Inhibition of LDL Oxidation In Vitro but not Ex Vivo by Troglitazone

Richard S. Crawford, Sunder R. Mudaliar, Robert R. Henry, and Alan Chait

Diabetic subjects are at increased risk for developing coronary artery disease, in part because of increased oxidation of LDL, which promotes atherogenesis. Troglitazone, a new antidiabetic drug of the thiazolidinedione class, acts as an insulin sensitizer and improves hyperglycemia. Structurally, it contains a tocopherol moiety similar to vitamin E and has been shown to have antioxidant properties in vitro. Therefore, we evaluated whether troglitazone inhibited LDL oxidation both in vitro and in type 2 diabetic subjects ex vivo. Troglitazone inhibited oxidation of LDL induced by Cu^{2+} or 2′2′-azobis-2-amidinopropane hydrochloride (AAPH) with 50% inhibition at 1 μmol/l and 100% inhibition at 5–10 μmol/l troglitazone. The inhibition of LDL oxidation by troglitazone also was time dependent. In addition, troglitazone inhibited oxidation of ^{125}I-labeled LDL and its subsequent uptake and degradation by macrophages. To determine whether troglitazone was incorporated into LDL particles or acted in the aqueous milieu, troglitazone was incubated overnight at 37°C with LDL or plasma before LDL re-isolation. After re-isolation, LDL that was incubated with troglitazone was no longer protected from oxidation, compared with probucol-treated LDL, which remained protected. Further, [^{14}C]troglitazone did not get incorporated into LDL. This suggests that troglitazone exerts its antioxidant effect in the aqueous milieu of LDL. Consistent with this was the observation that the lag phases of copper-induced conjugated diene formation, a measure of the susceptibility in vivo, was similar for subjects taking troglitazone (76 ± 5 min, n = 9) to subjects not taking the drug (77 ± 3 min, n = 11; NS). Thus, troglitazone may be of value as an aqueous-phase antioxidant in addition to its effect on glucose homeostasis. *Diabetes* 48:783–790, 1999

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AAPH, 2′2′-azobis-2-amidinopropane hydrochloride; BHT, butylated hydroxytoluene; FPLC, fast protein liquid chromatograph; MDA, malondialdehyde; PBS, phosphate-buffered saline; TBARS, thiobarbituric substance; TEC, trolox equivalent concentration.

Macrovascular disease is increased markedly in patients with type 2 diabetes (1). One of the underlying reasons is that diabetic subjects are under increased substrate-mediated oxidative stress (2), which may in part account for the increased susceptibility of LDL from subjects with poorly controlled diabetes to oxidative modification (3). Oxidative modification of LDL has been implicated in the development of atherosclerosis through a variety of mechanisms including enhanced cytotoxicity (4), stimulation of monocyte adhesion and chemotaxis (5), uptake by macrophage scavenger receptors leading to foam cell formation (6), modulation of the expression of several cytokines in growth factors (7,8), and stimulation of plasminogen activator inhibitor-1 expression (9). Conversely, antioxidants including vitamin E and probucol have been shown to inhibit LDL oxidation (10,11) and retard the development of atherosclerosis in hyperlipidemic animal models (12–14). It therefore is conceivable that agents that reduce the oxidant stress in diabetic subjects might reduce the susceptibility of LDL to oxidation and the development of atherosclerosis.

Troglitazone is a thiazolidinedione antidiabetic agent that acts as an insulin sensitizer (15,16). Other reported effects of troglitazone include an antihypertensive effect (17) and an ability to reduce plasma triglyceride levels (18,19). More recently, troglitazone, which is structurally similar to vitamin E, has been shown to have antioxidant properties (20,21). It is converted into three major metabolites, a sulfate, glucuronide, and quinone (22), the structures of which do not predict that they would have antioxidant properties.

This study was undertaken to determine whether the antioxidant effects of troglitazone might be important in reducing the susceptibility of LDL to oxidation. Specifically, the aims of this study were to examine whether troglitazone inhibited LDL oxidation in vitro, to determine if troglitazone acted as antioxidant within the LDL particle or within the aqueous milieu, and finally, to assess whether troglitazone could inhibit LDL oxidation ex vivo in plasma isolated from subjects with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Materials. RPMI-1640 medium was supplied by GIBCO (Gaithersburg, MD) and disposable cell culture wares by Falcon Labware (Becton, Dickinson, Oxnard, CA). Ficoll Plaque and Superose 6 were from Pharmacia LKB (Biotechnology, Piscataway, NJ). Carrier-free Na ^{125}I was supplied by Dupont New England Nuclear (Boston, MA). Troglitazone was a gift from Parke-Davis Pharmaceutical Research (Ann Arbor, MI), and 100 mmol/l stock solutions were prepared fresh daily by dissolving troglitazone into DMSO. Serial dilutions with DMSO were performed with final concentration of DMSO of 1%. Probucol was supplied by Sigma (St. Louis, MO). Stock solutions were dissolved in ethanol and diluted to appropriate concentrations for use in experiments. Final ethanol concentra-

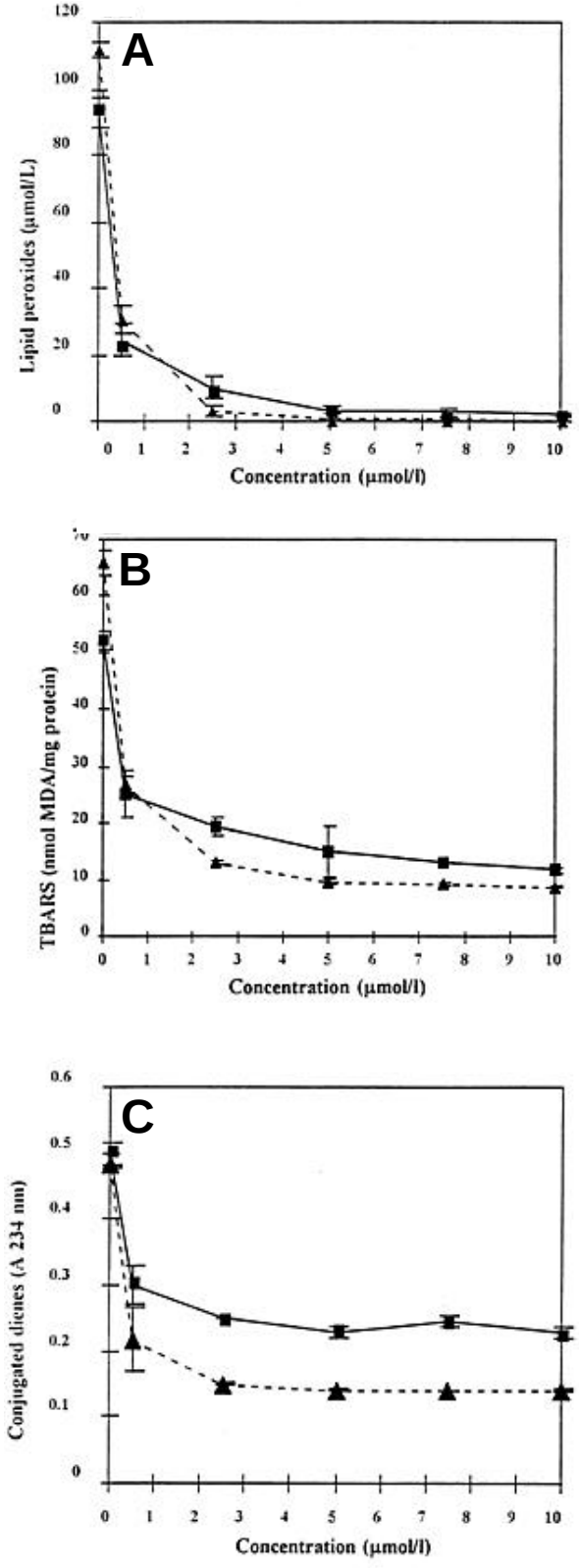

FIG. 1. Inhibition of LDL oxidation by troglitazone and probucol, the dose-response curve. LDL (200 mg/ml, final concentration) was incubated with troglitazone (■) or probucol (▲) at the indicated concentrations. Oxidation was initiated by addition of Cu^{2+} (1 μmol/l), and samples were incubated at 37°C for 2 h. LDL oxidation was terminated by the addition of BHT (25 μmol/l). Lipid oxidation was assessed by measurement of lipid peroxides (*A*), TBARS (*B*), and conjugated dienes (*C*), as described in METHODS.

tions were 1%. This concentration of DMSO was shown to have minimal antioxidant properties. This concentration of alcohol was shown to be without effect on the variables measured.

Isolation of LDL. To minimize variability between LDL preparations, LDL was isolated from pooled plasma from five to six normolipidemic (plasma cholesterol and triglyceride <200 mg/dl) individuals without increased lipoprotein(a) levels, as determined by a sinking pre-beta bands on lipoprotein electrophoresis. Blood was collected into EDTA (4 mmol/l final concentration), and plasma was separated by low-speed centrifugation for separation of LDL by a single vertical spin in a vertical ultracentrifuge rotor (Beckman Vti-50, Beckman, Fullerton, CA), using a method modified from Chung et al. (23). Briefly, a discontinuous NaCl/KBr density gradient was formed by adjusting the plasma to 1.3 g/ml with KBr, which then was overlaid with normal saline (d = 1.006 g/ml). Tubes were centrifuged in a Beckman Vti-50 vertical ultracentrifuge rotor for 2.5 h at 50,000 rpm at 10°C. The LDL band was localized by inspection and aspirated after tube slicing. It then was recentrifuged twice at d = 1.019 and d = 1.063 g/ml, as described previously (24). The isolated LDL (d = 1.019–1.063 g/ml) was dialyzed for 48 h against 0.15 mol/l NaCl containing EDTA (1 mmol/l final concentration). The protein content of the LDL was measured by a modification of the technique of Lowry (25) using albumin as standard. LDL was sterilized by passage through a 22-μm millipore filter, stored in the dark under N_2 and used within 2 weeks of preparation. For evaluation of uptake and degradation by macrophages, LDL was radiolabeled with Na ^{125}I, using the iodine monochloride method as previously described (26).

LDL oxidation in vitro. Before oxidation, LDL was dialyzed for 24 h against phosphate-buffered saline (PBS) to remove EDTA. LDL protein was quantified using the modified Lowry method and diluted with PBS to a final concentration of 200 μg/ml. LDL was mixed with either vehicle alone, troglitazone, or probucol at varied concentrations (Figs. 1–5), in a cell-free system. At time 0, Cu_2SO_4 (1 μmol/l final) was added to the LDL-containing solutions and incubated at 37°C. After incubation at 37°C for the indicated times, further oxidative modification was inhibited by the addition of butylated hydroxytoluene (BHT) to a final concentration of 25 μmol/l. The samples were stored under N_2 at 4°C and analyzed within 1 day. Malondialdehyde (MDA) and other products of lipid peroxidation were measured as thiobarbituric substances (TBARSs) by a modification of the method described by Buege and Aust (27). Results were reported as MDA equivalents (nmol MDA/mg LDL protein), using an extinction coefficient determined from MDA prepared by acid hydrolysis of malondialdehyde tetramethyl acetal. Lipid peroxides were quantified using methods previously described (28).

The susceptibility of LDL to oxidation was assessed by determining the lag phase of conjugated diene formation using a modification of the method of Esterbauer et al. (29). This method has been used to assess the susceptibility of isolated LDL to oxidative modification in vivo and is in part related to the endogenous antioxidant content of the LDL (30). Briefly, aliquots of LDL were mixed with Cu^{2+} (final concentration 5 μmol/l) or 2′2′-azobis-2-amidinopropane hydrochloride (AAPH). The appearance of conjugated dienes was measured by continuously monitoring absorbance at 234 nm in a Varian Cary 1-E spectrophotometer (Varian, Mulgrave, Victoria, Australia) equipped with a 12-position automatic sample changer for 8 h at 37°C. Results were expressed as the lag phase and rates of conjugated diene formation. The lag phase was defined as the interval between the intercept of the tangent of the slope of the curve with the time expressed in minutes. Rate of conjugated diene formation was defined as maximum change in absorbance at 234 nm/min.

The [^{14}C]troglitazone experiments. To determine whether troglitazone is incorporated into the LDL particle or in the aqueous milieu, human plasma was incubated with [^{14}C]troglitazone and subjected to density-gradient ultracentrifugation to isolate LDL, as described earlier. A total of 20 fractions were collected and assessed for protein, total cholesterol and triglyceride, and radioactive content. The ^{14}C-labeled samples were compared with control plasma that had been incubated with similar amounts of unlabeled troglitazone and subjected to a similar density-gradient ultracentrifugation for determination of cholesterol, triglyceride, and protein content of the individual fractions.

In separate experiments, human plasma was incubated overnight with [^{14}C]troglitazone at 37°C and subjected to fast protein liquid chromatograph (FPLC) analysis as previously described (31), with minor modifications. Briefly, 500 μl of sample was applied to a Superose 6 column (Pharmacia) in PBS. Sixty 2.5-ml fractions were collected and analyzed for cholesterol and protein content and radioactivity.

Effect of troglitazone on LDL oxidation after re-isolation of LDL. An alternative approach to determine whether troglitazone is incorporated into LDL was tested by performing oxidation experiments before and after re-isolation of LDL. Human LDL (containing 4 mmol/l EDTA) was incubated overnight at 37°C with either 10 μmol/l (final concentration) troglitazone or probucol, a lipophilic antioxidant that is incorporated into the core of LDL (11). Control LDL that contained neither troglitazone nor probucol was incubated in parallel. The next day, LDL was re-isolated from these samples by ultracentrifugation, and the LDL fractions were subsequently passed over sephracyl-300 gel columns to remove KBr, EDTA, and albumin. Samples then were quantified for cholesterol content and pro-

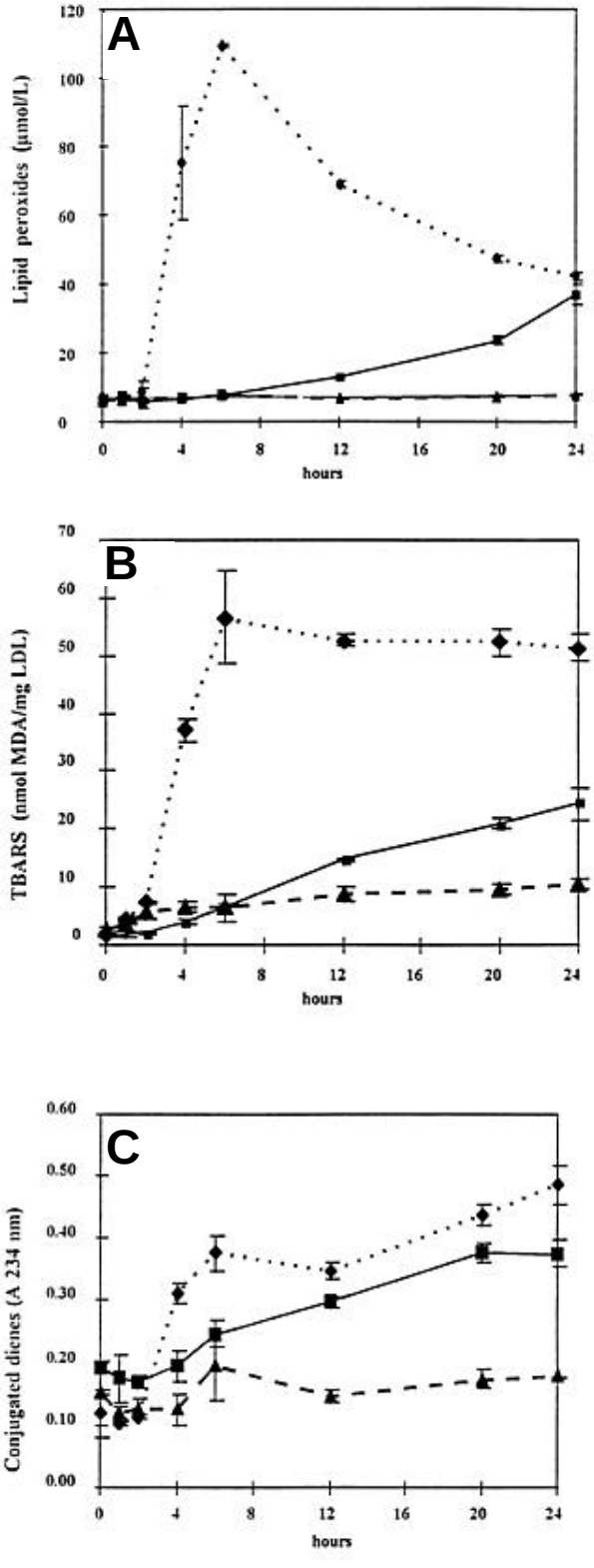

FIG. 2. Inhibition of LDL oxidation by troglitazone probucol, the time course. LDL (200 mg/ml, final concentration) was incubated either alone (◆), with troglitazone (■) or probucol (▲) (both 10 µmol/l). Oxidation was initiated by adding Cu^{2+} (1 µmol/l) to samples and incubating at 37°C for the time periods shown. Oxidation was stopped at each time point by adding BHT (25 µmol/l). Lipid oxidation was assessed by measurement of lipid peroxides (*A*), TBARS (*B*), and conjugated dienes (*C*).

tein content by colorimetric assay using available commercial kits (32). LDL samples were diluted to a final concentration of 200 µg/ml protein. All samples were oxidized by the addition of Cu_2SO_4 (5 µmol/l final concentration) and continuously monitored for formation of conjugated dienes, as described previously (29). Results were reported as lag phase of conjugate diene formation.

Additional oxidation experiments were performed by incubating plasma instead of LDL with troglitazone or probucol overnight at 37°C. The next day, LDL was isolated from plasma by density-gradient ultracentrifugation, passed over sephracryl-300 columns and subjected to copper-mediated oxidation as described above.

Cellular metabolism of ^{125}I-labeled modified lipoproteins. Human monocyte-derived macrophages were isolated by the method of Böyum (33), as described previously. The mixed mononuclear cells were plated in RPMI-1640 medium for 2 h to allow monocytes to adhere. After removal of nonadherent cells, the medium was switched to RPMI-1640 containing 20% autologous serum. Monocyte-macrophages were grown for 7–14 days in the same medium, which was changed twice weekly. For measurement of lipoprotein uptake and degradation, the medium was removed and the cells were washed twice with serum-free RPMI medium that was then replaced with serum-free medium containing native or modified ^{125}I-lipoproteins that previously had been oxidized in the presence or absence of varying concentrations of troglitazone, probucol, or vehicle. After incubation for 2 h, the medium was removed for determination of trichloroacetic-soluble radioactivity that was not free iodide (34). Degradation rates were corrected for cell-free controls incubated in parallel. Cell protein content was measured by the technique of Lowry et al. (25) after extraction of the cells with sodium hydroxide. Lipoprotein degradation was expressed as nanograms lipoprotein degraded per millgram cell protein.

Ex vivo studies

Human subjects. The study groups consisted of 22 obese subjects with type 2 diabetes, of whom 10 subjects were on insulin and troglitazone (200–400 mg/day) and 12 subjects were on insulin alone for glycemic control for a minimum of 6 months. Subjects were excluded if, during the preceding 6 months, they had symptomatic coronary heart disease (with a history of myocardial infarction or any revascularization procedure) or a history of a vaso-occlusive event. Subjects also were excluded if they had a history of any cancer in the previous 6 months, serum transaminase levels greater than twice the upper limits of normal, or a serum creatinine >2 mg/dl. The purpose, nature, and potential risks of the study were explained to all subjects before obtaining their written consent. The study protocol was approved by the Human Subjects Committee of the University of California, San Diego.

Ex vivo LDL oxidation. Plasma was obtained 24 h after the last dose of troglitazone, and stored at –70°C under nitrogen for 6–12 weeks before assay. Just before measurement of the susceptibility of LDL to oxidation, LDL was isolated by density-gradient ultracentrifugation as described earlier. LDL (100 µg/ml protein) in PBS was subjected to ex vivo oxidation mediated by the addition of copper (5 µmol/l final concentration) and monitored for the formation of conjugated dienes. Results were recorded as lag phase and rates of conjugated diene formation.

Ex vivo total plasma antioxidant capacity. Plasma samples from these human subjects also were assessed for total plasma antioxidant capacity using an assay adapted from Rice-Evans et al. (35). Briefly, 8 µl of plasma sample was mixed with metmyoglobin (2.5 µmol/l final concentration) and 2′2′-azobis-2-amidinopropane hydrochloride (ABTS) (150 µmol/l final concentration). The samples then were placed in quartz cuvettes preheated to 37°C. H_2O_2 (150 µmol/l final concentration) was added and the samples were monitored for absorbance at 730 nm. Results were compared with a standard of trolox, a water-soluble analog of vitamin E, and reported as trolox equivalent concentration (TEC) values.

Statistical analysis. Results are reported as mean ± SE. Student's *t* tests were used to detect statistical differences. A P value of <0.05 was considered statistically significant.

RESULTS

In vitro studies

Dose-response experiments. LDL incubated with concentrations of troglitazone varying between 0 and 10 µmol/l, or with probucol at similar concentrations, was subjected to copper-mediated oxidation and monitored for the formation of lipid peroxides, conjugate dienes, and TBARS. There was marked inhibition of LDL oxidation, with >50% inhibition of oxidation at concentrations as low as 0.05 µmol/l of troglitazone. Similar results were seen with probucol. There was near-complete inhibition of LDL oxidation at 5 µmol/l of troglitazone or probucol (Fig. 1). Similar results were seen with AAPH-mediated LDL oxidation (data not shown).

Time course experiments. LDL was incubated alone or in the presence of troglitazone or probucol (10 μmol/l final concentration) and subjected to copper-mediated oxidation for measurement of the lipid peroxides, TBARS, and conjugated dienes at various time points over 24 h. In the absence of added antioxidants, there was a peak formation of oxidative products at 6 h, then a gradual fall-off thereafter caused by degradation of these oxidation products. The formation of oxidation products in LDL was markedly inhibited by both troglitazone and probucol. In the troglitazone-treated samples, low levels of oxidation products were observed at the end of 24 h, whereas the probucol-treated samples showed near-complete inhibition of formation of oxidative products (Fig. 2). Similar effects were observed when oxidation was mediated by AAPH instead of Cu_2SO_4 (data not shown).

Degradation of oxidized ^{125}I-LDL by macrophages. The ^{125}I-LDL that had been oxidized by copper in the presence or absence of troglitazone or probucol was incubated with macrophages for measurement of its uptake and degradation. Compared with unoxidized LDL, the copper-oxidized LDL showed about a fourfold increase in degradation, consistent with uptake of the oxidized LDL by the scavenger receptor. In contrast, LDL that had been oxidized in the presence of troglitazone or probucol showed no increased uptake and degradation by macrophages, with degradation values similar to that of unoxidized LDL (Fig. 3). Thus, inhibition of LDL oxidation by troglitazone or probucol prevented its subsequent uptake and degradation by macrophages.

Effect of re-isolation of LDL on inhibition of copper-mediated oxidation by troglitazone and probucol. To determine whether troglitazone exerted its antioxidant effects within the LDL particle or in the aqueous milieu, LDL was incubated with troglitazone or probucol before or after its re-isolation. Samples of LDL before and after re-isolation were subjected to copper-mediated oxidation and monitored for formation of conjugated dienes. Native LDL showed a short lag phase (59 ± 6 min), which did not change after re-isolation (46 ± 10; NS). Before re-isolation, both the troglitazone- and probucol-treated samples showed a prolonged lag phase (120 ± 8, 138 ± 6, respectively), consistent with the antioxidant properties of these compounds. The probucol-treated samples continued to show a prolonged lag phase after re-isolation (118 ± 10; NS), consistent with the prior observation that probucol is incorporated to the LDL particle and can reduce the susceptibility of LDL to oxidation even after its re-isolation (30). Conversely, the lag phase of the troglitazone-treated LDL after re-isolation was significantly lower (66 ± 7; $P < 0.05$) than at baseline (Fig. 4), suggesting the troglitazone was not incorporated into the LDL particle and therefore did not protect LDL from oxidation after re-isolation.

The [^{14}C]troglitazone studies. To further determine whether troglitazone was incorporated into LDL particles, [^{14}C]troglitazone was incubated with either plasma or LDL, after which the LDL was re-isolated by density-gradient ultracentrifugation. The profile of radioactivity corresponded only minimally with that of cholesterol, which indicates that the radiolabeled troglitazone was not significantly incorporated into LDL (Fig. 5*A*). Rather, it was present in the cholesterol-free fractions containing the highest content of protein (i.e., fraction corresponding to plasma proteins of higher density than lipoproteins).

To further characterize the distribution of troglitazone in plasma, analysis of the distribution of troglitazone incubated in plasma was carried out by FPLC analysis. The FPLC profile showed only weak association of [^{14}C]troglitazone with LDL and HDL, the majority of the radiolabel being present in fractions other than those containing lipoproteins (Fig. 5*B*).

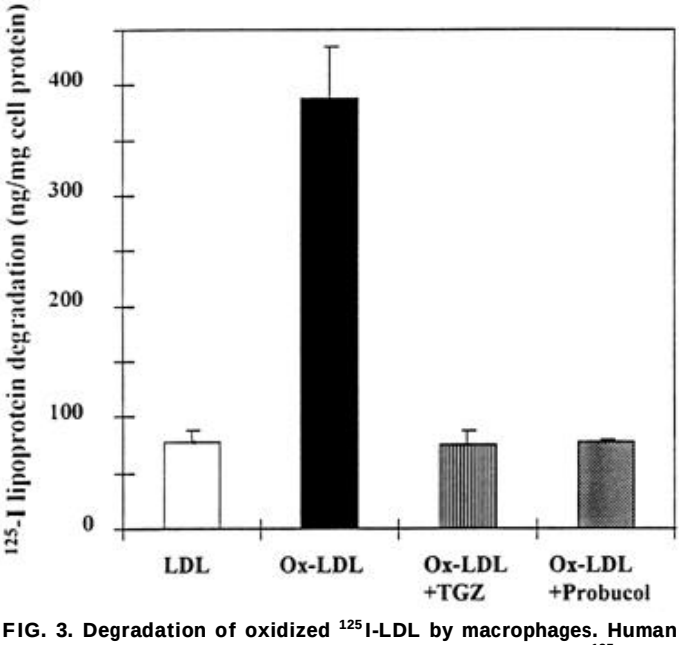

FIG. 3. Degradation of oxidized ^{125}I-LDL by macrophages. Human monocyte-derived macrophages were incubated with native ^{125}I-LDL, or ^{125}I-LDL that previously had been oxidized by adding 5 μmol/l Cu^{2+} in the absence or presence of varying concentrations of troglitazone or probucol (10 μmol/l), for measurement of lipoprotein degradation products, as described in METHODS.

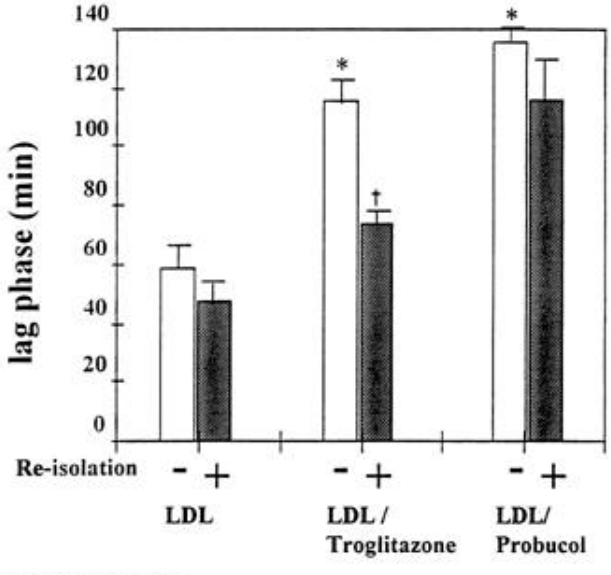

FIG. 4. Lack of protection of troglitazone against LDL oxidation after re-isolation of LDL. LDL was oxidized by the addition of Cu^{2+} (5 μmol/l) in the presence of troglitazone or probucol before (–) or after (+) re-isolation of LDL by ultracentrifugation for 24 h at 37°C. Lipid oxidation was assessed by continuously monitoring conjugated diene formation. Results are expressed as the lag phase of conjugated diene formation. *$P < 0.05$ vs. control LDL; †$P < 0.05$ vs. before reisolation.

Evaluation of the susceptibility to oxidation of LDL from diabetic subjects being treated with troglitazone. Table 1 demonstrates the baseline characteristics of the study subjects. The troglitazone plus insulin-treated group had slightly higher glycosylated hemoglobin values than subjects receiving insulin alone (8.5 ± 0.4 vs. 7.4 ± 0.4%; $P = 0.05$). Total cholesterol values were similar in the troglitazone plus insulin group and the insulin-alone group (5.1 ± 0.3 vs. 4.3 ± 0.3 mg/dl; NS). HDL cholesterol levels were similar in the troglitazone plus insulin group and the insulin-alone group (1.0 ± 0.1 and 0.9 ± 0.2 mg/dl, respectively; NS). LDL cholesterol values were higher in the troglitazone plus insulin-treated group (3.2 ± 0.2 mg/dl), as compared with the insulin alone-treated group (2.4 ± 0.3; $P = 0.03$). Triglyceride levels were similar between the troglitazone plus insulin group and the insulin-alone group (2.0 ± 0.3 vs. 2.1 ± 0.5 mg/dl; NS).

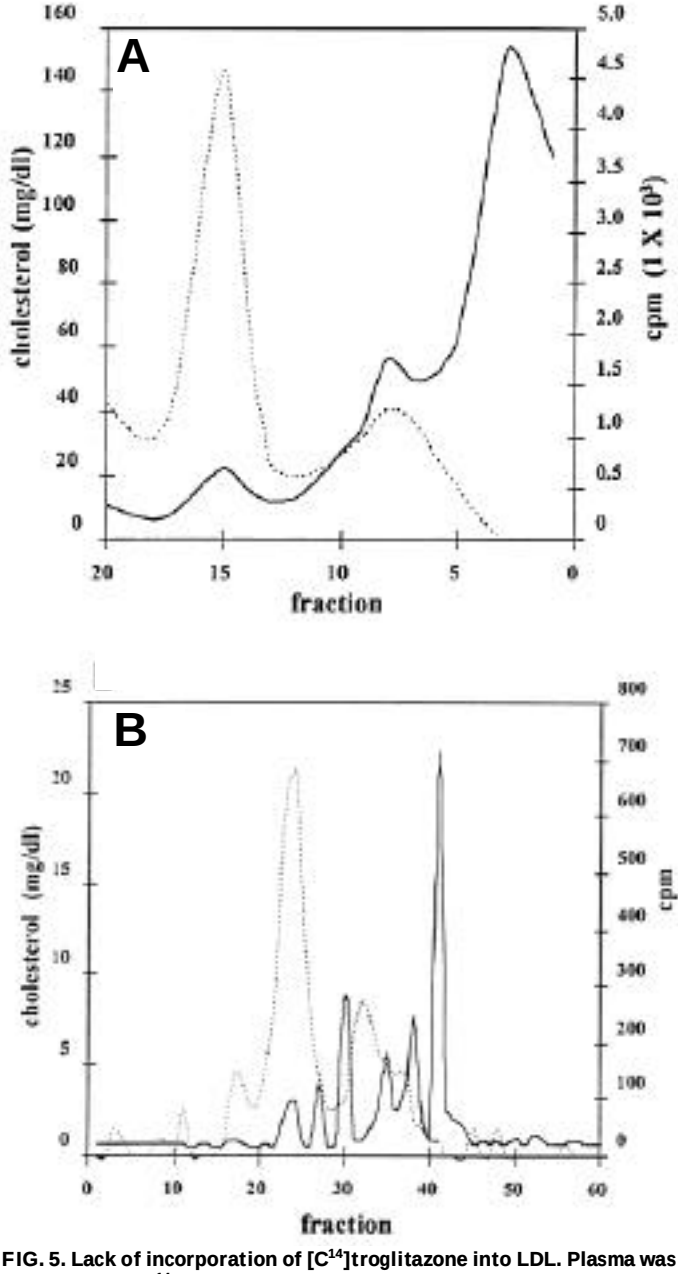

FIG. 5. Lack of incorporation of [C^{14}]troglitazone into LDL. Plasma was incubated with [^{14}C]troglitazone overnight at 37°C. *A*: Plasma then was subjected to density-gradient ultracentrifugation and 38 fractions were quantified for cholesterol content (- - -) and radioactivity (——). *B*: Plasma then was subjected to FPLC and 60 fractions were quantified for cholesterol content (- - -) and radioactivity (——).

The lag phases of conjugated diene formation in the insulin group and the insulin plus troglitazone-treated group were similar, with mean values of 77 ± 3 and 76 ± 5 min, respectively (NS) (Table 2). In addition, no differences were observed in the rates of formation of conjugated dienes. Thus, the therapeutic use of troglitazone did not appear to reduce the susceptibility of LDL to oxidation that had been isolated from plasma and subjected to an oxidative stress.

The total antioxidant capacity of plasma also was compared in these subjects and was expressed as TEC, defined previously. TEC values were 0.59 ± 0.06 and 0.54 ± 0.06 µmol/l in the insulin-alone group and troglitazone plus insulin group, respectively (NS). Thus, there was no difference in total antioxidant capacity of plasma in the insulin-alone group as compared with the troglitazone plus insulin-treated group. This suggests that troglitazone did not afford additional protection against oxidation over and above other antioxidants present in plasma.

DISCUSSION

Diabetic subjects are at increased risk for macrovascular disease and its complications (2,36). One hypothesis to explain this increased risk is that diabetic subjects are under increased oxidative stress. This could result in increased oxidation of LDL (37), which is believed to play an important role in atherogenesis (2). Several mechanisms may lead to increased oxidative stress in diabetes. First, hyperglycemia may increase the generation of free radicals through the ability of glucose to enolize and yield oxidizing intermediates such as superoxide anion, hydroxyl radicals, and hydrogen peroxide (38,39). All of these intermediate products can lead to lipid peroxidation and cellular damage, resulting in atherosclerotic lesion formation. Next, antioxidant defenses are reduced in diabetes (3,37). Moreover, type 2 diabetes often is associated with various metabolic derangements including central obesity, insulin resistance and hyperinsulinemia, hypertension, and dyslipidemia (40). A feature of the dyslipidemia in type 2 diabetes is the presence of small dense LDL, which is prone to oxidative damage, which might in part explain its atherogenicity (41). Troglitazone has been shown to improve metabolic disturbances associated with type 2 diabetes, including hyperglycemia and hyperinsulinemia (42). Because troglitazone increases LDL concentrations without changing apo B concentrations (43), the composition of LDL is altered by troglitazone toward particles of larger size and decreased density (44,45). All of these may reduce the oxidant stress in type 2 diabetic subjects and therefore potentially reduce the risk for atherogenesis.

The results of this study suggest that troglitazone acts directly as an antioxidant and is a potent inhibitor of LDL oxidation in vitro. At micromolar concentrations, troglitazone inhibited LDL oxidation significantly, compared with controls, with a dose-response similar to probucol. These concentrations of troglitazone that inhibit LDL oxidation in vitro correspond to pharmacological levels seen in human subjects treated with troglitazone (46). Inhibition of LDL oxidation by troglitazone was seen whether oxidation was mediated by either copper or AAPH. These results are in agreement with two earlier studies that tested the effect of troglitazone on LDL

TABLE 1
Subject characteristics

Variable	Insulin	Insulin + troglitazone
Age of subjects (years)	61 ± 2.0	60 ± 3.0
BMI (kg/m^2)	30.1 ± 1.6	31.1 ± 0.8
Waist-to-hip ratio	0.98 ± 0.01	1.01 ± 0.01
Fasting glucose (mmol/l)	9.1 ± 1.0	9.4 ± 0.9
HbA_{1c} (%)	7.4 ± 0.4	8.5 ± 0.4*
Total cholesterol (mmol/l)	4.3 ± 0.3	5.1 ± 0.3
Triglyceride (mmol/l)	2.1 ± 0.5	2.0 ± 0.3
HDL cholesterol (mmol/l)	1.0 ± 0.2	1.0 ± 0.1
LDL cholesterol (mmol/l)	2.4 ± 0.3	3.2 ± 0.2†

Data are means ± SE. *P = 0.05 vs. insulin group; †P < 0.05 vs. insulin group.

oxidation in vitro (21,47). The likely reason for the antioxidant properties of troglitazone is related to its structural similarity to vitamin E. Both troglitazone and vitamin E contain a tocopherol moiety, which is a potent inhibitor of free-radical formation and oxidative processes (48). Thus, other thiazoladinediones, which do not contain a tocopherol moiety, are unlikely to have this anti-oxidant effect.

However, unlike vitamin E, troglitazone does not appear to be readily incorporated into the LDL particle. The observation that the ultracentrifugal re-isolation of LDL that had been incubated with troglitazone resulted in loss of protection against LDL oxidation suggests that troglitazone was not incorporated into the LDL particle. This is in contrast to probucol, a lipophilic antioxidant, which previously has been described to be readily incorporated into the LDL particle (30). Probucol retained its ability to protect LDL against oxidation even after re-isolation of the LDL. Additional evidence for the lack of incorporation of troglitazone into the LDL particle comes from the [^{14}C]troglitazone experiments. The [^{14}C]troglitazone did not appear to be incorporated into the LDL particles when incubated with either serum or LDL directly. Therefore, it is conceivable that troglitazone may exert its antioxidant effects in vivo in the aqueous milieu like vitamin C by regenerating the antioxidant potential of vitamin E (49).

These findings are in contrast to those of Cominacini et al. (21), who found that troglitazone was incorporated into the LDL particle. In that study, troglitazone was detected in LDL isolated after 12-h incubation in plasma. Troglitazone was detectable in LDL by high-performance liquid chromatograph (HPLC) in concentrations proportional to the amount of troglitazone initially added to the plasma. That study also found that LDL isolated from plasma that previously had been incubated with troglitazone was protected against oxidation, also suggesting that troglitazone is incorporated into the LDL particle in vitro (21). The reason for the difference between the study of Cominacini et al. (21) is not clear, but may be the result of different conditions under which the in vitro experiments were performed. In Cominacini et al. (21), the vehicle in which troglitazone was dissolved was not specified. While the DMSO used in the present study solubilized the troglitazone, it may have prevented its adequate incorporation into the LDL particle. The significance of differences seen between these in vitro studies and its relevance to potential antioxidant effect of troglitazone in vivo remain unclear and require further investigation.

In contrast to the ability of troglitazone to inhibit LDL oxidation in vitro in the present study, it did not appear to reduce the susceptibility of LDL from human subjects treated with troglitazone to copper-mediated oxidation ex vivo. Matched subjects treated with troglitazone plus insulin had no difference in either the lag phase or rate of conjugated diene formation as compared with subjects treated with insulin alone. This would be expected if the troglitazone does not get incorporated into LDL in vivo. Because LDL is removed from its aqueous milieu during its isolation from plasma, the LDL would not be exposed to troglitazone during its oxidation ex vivo. However, when total plasma antioxidant capacity was assessed, no difference between the insulin-treated and the insulin plus troglitazone-treated groups was observed. This may be due to the fact that troglitazone is extensively metabolized to forms that are unlikely to exert antioxidant effects. Alternatively, this method of assessing total plasma antioxidant capacity measures the net effect of all plasma antioxidants, including albumin, urea,

TABLE 2
Lag phase and rates of conjugated diene formation and total plasma antioxidant capacity

	Insulin	Insulin + troglitazone
n	11	9
Lag phase (min)	77 ± 3	76 ± 5
Rate (OD/h)	3.2 ± 0.1	3.0 ± 0.1
TEC (μmol/l)	0.59 ± 0.06	0.54 ± 0.06

Data are means ± SE. OD, optical density.

endogenous vitamin C and vitamin E, among others. It therefore might be unable to distinguish potential protective effects of troglitazone against the background of these other antioxidants.

In conclusion, troglitazone is a potent inhibitor of LDL oxidation in vitro. However, it does not appear to be readily incorporated into the LDL particle, consistent with an inability to detect the difference in the susceptibility to oxidation of LDL isolated from human subjects treated with troglitazone. Nonetheless, troglitazone may exert its antioxidant properties in the aqueous milieu, and may thereby play a role in inhibiting LDL oxidation and other processes in vivo.

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