ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 2, 2000
Mary Ann Liebert, Inc.

Forum Original Research Communication

Troglitazone Protects Human Erythrocytes from Oxidant Damage

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ABSTRACT

The antidiabetic drug troglitazone contains the active chromanol ring of α-tocopherol, which should give it antioxidant properties within cells. In these studies, the antioxidant effects of troglitazone were tested in human erythrocytes and in their ghosts. Troglitazone bound to erythrocyte ghosts in a linear manner and was retained even after centrifugation washes. In response to an oxidant stress generated by a water-soluble free radical initiator, troglitazone that was bound to erythrocyte ghosts was oxidized, but induced a lag-phase in the disappearance of endogenous α-tocopherol and in the appearance of lipid hydroperoxides. Troglitazone also delayed loss of endogenous α-tocopherol and hemolysis in washed intact erythrocytes in response to free radical-induced extracellular oxidant stress. To mimic exposure of erythrocytes to lipid hydroperoxides *in vivo*, erythrocytes were incubated with phospholipid liposomes that contained small amounts of preformed lipid hydroperoxides. This induced an oxidant stress in both the liposomes and cells. Troglitazone in concentrations above 4 μ*M* almost completely prevented further appearance of lipid hydroperoxides in the liposomes, and also completely preserved α-tocopherol in the erythrocytes. The present results suggest that troglitazone will help to prevent peroxidative damage to erythrocytes in areas of excessive oxidant stress in the vascular bed. Antiox. Redox Signal. 2, 243–250.

INTRODUCTION

TROGLITAZONE, a novel oral hypoglycemic agent, was initially synthesized to contain both a thiazolidinedione moeity and the active chromanol ring of α-tocopherol (Fig. 1) (Yoshioka *et al.*, 1989). The former makes it an inhibitor of the nuclear peroxisome proliferator activated receptor-γ (PPAR-γ) (Spiegelman 1998), and the latter gives it potential as an antioxidant (Yoshioka *et al.*, 1989). It is clear from clinical studies that this drug improves insulin resistance and glycemic control in patients with the metabolic syndrome of insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM) (Saltiel & Olefsky 1996; Maggs *et al.*, 1998; Schwartz *et al.*, 1998). There is also accumulating evidence that this agent can function as an antioxidant to increase the resistance of low-density lipoprotein (LDL) to oxidative damage, both *in vitro* (Cominacini *et al.*, 1997a; Noguchi *et al.*, 1996), in nondiabetics (Cominacini *et al.*, 1997b), and in patients with NIDDM (Cominacini *et al.*, 1998). Oxidized LDL has been linked to the pathogenesis of atherosclerosis (Diaz *et al.*, 1997). The antioxidant effects of troglitazone on LDL oxidation may be especially relevant for diabetics, because LDL from diabetics has been reported to have enhanced susceptibility to oxidation (Cominacini *et al.*, 1994), and atherosclerosis is the major cause of mortality in NIDDM (Baynes, 1991).

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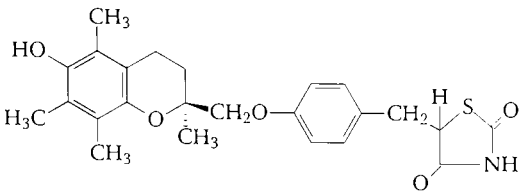
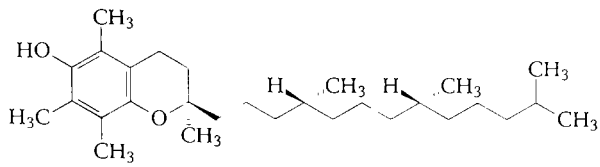


Troglitazone



α-Tocopherol

FIG. 1. Chemical structures of troglitazone and α-tocopherol.

Concentrations of troglitazone in blood from humans on therapeutic doses of the drug range from 2 to 6 μM (Loi *et al.*, 1997). In blood, troglitazone is mostly bound to albumin (Shibukawa *et al.*, 1995), and, because of its hydrophobicity, should also bind to erythrocytes. Because erythrocytes are exposed to oxidant stress in areas of atherosclerosis and inflammation, the question arises as to whether this drug can also protect erythrocytes from lipid peroxidative damage and hemolysis. It has long been known that the hemolytic sensitivity of erythrocytes is inversely proportional to the α-tocopherol content of the cell membrane (Horwitt *et al.*, 1968; Bieri *et al.*, 1976; Mino *et al.*, 1978). Before direct and sensitive assays for α-tocopherol were available, the hemolytic response of erythrocytes to hydrogen peroxide was even used as a measure of the membrane content of α-tocopherol (Horwitt *et al.*, 1968; Mino *et al.*, 1978). If the antioxidant function of α-tocopherol helps to protect the membrane from lipid peroxidative damage, then troglitazone bound to the erythrocyte membrane might have a similar property. The present studies were carried out to assess whether troglitazone binds to the cell membrane of human erythrocytes, and, if so, whether it serves to protect the cell against exogenous oxidant stress.

MATERIALS AND METHODS

Materials

Troglitazone (±-5-[4-(6-hyroxy-2,5,7,8-tetramethylchroman-2-yl)methoxy]-benzyl]-2,4-thiazolidinedione), was obtained from Parke-Davis (Ann Arbor, MI). Troglitazone was initially dissolved in dimethylsulfoxide and diluted such that the concentration of dimethylsulfoxide in incubations containing 57 μM troglitazone was 0.8% (vol/vol). The water-soluble free radical initiator 2,2'-azobis(amidinopropane)dihydrochloride (AAPH) was obtained from Wako Chemical Co. (Richmond, VA). Other analytical grade reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of human erythrocytes

Blood was drawn from normal human volunteers by venipuncture, anticoagulated with heparin, and washed three times by centrifugation in 10 volumes of phosphate-buffered saline (PBS) with careful removal of the buffy coat of leukocytes. PBS was composed of 140 mM NaCl and 12.5 mM Na_2HPO_4, adjusted to pH 7.4. This study was approved by the Vanderbilt University Institutional Review Board and subjects gave their written consent.

Preparation of liposomes and emulsions

Small unilamellar liposomes were prepared as previously described from crude soybean phosphatidylcholine (Sigma, Type II) (Waters *et al.*, 1997). Briefly, the lipid was dissolved in a minimal volume of chloroform, dried under nitrogen to a thin film, reconstituted in PBS by vigorous mixing, and sonicated to an opalescent solution (May *et al.*, 1998).

Preparation of white erythrocyte ghosts

Washed erythrocytes were hemolyzed in 40 volumes of hypotonic 5 mM sodium phosphate buffer, pH 8.0, and leaky or white ghosts were prepared by three to five centrifugation washes in the 5 mM sodium phosphate buffer as described by Steck and Kant (1974). Ghosts were stored at −20°C until use within 1–2 weeks. Results for ghosts are normalized to their protein

content, which was measured by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL).

Assay of troglitazone

Erythrocyte ghosts (32 μg of protein) in a microfuge pellet or 0.1 ml of liposomes in the supernatant from cells (65 nmol of phospholipid/ml) were diluted with 0.5 ml of ice-cold methanol, mixed, and incubated on ice for 5 min. The samples were microfuged at 13,000 × *g* for 1 min, and an aliquot of the supernatant was taken for assay of troglitazone by high-performance liquid chromatography (HPLC). It was not found necessary to add an antioxidant such as pyrogallol to the sample during extraction. However, this method was not suitable for adequate precipitation of interfering proteins from intact human erythrocytes. Reverse-phase HPLC was carried out using a 4.6 × 150-mm Bio-Rad Clinical C-8 HPLC column (Part number 195 5028) in 70% methanol:water (vol/vol) that contained a final concentration of 20 m*M* sodium perchlorate. Detection of troglitazone was carried out by a simplification of the reduction-oxidation method described by Takeda *et al.* (1996) for α-tocopherol, with some modifications (May *et al.*, 1998) Briefly, following separation on the column, the eluate was reduced by an ESA model 5020 guard cell (ESA, Inc., Chelmsford, MA) that was set in the reducing mode at −0.5 volts. Troglitazone was detected with an ESA model 5011 analytical cell, with the first electrode in the analytical cell set at −0.5 volts, and the second detecting electrode set at +0.6 volts. At a flow rate of 1 ml/min, troglitazone eluted at 6–7 min in this system. Sensitivity for troglitazone in the assay was 2 pmol.

Analytical assays

The content of α-tocopherol in erythrocyte ghosts and in liposomes to which α-tocopherol had been added was measured by HPLC using electrochemical detection (May *et al.*, 1998). Results for α-tocopherol are expressed per milliliter of erythrocyte cytosol, which was taken as 70% of the packed erythrocyte cell volume (Orringer and Roer, 1979). Lipid hydroperoxides in ghosts and liposomes were measured using the ferrous oxidation of xylenol orange (Fox-2) assay without modification (Wolff, 1994). Specificity for hydroperoxides was confirmed using a paired control sample that was incubated with triphenylphosphine (Nourooz-Zadeh *et al.*, 1994), and the resulting value was subtracted from the average of duplicate readings taken in the absence of triphenylphosphine. Hemolysis was measured as the increase in absorbance at 540 nm of erythrocyte supernatants compared to absorbance produced by hemolysis of a known volume of erythrocytes in water. Phospholipids were determined by their phosphorus content (Ames 1966).

RESULTS

To determine whether troglitazone can bind to and be retained by the erythrocyte membrane, erythrocyte ghosts were incubated with increasing concentrations of the drug, washed by centrifugation, and assayed for their troglitazone content. The troglitazone content of ghost membranes increased linearly with increasing loading concentrations of the drug (Fig. 2), suggesting that it bound to or was incorporated into the membrane. On average, the

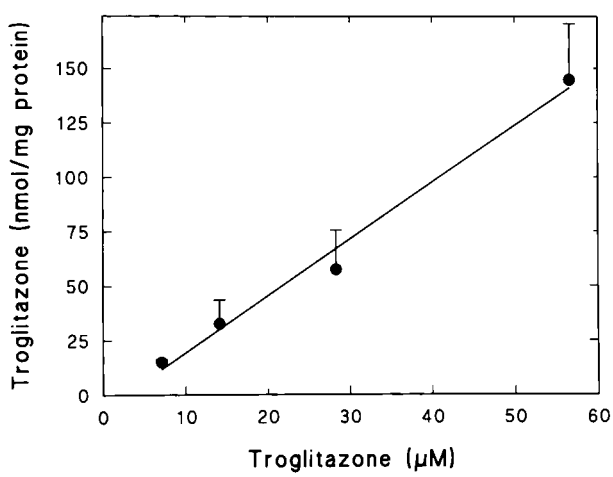


FIG. 2. Loading of erythrocyte ghosts with troglitazone. Erythrocyte ghosts (0.16 mg/ml) were incubated in PBS with the indicated concentration of troglitazone for 20 min at 37°C, washed twice in 1 ml of PBS in a microfuge at 13,000 × *g*, and assayed for troglitazone. Data are shown as mean + SE from three experiments, with a linear fit indicated by the solid line ($r = 0.996$).

ghosts retained 33% of the troglitazone added to the incubation. As shown in Fig. 3, troglitazone bound to erythrocyte ghosts protected those ghosts from peroxidative damage by the free radical initiator AAPH. In the upper panel of Fig. 3, it can be seen that AAPH treatment decreased the amount of troglitazone in the ghosts with time, and that most loss was evident over the first 15–30 min of incubation. Ghosts loaded with the highest troglitazone concentration retained almost 20% of the loaded troglitazone during the incubation, whereas ghosts loaded with lower concentrations were nearly depleted of the agent. Nonetheless, as shown in the middle panel of Fig. 3, the ability of troglitazone to spare α-tocopherol paralleled the loading concentration of the drug, with a lag-phase evident at the highest loading concentration. Associated with sparing of α-tocopherol was a delay in both onset and severity of lipid hydroperoxide formation in the ghosts during AAPH oxidation, as shown in the lower panel of Fig. 3. A lag-phase in hydroperoxide formation was again observed that was proportional to the loading concentration of troglitazone. Thus, troglitazone binds to ghosts, and protects the ghosts against both oxidative loss of α-tocopherol and lipid hydroperoxide formation.

The sparing effect of troglitazone on α-tocopherol was also apparent in intact erythrocytes. When erythrocytes were loaded directly with increasing concentrations of troglitazone, washed by centrifugation, and then incubated with AAPH, troglitazone protected erythrocyte α-tocopherol in a dose-dependent manner, as shown in the upper panel of Fig. 4. These concentrations of troglitazone also protected the cells against AAPH-induced hemolysis, as shown in the lower panel of Fig. 4. The ability of troglitazone to prevent loss of α-tocopherol and hemolysis in response to an external oxidant stress shows that the ability of troglitazone to protect α-tocopherol in ghosts also extends to intact cells.

To simulate an oxidant stress more likely to be encountered in erythrocytes *in vivo*, their interactions with liposomes containing small amounts of preformed lipid hydroperoxides were evaluated. It has been determined that

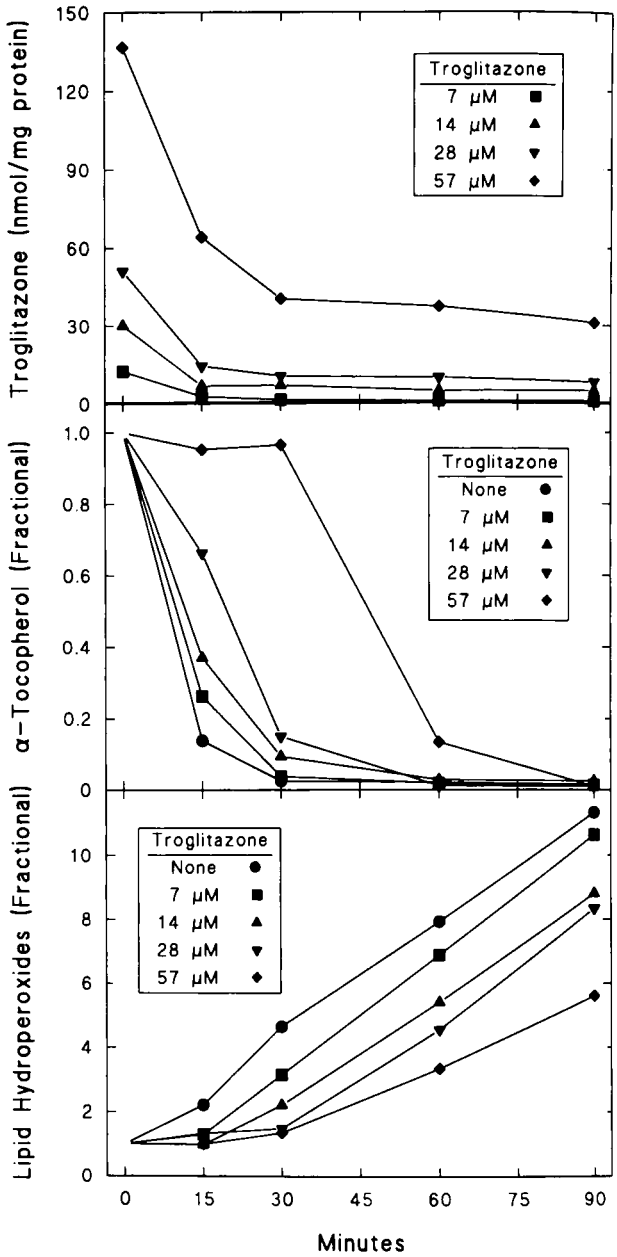


FIG. 3. Protection of erythrocyte ghosts from AAPH-induced lipid peroxidation. Erythrocyte ghosts (0.16 mg/ml) were incubated in PBS for 20 min at 37°C with the indicated concentration of troglitazone, washed twice as described in the legend to Fig. 2, and suspended to the same ghost concentration in PBS that contained 50 m*M* AAPH. After incubation for the indicated times at 37°C, aliquots of the mixed ghosts were taken for assay of troglitazone (upper panel, $n = 4$), α-tocopherol content (middle panel, $n = 3$), and lipid hydroperoxide formation (lower panel, $n = 5$). The data for α-tocopherol and lipid hydroperoxides are expressed as a fraction of the values at zero-time, which were 290 ± 15 pmol/mg protein for α-tocopherol, and 312 ± 72 nmol/mg of ghost protein for lipid hydroperoxides. Error bars are omitted for clarity of presentation.

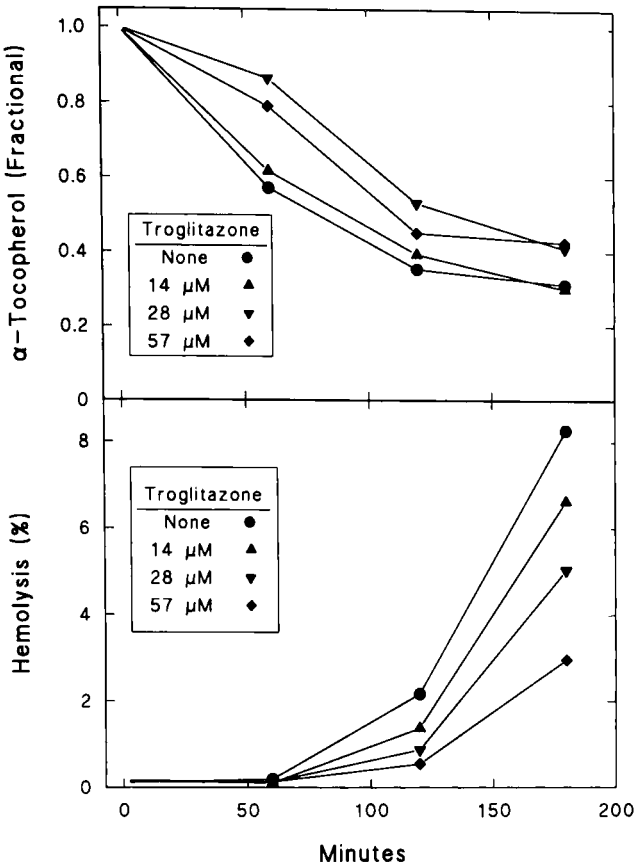


FIG. 4. Protection of α-tocopherol in intact erythrocytes from oxidation by AAPH. Erythrocytes at a 20% hematocrit were incubated with the indicated concentration of troglitazone for 20 min at 37°C. The cells were washed three times by centrifugation in 3 volumes of PBS, and suspended to the same hematocrit in PBS that contained AAPH at an extracellular concentration of 63 m*M*. After incubation for the indicated times at 37°C, aliquots of mixed cells and buffer were taken for assay of α-tocopherol (upper panel, $n = 3$) and for hemolysis (lower panel, $n = 4$). Data for α-tocopherol are expressed as a fraction of the zero-time value of α-tocopherol, which was 5.6 ± 1 nmol/ml of erythrocytes.

such liposomes induce an oxidant stress in the cells, which in turn causes further lipid hydroperoxide formation in the liposomes (Kobayashi *et al.*, 1985; May *et al.*, 1998). As shown in Fig. 5, incubation of erythrocytes with hydroperoxide-containing liposomes resulted in a progressive increase in the lipid hydroperoxide content of the liposomes over about 40 min. When 7 μ*M* troglitazone was present in the incubation, the rise in lipid hydroperoxides in the liposomes was completely prevented. Troglitazone could be detected in the supernatant that contained the liposomes, and was decreased in the presence of cells (Fig. 6, upper panel), which could indicate either oxidative loss or loss from binding of troglitazone to the cells. The α-tocopherol content of erythrocytes was decreased to about 25% of control in the presence of liposomes, but this decrease was completely prevented by initial concentrations of troglitazone of 3.5 μ*M* and higher (Fig. 6, middle panel). The average baseline erythrocyte α-tocopherol concentration was about half that found in the cells from the experiments of Fig. 4, due to the use of different donors. At 3.5 μ*M* troglitazone, only trace amounts of the agent could be detected in the liposomal supernatant from cell incubations (Fig. 6, upper panel). Changes in lipid hydroperoxides in the liposomes correlated with the loss of α-tocopherol in the cells, and began

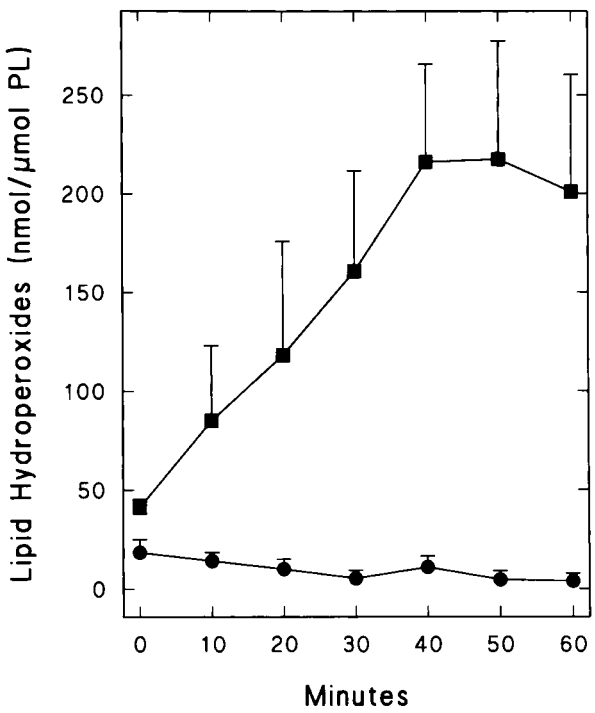


FIG. 5. Prevention of lipid hydroperoxide formation by troglitazone in liposomes incubated with erythrocytes. Erythrocytes at a 20% hematocrit were incubated at 37°C in PBS with phospholipid liposomes (0.65 μmol phosphatidylcholine/ml) and 5 m*M* glucose in the absence (■) or presence (●) of 7 μ*M* troglitazone. At the indicated times, aliquots of the mixed cells and liposomes were removed and centrifuged in the microfuge to pellet the cells. Portions of the supernatant were assayed for lipid hydroperoxides in the liposomes (PL). Data are shown as mean + SE from three experiments.

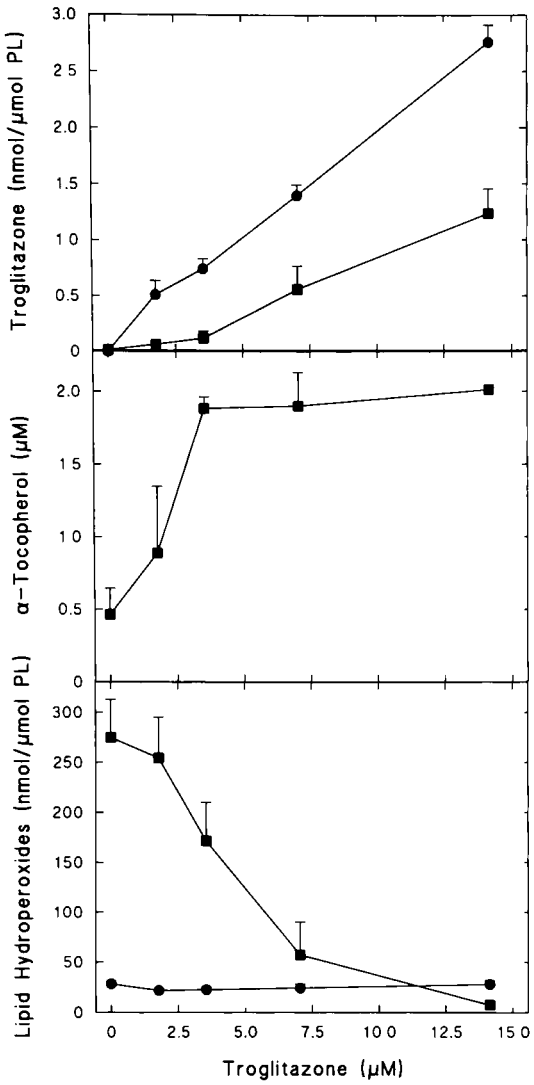


FIG. 6. Effects on increasing amounts of troglitazone on erythrocyte-induced liposome peroxidation. Liposomes (0.65 μmol phosphatidylcholine/ml) were incubated at 37°C in PBS that contained 5 m*M* D-glucose and the indicated concentration of troglitazone in the absence (●) or presence (■) of erythrocytes at a 20% hematocrit. After 1 hr, the cells were pelleted in a microfuge and assayed for their content of α-tocopherol (middle panel). Aliquots of the supernatant were taken for assay of troglitazone (upper panel) and lipid hydroperoxides (lower panel). Data are shown as mean + SE from three experiments for α-tocopherol, five experiments for troglitazone, and eight experiments for lipid hydroperoxides.

to decrease at concentrations of troglitazone of 3.5 μ*M* and higher. The half-maximal troglitazone concentration for this protection was 4 μ*M*. No increase in lipid hydroperoxides occurred in liposomes incubated without cells (Fig. 6, lower panel). Inclusion of dimethylsulfoxide at the concentrations present in the experiments of Fig. 6 was without effect on the loss of α-tocopherol in the cells or rise in lipid hydroperoxides in the liposomes (results not shown). Thus, the presence of troglitazone the incubation containing cells and liposomes protected cellular α-tocopherol, and this was associated with a concomitant decrease in formation of lipid hydroperoxides in the liposomes.

DISCUSSION

The erythrocyte provides a good model for testing effects of antioxidants in blood, because it is exposed to the same concentrations of an oxidant as plasma and LDL, and because it is likely to be more than an innocent bystander in the peroxidative damage associated with the atherosclerotic process. Regarding the latter, should the erythrocyte undergo lysis and release of hemin, it could act as a pro-oxidant with regard to LDL (Miller *et al.*, 1995). On the other hand, intact erythrocytes can protect against oxidant damage. For example, these cells rapidly recycle dehydroascorbic acid, the two-electron-oxidized form of ascorbate to ascorbate (Mendiratta *et al.*, 1998), and thus may help maintain plasma and interstitial concentrations of the vitamin. Ascorbate can in turn regenerate oxidized α-tocopherol in LDL (Maxwell and Lip, 1997) and in erythrocytes (May *et al.*, 1998). Resistance of LDL to oxidation should retard deposition of oxidized LDL in the atherosclerotic lesion, and maintenance of α-tocopherol in the erythrocyte membrane will prevent hemolysis in areas of high oxidant stress (Horwitt *et al.*, 1968; Bieri *et al.*, 1976; Mino *et al.*, 1978). By analogy with ascorbate, an agent that spares α-tocopherol and delays lipid hydroperoxide formation in the erythrocyte membrane therefore has the potential to retard the progression of oxidant-induced atherosclerosis. In this work, we show for the first time that the antidiabetic agent troglitazone has these attributes.

Over the concentration range of 4–57 μ*M* (2–25 μg/ml), troglitazone delayed or prevented several aspects of lipid peroxidative damage in human erythrocytes and their ghosts. Troglitazone bound to the human erythrocyte cell membrane, despite dilutions

and centrifugation washes of the ghosts (Fig. 2). These observations are in accord with the results of studies in which incubation of troglitazone in plasma resulted in LDL prepared from plasma that contained troglitazone (Cominacini *et al.*, 1997a). In the face of an oxidant stress induced by the free radical initiator AAPH, enough troglitazone remained bound to the washed cells to retard loss of α-tocopherol (Fig. 4) and enough was retained by washed ghosts to both spare α-tocopherol and delay the appearance of lipid hydroperoxides (Fig. 3).

Troglitazone also reduced the oxidative interaction between human erythrocytes and liposomes that contained small amounts of preformed lipid hydroperoxides, a model designed to reflect what erythrocytes might naturally encounter in areas of oxidant stress in the vascular bed. Although the mechanism by which liposomes exert an oxidant stress on erythrocytes has not been established with certainty, it clearly depends on the presence of lipid hydroperoxides in the liposomes (Kobayashi *et al.*, 1985; May *et al.*, 1998). Thus, neither LDL containing endogenous α-tocopherol nor liposomes prepared to contain exogenous α-tocopherol induce an oxidant stress in erythrocytes (Mendiratta *et al.*, 1998). In the present studies, protection against oxidative loss of α-tocopherol in erythrocytes and decreased lipid hydroperoxide formation in liposomes was observed at initial troglitazone concentrations as low as 2–4 μM (Fig. 6). Measured maximum plasma concentrations of troglitazone are from 2 to 6 μM in subjects taking 400 mg of the drug each day (Loi *et al.*, 1997). Whereas troglitazone in plasma is largely bound to albumin (Shibukawa *et al.*, 1995) and thus poorly accessible, our finding that the drug incorporates into the erythrocyte membrane (Fig. 2) could allow it to function as an antioxidant in the manner of α-tocopherol.

The present results extend to cells and cell membranes the findings of previous studies, which showed that the same concentration range of troglitazone protected human LDL from Cu^{2+}-induced oxidation (Cominacini *et al.*, 1997a). The latter findings have also been confirmed in LDL from normal volunteers (Cominacini *et al.*, 1997b), obese subjects (Tack *et al.*, 1998), and diabetics (Cominacini *et al.*, 1998) taking therapeutic amounts of the drug. The ability of troglitazone both to spare erythrocyte α-tocopherol and to lessen the oxidant interaction of erythrocytes and hydroperoxide-containing liposomes may predict its effects *in vivo* when erythrocytes are exposed to lipid hydroperoxide-containing LDL in areas of oxidant damage.

ACKNOWLEDGMENT

This work was supported by a grant-in-aid from Parke-Davis, Warner-Lambert.

ABBREVIATIONS

AAPH, 2,2′-Azobis(amidinopropane)dihydrochloride; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; PBS, phosphate-buffered saline; PPAR-γ, peroxisome proliferator activated receptor-γ.

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Received for publication December 20, 1999; accepted February 13, 2000.

This article has been cited by:

1. Samuel Rahbar, James L Figarola. 2003. Novel inhibitors of advanced glycation endproducts. *Archives of Biochemistry and Biophysics* **419**:1, 63-79. [CrossRef]