resulted in denaturation of apolipoprotein AI, with an increased negative charge [29]. Interestingly, this modified HDL lost its ability to stimulate efflux of cholesterol from foam cells, thus suggesting that oxidative modification of HDL may stimulate development of atherosclerosis by limiting this efflux [29]. Further studies on patients at risk for atherosclerosis are needed to establish whether susceptibility of HDL to in vitro modification is an important risk factor in atherogenesis.

The results of this study also show that LDL vitamin E levels obtained at the times of incubation with Cu^{++} and HUVEC fitted a single exponential decay model.

At least for Cu^{++}-induced LDL oxidation, this is in agreement with previously reported data [42]. Troglitazone added exogenously to LDL determined dose-dependent reduction of the vitamin E initial decay rate, and thus of LDL vitamin E consumption. Even if the mechanism underlying this protective effect of troglitazone on vitamin E is unknown, one hypothesis proposed by Thomas et al. [49] is that troglitazone functions as a coantioxidant. In the model proposed by these authors, vitamin E does not act as a chain-breaking antioxidant, but facilitates the transfer of radical reactions from the aqueous phase into LDL and mediates radical chain reactions within the lipoprotein particle. The vitamin thus exhibits prooxidant activity. Since it is the isolation of the vitamin $E^{\cdot}$ within a lipoprotein undergoing oxidation that forces it to react with the polyunsaturated fatty acids of LDL, prevention of this oxidation depends on the rapid destruction of vitamin $E^{\cdot}$. This implies that a compound must have a high capacity to reduce and interact with LDL-associated vitamin $E^{\cdot}$. The conversion of the lipophilic vitamin $E^{\cdot}$ into a harmless aqueous radical is the process that actually prevents lipid hydroperoxide formation.

In this study we also demonstrated that, under equal oxidative stress, troglitazone was a much more potent radical scavenger than vitamin E. Our results agree with those obtained under different experimental conditions by Nagasaka et al. [50], who studied the

effect of troglitazone and vitamin E on LDL lipid peroxidation by 2,2′-azobis(2-aminopropane) dihydrochloride. These authors found that the 50 % inhibitory concentrations of troglitazone and vitamin E were 10 μmol/l and 25 μmol/l, respectively. Although the mechanism of enhanced antioxidative activity of troglitazone is not clear, it may be related to its structure. The hindered phenol of vitamin E acts as a chain-breaking antioxidant, whereas the major function of the phytyl side chain is to retain the molecule in LDL [44]. The thiazolidine ring of troglitazone may affect its stability and thus its affinity for LDL and hence its antioxidant capacity. Of course the present results do not preclude that, under our experimental conditions, troglitazone may protect vitamin E by scavenging peroxyl radicals faster than vitamin E, or that vitamin E can be regenerated by troglitazone as, for instance, by vitamin C [51].

Troglitazone was also shown to be much more potent than vitamin E when LDL were oxidized by HUVEC than by Cu^{++}. This finding raises new questions as to how HUVEC modify LDL. If the contribution of the modifying cells is only to favour LDL lipid peroxidation followed by metal-catalysed propagation, the potency of troglitazone in the Cu^{++} and HUVEC model might be of the same order. If, as recently proposed [32, 48], the modifying cells directly provide peroxidative products to the LDL, a possible effect of troglitazone on production of these compounds cannot be ruled out. The present data allow no definitive conclusion on the matter and this point requires further study.

In conclusion, the results of this study demonstrate that troglitazone, in addition to ensuring blood glucose control, may be useful in inhibiting lipoprotein peroxidation, and thus slow the development of atherosclerosis in diabetes. Further studies are needed to confirm these results in the lipoproteins of diabetic patients.

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