Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 307–313
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effects of troglitazone on intracellular cholesterol distribution and cholesterol-dependent cell functions in MA-10 Leydig tumor cells

Dale A. Freeman*, Angela Romero

Department of Internal Medicine, University of Louisville School of Medicine and Department of Veterans Affairs Medical Center (11), 800 Zorn Avenue, Louisville, KY 40206, USA

Received 3 December 2002; accepted 21 March 2003

Abstract

Troglitazone treatment of MA-10 Leydig tumor cells resulted in cellular cholesteryl esters decreasing and cell free cholesterol increasing. This was not an effect unique to this chemical entity; rosiglitazone and pioglitazone caused these changes also. The excess free cholesterol was recovered largely in the cholesterol oxidase susceptible, plasma membrane cholesterol pool. This effect of troglitazone probably is not mediated by activation of peroxisome proliferator activated receptors since it immediately reversed with washing and did not occur at all in cells treated with the peroxisome proliferator activated receptor agonist, 15-deoxy Δ 12,14 prostaglandin J-2. Plasma membrane cholesterol esterification was inhibited by troglitazone in a dose-dependent manner. Plasma membrane cholesterol esterification was inhibited half-maximally by 14 μM troglitazone and by more than 90% by 40 μM troglitazone. This effect was not unique for MA-10 cells. Similar results were found using fibroblasts. Troglitazone was not simply inhibiting internalization of plasma membrane cholesterol. Dibutyryl-cAMP stimulation of troglitazone-treated cells resulted in more progesterone synthesis than in stimulated control cells; moreover, radioactive plasma membrane cholesterol was readily converted into progesterone in troglitazone-treated cells. Studies of LDL uptake in troglitazone-treated cells indicated that intracellular membranes were cholesterol replete. Troglitazone inhibited plasma membrane cholesterol esterification with kinetics similar to 58-035, a known inhibitor of the acyl coenzyme A: cholesterol acyltranserase (ACAT) enzyme. It is not likely an ACAT inhibitor since troglitazone did not block incorporation of exogenous free fatty acids into cholesteryl esters. Thus, it appears that troglitazone prevented presentation of free fatty acid to the ACAT enzyme.

Keywords: Troglitazone; MA-10 Leydig tumor cells; Cellular cholesteryl esters; Thiazolidinedione; Cholesterol metabolism

1. Introduction

Thiazolidinediones are an important class of drugs that have found utility for treating patients with diabetes and insulin resistance. This class of drugs acts by improving cellular sensitivity to insulin. These agents also are agonists for the peroxisome proliferator activated receptors (PPARs). It is thought that PPAR effects underlie the beneficial effects of the thiazolidinediones.

Some effects of thiazolidinediones may not be identical for all drugs either because different drugs activate the several PPARs to different extents or because some drug effects are mediated totally independently of the PPAR system. Example of the first type of differentiated regulation has been demonstrated for the PPARγ activation by troglitazone and rosiglitazone [1]. The second type of effect has been shown to occur in troglitazone-treated cells. Troglitazone, but not rosiglitazone, pioglitazone, or 15-deoxy Δ 12,14 prostaglandin J-2, inhibits *de novo* synthesis of cholesterol in several cell types [2]. This effect has a very rapid onset of action, is readily reversed, and only caused by certain thiazolidinediones.

Effects that differ between thiazolidinediones may prove to be quite important. An effect on cholesterol synthesis, for example, might provide an important hypolipidemic effect. The pattern of PPAR activation may ultimately prove important in determining the therapeutic value of

* Corresponding author. Tel.: +1-502-894-6203; fax: +1-502-894-6225.
E-mail address: dale.freeman@med.va.gov (D.A. Freeman).

Abbreviations: PPAR, peroxisome proliferator activated receptor; LDL, low density lipoprotein; ACAT, acyl CoA: cholesterol acyltransferase; SREBP, sterol regulatory element binding protein.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00234-X

a drug or may be the cause of toxicity of individual drugs. These unique and/or PPAR-independent drug effects may provide a basis for choosing one drug over another in that some drugs may possess valuable anti-atherogenic effects or greater toxicities than other drugs.

In the present studies, we investigated the underlying mechanisms and consequences of an apparently PPAR-independent effect of troglitazone. Troglitazone or other thiazolidinediones blocked cholesterol esterification in MA-10 Leydig tumor cells and in human fibroblasts. Free cholesterol accumulated mainly in the plasma membrane of treated cells. Troglitazone was not preventing internalization of plasma membrane cholesterol since steroid hormone synthesis was not inhibited and LDL uptake was downregulated indicating that troglitazone produced cholesterol flooding of internal membranes. Troglitazone prevented cholesterol esterification by sequestration of free fatty acid used for cholesterol esterification.

2. Material and methods

2.1. Materials

[1,2-^{3}H]Cholesterol (65 Ci/mmol and [1,2,6,7-^{3}H]pro- Ci/mmol) were from Dupont/NEN. 58-035 was a gift of John Heider of Sandoz, Inc. Troglitazone was a gift of Parke-Davis, rosiglitazone a gift from SmithKline Beecham. 15-Deoxy Δ 12,14 prostaglandin J-2 was purchased from Cayman Biochemical. Cholesterol, 7-ketocholesterol, cholesteryl oleate, cholestenone, cholesteryl butyrate, and progesterone were purchased from Steroiloids. Cholesterol oxidase was purchased from Bechman Instruments. Dibutyryl-cAMP was purchased from Sigma.

2.2. Methods

2.2.1. Cells and general experimental protocols

Growth conditions for MA-10 cells were the same as described previously [3]. Experiments were performed in assay medium which was growth medium without serum but containing 1 mg/mL BSA. Thiazolidinediones were added as 200-fold concentrated solutions in DMSO while 58-035 was added as a 200-fold concentrate in ethanol. Plasma membrane cholesterol was radiolabeled by exposing cells to assay medium containing [^{3}H]chol- for 2 hr after which the dishes were washed and placed back in assay medium without radioactivity but containing any experimental additions. Cell surface cholesterol was converted to cholestenone by treatment of cell monolayers with cholesterol oxidase following exactly the protocols described previously [4]. Decay of preformed cholesteryl esters was measured in cells preincubated overnight with 50 μg/mL LDL and [^{3}H]cholesterol and then washed and incubated with 58-035 and/or other additions.

2.2.2. Lipid methods

Cell monolayers were scraped from dishes and the lipids extracted as described earlier [5]. Radioactivity associated with cholesterol and cholesteryl esters was quantified by liquid scintillation counting of the area of thin layer chromatography sheets developed in heptane:diethyl ether:acetic acid 85:15:1 [5]. Radioactivity present in the medium as progesterone was quantified after separation from cholesterol radioactivity as described previously [6]. Quantification of cholesterol, cholestenone, cholesteryl ester, and triglyceride mass was by gas–liquid chromatography using cholesteryl butyrate as an internal recovery standard and using the columns and methods employed previously [7].

2.3. Other methods

Esterification of exogenous free fatty acids made up using [^{14}C]oleate bound to BSA prepared as described by Van Harkin [8]. Progesterone was quantified by specific radioimmunoassay of unextracted assay medium [3].

Statistical significance was determined by *t*-test using the GraphPad Prism version 1 Program.

3. Results

3.1. Troglitazone causes cellular cholesteryl ester content to decrease and cellular free cholesterol levels to increase

Troglitazone blocks *de novo* synthesis of cholesterol in several cell types [2]. In initial experiments, we found that total cholesterol was decreased little to none by troglitazone treatment, but that the distribution of cholesterol was markedly altered. The data of Table 1 show experiments where troglitazone was added to MA-10 cells or to MA-10 cells that had been preincubated with LDL to augment the normally small stores of cholesteryl esters found in these cells [4]. Since basal MA-10 cells have such small stores of cholesteryl esters, the ester-augmented cells were used so that it would be possible to determine if the cholesteryl esters lost were recovered as cell free cholesterol. In basal MA-10 cells troglitazone treatment caused cell cholesteryl ester levels to fall from 8.8 to 3.2 μg/dish or from 22.3% of total cholesterol to 7.2% of total cholesterol. Free cholesterol levels increased slightly but not significantly. The cells preincubated with LDL had markedly greater cell content of total cholesterol mainly because cholesteryl esters had increased more than 3.4-fold above basal. In these cells, ester cholesterol decreased from 30.3 to 18.4 μg/dish with troglitazone treatment while cell free cholesterol increased from 40.5 to 47.7 μg/dish; all changes were statistically significant. In this experiment, the molar loss of cholesteryl ester is quantitatively recovered as cell free cholesterol.

Table 1
Effect of troglitazone on cellular mass of cholesterol and cholesteryl esters[a]

Additions	Free cholesterol (μg/dish)	Cholesteryl esters (μg/dish)	Total (μg/dish)	Percent cholesteryl esters
None	33.8 ± 4.1	8.8 ± 3.0	42.1 ± 7.0	22.3 ± 4.0
20 μM troglitazone	37.2 ± 8.5	3.2 ± 1.1*	43.8 ± 5.8	7.2 ± 1.6*
50 μg/mL LDL	40.5 ± 1.2	30.3 ± 3.0	70.7 ± 1.3	42.8 ± 2.3
50 μg/mL LDL + 20 μM troglitazone	47.7 ± 1.4**	18.4 ± 5.2**	66.1 ± 4.9	27.5 ± 5.5***

[a] 60 × 15 mm dishes of MA-10 cells were incubated for 8 hr in serum free defined medium containing either troglitazone added from a 200-fold concentrated solution in DMSO or the same volume of DMSO alone. Other dishes were incubated with 50 μg/mL LDL cholesterol as well. After 4 hr dishes were washed twice with assay medium and the cells scraped from the dishes and extracted for analysis of lipid mass. Mass was determined by gas–liquid chromatography using cholesteryl butyrate as an internal recovery standard.

* Significantly different from control at $P < 0.04$.

** Significantly different than control at $P < 0.03$.

*** Significantly different from control at $P < 0.02$.

3.2. *Free cholesterol derived from cholesteryl esters of troglitazone-treated cells is largely recovered as plasma membrane free cholesterol*

Most cells store the bulk of the cholesterol in the plasma membrane [9]. In MA-10 cells, about 60–70% of total cell free cholesterol is found in this membrane [4]. Since troglitazone appeared to have caused a substantial shift of cholesterol within the cell, it was of interest to determine where the cholesterol derived from the cholesteryl esters would be stored in the cells. The data of Table 2 show experiments where cholesteryl oxidase was used to differentiate cell surface cholesterol from intracellular cholesterol. Treatment of the cells with this enzyme converts the plasma membrane cholesterol to cholestenone. In cells with basal levels of cholesteryl esters, troglitazone caused significant reduction in the percentage of total cholesterol in cholesteryl ester (20.4–6.2%) and small and statistically insignificant increases in the percentage of both intracellular cholesterol and cell surface cholestenone. In cholesteryl ester-loaded cells, troglitazone caused the percent cholesterol present as ester to decrease significantly from 45.5 to 31.7% and also caused a significant increase in the percentage of cholesterol recovered in the plasma membrane from 41.1 to 57% of total cell cholesterol. To determine whether the endoplasmic reticulum, the major intracellular membrane, also became cholesterol loaded in troglitazone-treated cells, the experiments of Table 3 were performed. These experiments use LDL cholesterol uptake as a measure of endoplasmic reticulum cholesterol content. LDL uptake is directly proportional to LDL receptor expression which is in turn regulated by the endoplasmic reticulum cholesterol sensing proteins, the sterol regulatory element binding proteins [10]. Cholesterol loading of the endoplasmic reticulum should downregulate LDL receptors and prevent uptake of LDL cholesterol. In these experiments, cells inhibited with 7-ketocholesterol and cholesterol were used as a positive control since it is

Table 2
Effect of troglitazone on cellular distribution of cholesterol and content of cholesteryl esters[a]

Additions	Percentage of total cell cholesterol		
	Cholesterol	Cholestenone	Cholesteryl esters
None	15.2 ± 5.7	64.3 ± 6.7	20.4 ± 1.3
20 μM troglitazone	22.0 ± 3.7	71.7 ± 4.5	6.2 ± 1.2**
50 μg/mL LDL	14.1 ± 3.5	41.1 ± 6.9	45.5 ± 3.2
50 μg/mL LDL + 20 μM troglitazone	11.7 ± 5.0	57.0 ± 5.3*	31.7 ± 3.8**

The percentage of cell surface cholestenone (*) is significantly different between control and troglitazone-treated cells at $P < 0.04$. The difference in the percentage of cholesterol as cholesteryl esters (**) is significantly different between control and troglitazone at $P < 0.008$.

[a] 60 × 15 mm dishes of MA-10 cells were incubated for 8 hr in serum free defined medium containing the indicated concentrations of troglitazone and/or LDL. At $t = 8$ hr, all dishes were washed and treated with cholesterol oxidase to convert cell surface cholesterol to cholestenone. After extraction of cellular lipids the cell content of cholesterol, cholestenone, and cholesteryl esters was determined by gas–liquid chromatography using cholesteryl butyrate as an internal recovery standard.

Table 3
Effect of 7-ketocholesterol and troglitazone on LDL cholesterol uptake[a]

Treatment	LDL	Total cholesterol (μg/dish)	
		Experiment 1	Experiment 2
Control	−	24.8 ± 4.6	16.1 ± 0.7
	+	43.3 ± 10.8 (1.8×)*	28.1 ± 5.4 (1.7×)*
7-Ketocholesterol	−	29.1 ± 3.8	16.4 ± 1.7
	+	31.1 ± 7.9 (1.1×)	24.7 ± 2.0 (1.5×)*
Troglitazone	−	22.1 ± 2.3	15.7 ± 2.4
	+	22.4 ± 1.8 (1.0×)**	19.1 ± 1.0 (1.2×)**

[a] 60 × 15 mm dishes of cells were incubated for 24 hr in complete growth medium alone, with 5 μg/mL 7-ketocholesterol + 12 μg/mL cholesterol or with 40 μM troglitazone. At this time the dishes were washed and placed in new assay medium (no serum but 1 mg/mL BSA) containing 10 μg/mL of LDL. After 8 hr at 37°, the dishes were washed twice, cells were scraped from the plate, and the cellular lipid extracted. The cell content of cholesterol (free + esterified cholesterol) was determined using gas–liquid chromatography with cholesteryl butyrate as an internal recovery standard.

* $P < 0.05$ more than control minus LDL.

** $P < 0.05$ less than control plus LDL.

known that this treatment will downregulate LDL receptor in these [11] and other cells [12]. Incubating control cultures with LDL resulted in a 1.8-fold increase in total cell cholesterol in the first experiment and a 1.7-fold increase in the second experiment. Treatment with 7-ketocholesterol decreased LDL uptake to 1.1- and 1.5-fold control values while troglitazone treatment decreased LDL uptake to either control values in one experiment and to 1.2-fold control values in the second experiment. All of these experiments had been done over an 8-hr time course indicating that this effect of troglitazone was rapid. To get an idea whether this was an unusually rapid genomic effect mediated by PPARs or a PPAR-independent effect, two types of experiments were done.

3.3. The effect of troglitazone on intracellular cholesterol distribution does not persist after troglitazone is removed and cannot be duplicated by a non-thiazolidinedione PPAR agonist

The data of Table 4 show experiments where cells preincubated for 24 hr with troglitazone and cells without any preincubations were subsequently washed and then incubated with or without troglitazone. In the second incubation, LDL was included in all groups so that changes in cell cholesterol distribution would be more apparent. Preincubation of cells with troglitazone did not cause a persistent effect after troglitazone was removed; the percent distribution of cholesterol is very similar to cells without troglitazone preincubation. Preincubation also did not reduce the effect of troglitazone in the second incubation. Thus, cells without any preincubation or with troglitazone in the preincubation both showed similar reduction in percent cell cholesterol as cholesteryl esters and increases in percent cell cholesterol as cell surface cholesterol. In both cases, cholesteryl esters fell to 24–25.3% while cholestenone increased from 51.4 to 53.1% of total cell cholesterol. In general, it would be surprising if a drug acting through a transcriptional regulator, such as the PPAR, responded so rapidly or turned off so quickly as was found in these experiments of cholesterol distribution. 15-Deoxy Δ 12,14 prostaglandin-J2 is a potent PPAR agonist but structurally unrelated to troglitazone. This agent, at concentrations up to 100 μM, had no effect on cell cholesterol distribution (data not shown).

Table 4
Troglitazone must be present to alter the distribution of cholesterol in cells; there is no residual effect from previous troglitazone incubation[a]

Preincubations	Additions	Percentage of total cell cholesterol		
		Cholesterol	Cholestenone	Cholesteryl esters
None	None	20.8 ± 1.3	30.6 ± 2.5	48.7 ± 4.2
None	20 μM troglitazone	21.2 ± 2.0	53.1 ± 13.2**	25.3 ± 12.9*
20 μM troglitazone	None	24.8 ± 8.3	33.5 ± 5.5	42.2 ± 6.0
20 μM troglitazone	20 μM troglitazone	23.2 ± 2.2	51.4 ± 2.6**	24.0 ± 3.7*

The percentage of cell cholesterol identified as cholesteryl esters (*) as well as that identified as cell surface cholestenone (**) was significantly different, $P < 0.05$ in either incubation containing troglitazone compared with control incubations. Preincubation of cells with troglitazone had no effect on any measured parameter (compare group 1 with group 3 and group 2 with group 4).

[a] 60 × 15 mm dishes of MA-10 cells were incubated for 24 hr alone ("None" preincubations) or with troglitazone. After this incubation the medium was removed from all cells, the cells were then washed twice with defined medium, after which defined medium containing 50 μg/mL of LDL cholesterol and either no troglitazone or troglitazone as indicated. After 8 hr at 37°, the medium was again removed, the cells treated with cholesterol oxidase to convert cell surface cholesterol into cholestenone. The cellular lipids were extracted from the cells and quantitated by gas–liquid chromatography using cholesteryl butyrate as an internal recovery standard.

3.4. Troglitazone blocks esterification of plasma membrane cholesterol

Plasma membrane cholesterol cycles into the cell and is esterified in several cell types [13–15]. The data of Table 5 show experiments where the effect of troglitazone to alter this process was measured. Cells were plasma membrane cholesterol labeled at 4° and then allowed to take up and esterify cell surface cholesterol at 37° in either the absence or presence of LDL. As expected from earlier experiments, addition of LDL caused more plasma membrane radioactivity to be esterified, 27.6% vs. 14.1% for cells incubated

Table 5
Effect of troglitazone on incorporation of plasma membrane cholesterol radioactivity into cholesterol esters[a]

Additions	DPM in cholesterol	DPM in cholesteryl esters	Percent cholesteryl esters	Percent inhibition
None	21568 ± 1835	3531 ± 423	14.1 ± 0.5	
20 μM troglitazone	20518 ± 885	1416 ± 467	6.6 ± 2.0*	47
LDL 50 μg/mL	16444 ± 2396	6245 ± 552	27.6 ± 1.6**	
LDL 50 μg/mL + 20 μm troglitazone	22008 ± 3580	3868 ± 1045	15.2 ± 5.2*	55

Troglitazone treatment (*) is significantly different than control at $P < 0.02$. LDL treatment (**) is significantly different than no LDL at $P < 0.002$.

[a] 30 × 15 mm of MA-10 cells were placed on ice, washed twice with 4° serum free assay medium, and then incubated for 4 hr with 1 mL of assay medium containing 0.5 μCi [^{3}H]cholesterol. At this time the dishes of cells were washed twice with 37° assay medium after which the medium was replaced with assay medium containing the indicated additions. After 4 hr at 37°, the dishes were again washed twice and the cells scraped from the dish. Lipids were extracted and free cholesterol radioactivity separated from esterified cholesterol radioactivity by thin layer chromatography. Radioactivity was quantitated by scintillation counting of the areas of the TLC plate that corresponded to cholesterol and to cholesteryl esters.

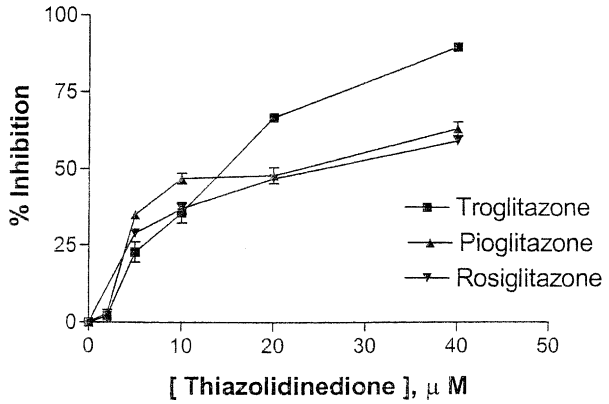

Fig. 1. Cells were incubated and treated exactly as described in Table 5 with the exception that they received the indicated concentration rather than the single concentration used in the table. Percentage inhibition is % cell cholestrol as ester in troglitazone-treated cells/% cholestrol as ester in control cells.

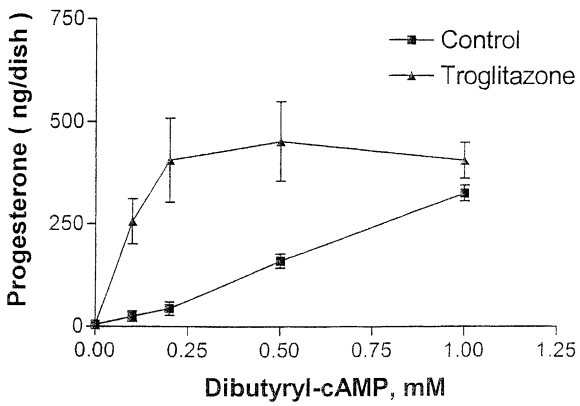

Fig. 2. Effect of troglitazone on cAMP-stimulated steroidogenesis. 30 × 15 cm dishes of cells without troglitazone or containing 40 μM triglitazone were incubated with the indicated concentration of dibutyryl-cAMP for 4 hr. At this time, medium was removed and assayed for progesterone content by radioimmunoassay.

without lipoproteins. Troglitazone, in either case, significantly inhibited plasma membrane cholesterol incorporation into cholesteryl esters. For cells incubated without lipoproteins, the incorporation was inhibited to 47% of control while for cells incubated with lipoproteins, the inhibition was to 55% of control. To confirm that the results were not a unique or unusual effect specific to MA-10 cells, experiments were performed with human fibroblasts as well. Plasma membrane cholesterol incorporation into intracellular esters was inhibited 93–100% by 40 μM troglitazone. A dose–response curve plotting inhibition as a function of troglitazone concentration is shown in Fig. 1. Inhibition to 50% was achieved with 14 μM troglitazone while incubation with 40 μM troglitazone inhibited esterification of plasma membrane cholesterol by 90%. Two other thiazolidinediones were tested as well, pioglitazone and rosiglitazone. Both compounds inhibited cholesterol incorporation into cholesteryl esters in a dose-dependent manner. These results might be explained by an effect of troglitazone to prevent plasma membrane internalization, transport through the cell, or esterification.

3.5. Steroidogenesis and the mobilization of steroidogenic plasma membrane cholesterone are not blocked by troglitazone

In MA-10 cells, most cholesterol substrate for short-term steroid hormone synthesis comes from free cholesterol stores [5]. The bulk of free cholesterol in MA-10 cells [4] as in all cells is in the plasma membrane [9]. It was reasoned then that if troglitazone treatment blocked internalization of plasma membrane cholesterol then it should significantly inhibit dibutyryl-cAMP-stimulated progesterone synthesis by the MA-10 cells. The data of Fig. 2 indicate that this clearly is not the case. In these experiments, MA-10 cells were stimulated by increasing concentrations of dibutyryl-cAMP up to the saturating concentration of 1 mM. Troglitazone-treated cells actually are much more sensitive to the cAMP analogue and achieve maximal activation of steroid synthesis at 0.2 mM vs. 1 mM in the control cells. Maximal activation is also slightly greater in troglitazone-treated than control cells. These experiments provided indirect evidence that plasma membrane cholesterol readily entered troglitazone-treated cells. To determine if this was in fact the case, the experiments of Fig. 3 were done. In these experiments, plasma membrane cholesterol was radiolabeled at 4°, and then the cells were washed and stimulated by dibutyryl-cAMP. After 2 and 4 hr, medium was removed and saved for determination of progesterone radioactivity while the cell monolayer was scraped from the dish and extracted for determination of cholesterol radioactivity. Control and

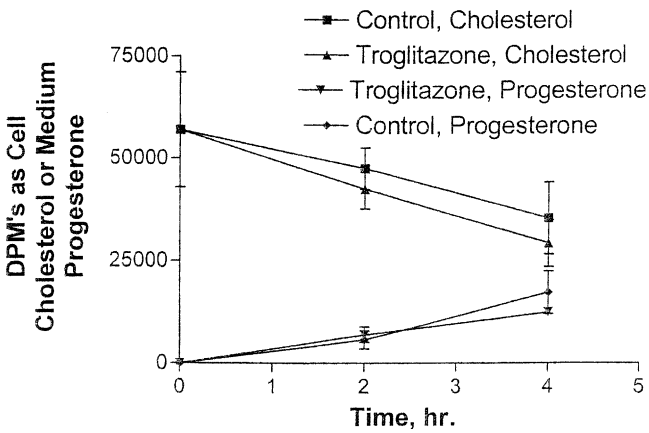

Fig. 3. Effect of troglitazone on utilization of plasma membrane cholesterol for steroid hormone synthesis. 60 × 15 mm dishes of cells were placed on ice, washed twice with 4° assay medium and then incubated for 2 hr at 4° with medium containing 1 μC [^{3}H]cholesterol. After this incubation, the cells were washed twice and placed back in medium containing 1 mM dibutyryl-cAMP alone or with dibutyryl-cAMP and 40 μM troglitazone. After 4 h at 37°, medium was collected and cells were scraped from the dish. Progesterone radioactivity was separated from other medium radioactivity by thin layer chromatography. Cholesterol radioactivity was separated from other lipid radioactivity by thin layer chromatography developed in heptane:diethyl ether:acetic acid 85:15:1.

troglitazone-treated cells were treated identically. In both groups, there is a time-dependent fall in cell free cholesterol activity and an increase in progesterone radioactivity in the medium. Progesterone had been purified from cholesterol and other steroids by thin layer chromatography so that the radioactivity appearing as progesterone was less than the radioactivity lost as cholesterol. This was not too surprising since some free cholesterol was lost to the medium and because progesterone is not the only steroid hormone product of MA-10 cells. Total medium radioactivity very closely matched cholesterol radioactivity lost. Most importantly, however, a defined steroid hormone product was clearly synthesized from plasma membrane cholesterol substrate indicating that cell surface cholesterol did enter the cell in troglitazone-treated cells.

3.6. Effect of troglitazone on the ACAT

Several steps may be required before a cholesterol molecule located in the plasma membrane is esterified. At minimum, plasma membrane must be (a) internalized into the cell, (b) moved through the cell, (c) delivered to the endoplasmic reticulum, (d) presented to the ACAT enzyme, (e) esterified, after which the cholsteryl ester is (f) transported to the lipid droplet. From the experiments above, it appeared that troglitazone was not inhibiting proximal steps. Another step that could be troglitazone sensitive and is relatively amendable to study is the ACAT reaction itself at the far distal end of the cholesterol internalization esterification pathways. The experiments of Fig. 4 compare the effects of 58-035, a well characterized ACAT inhibitor, and troglitazone on the cellular stores of cholesteryl esters. In cells incubated without inhibitors, cell stores of cholesteryl ester remained constant throughout the 8 hr of the experiment. With ACAT blocked by 58-035 or when cells were incubated with troglitazone, the cell content of cholesteryl esters decreased over the 8 hr so that about

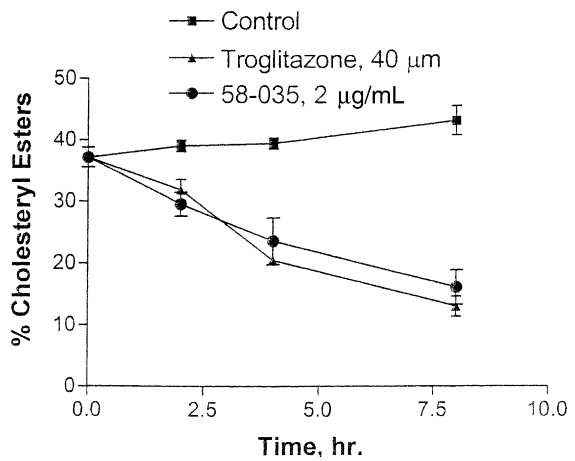

Fig. 4. Effect of 58-035 or troglitazone on cell cholesteryl ester stores. At $t = 0$ cells were placed in assay medium alone, containing 2 μg/mL 58-035 or containing 40 μM troglitazone. At the times indicated, cells were harvested, extracted, and the lipids quantified by gas–liquid chromatography.

Table 6
Troglitazone does not block the incorporation of oleic acid into cholesteryl esters[a]

Additions	Oleate incorporation into cholesteryl oleate (pmol/dish/hr)		
	Experiment 1	Experiment 2	Experiment 3
None	423 ± 42	315 ± 23	496 ± 25
20 μM troglitazone	483 ± 54	350 ± 68	461 ± 79
50 μg/mL LDL			1010 ± 206*
50 μg/mL LDL + 20 μM troglitazone			1153 ± 49*

[a] 60 × 15 mm dishes of MA-10 cells were washed twice with defined assay medium and then incubated with assay medium containing the indicated additions. After 2 hr at 37°, 400 nmol [^{14}C]oleate/BSA suspension was added and the incubation continued for another 2 hr. At this time the cells were scraped from the dish and the lipids extracted. Cholesteryl esters were separated from other lipids by thin layer chromatography developed with heptane:diethyl ether:acetic acid 85:15:1. Radioactivity corresponding to cholesteryl esters was quantitated by liquid scintillation counting. There was no significant difference in fatty acid incorporation into cholesteryl esters between cells incubated with or without troglitazone; however, incubation of cells with LDL caused the expected statistically significant.

* $P < 0.05$, increase in cholesterol esterification.

one half of cholesteryl esters were lost during the experiment. To determine whether troglitazone caused inhibition of the ACAT enzyme, the experiments of Table 6 were performed. In these experiments, cells were incubated with [^{14}C]oleate/BSA and allowed to synthesize radioactive cholesteryl esters. As expected, cells incubated with LDL had more cholesterol available for esterification and synthesized greater quantities of [^{14}C]cholesteryl oelate. Adding troglitazone either to control or LDL-treated cells had no effect on the amount of esters synthesized. Thus, when exogenous free fatty acid substrate was made available to the cell, the cell readily esterified cholesterol. This finding raised the possibility that troglitazone treatment was limiting the availability of the cell to provide free fatty acid substrate to the ACAT. In experiments supplying free fatty acid as oleate/BSA complexes, it was found that cholesterol esterification increased in both control and troglitazone-treated cells, but that free fatty acid treatment could not totally bypass the troglitazone block.

4. Discussion

The present experiments indicate that troglitazone and other thiazolidinediones inhibit esterification of cholesterol in MA-10 cells and in fibroblasts. This effect is likely PPAR independent since it is rapidly inducible, readily and rapidly reversed, and is not caused by a chemically distinct PPAR agonist, 15-deoxy Δ 12,14 prostaglandin J-2.

Three explanations may explain the effect of troglitazone on cholesteryl ester storage. Troglitazone could conceivably block cholesterol esterification by: (1) blocking cholesterol transport into the cell or movement to the site of

the ACAT enzyme, (2) by blocking the ACAT enzyme itself, or (3) by depriving the enzyme of essential substrate. The first possibility seems very unlikely since plasma membrane cholesterol was readily internalized and converted to steroid hormones and because even the endoplasmic reticulum membranes became cholesterol replete in troglitazone-treated cells. The second explanation seems unlikely as well. Measuring ACAT activity by quantifying incorporation of exogenous free fatty acid into cholesteryl esters showed no effect of troglitazone. Since the enzyme worked normally when supplied with exogenous fatty acid but did not function using the endogenous fatty acids present in troglitazone-treated cells, it seems that troglitazone deprives the ACAT enzyme of endogenous free fatty acid.

At least in the time range employed for the present studies, blocking cholesterol esterification had significant cellular effects. Cell free cholesterol levels increased. Most excess free cholesterol was susceptible to cholesterol oxidase implying that it was at the cell surface. The reduction in LDL uptake, however, implied that the intracellular membranes containing the SREBP were also cholesterol overloaded. LDL uptake was more suppressed by troglitazone treatment than by treatment with oxysterols. Likely, the greater plasma membrane free cholesterol markedly altered the responsiveness to cAMP analogues and even increased the maximal steroid response. In this regard, the MA-10 cells behaved much differently from granulosa cells where troglitazone treatment inhibits steroidogenesis largely through an effect on the 3-B-hydroxysteroid dehydrogenase Δ5Δ4 isomerase enzyme [16].

One must wonder about the net effect of these changes on cell function. Do the effects in aggregate produce toxicity or are they beneficial? If cholesterol esterification was prevented in monocytes/macrophages, these drugs may inhibit foam cell formation and hence alter atherosclerosis. Other data using macrophage-like cells suggest that the effects could even be more complex with some PPAR-mediated effects favoring foam cell formation [17,18] and the effect described here inhibiting formation. It is also possible that the cholesterol filling of membranes might lead to changes in enzyme activities and alteration in other cell functions since clearly the cholesterol environment of membrane-bound enzymes has marked effects on activity [19,20].

References

[1] Camp HS, Li O, Wise SC, et al. Differential activation of peroxisome proliferation-activated receptor-γ by troglitazone and rosiglitazone. Diabetes 2000;49:539–47.

[2] Wang M, Wise SC, Leff T, Su T-Z. Troglitazone, and antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of perosixome proliferator activated receptor-γ. Diabetes 1999;48:254–60.

[3] Ascoli M. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. Endocrinology 1981;108:88–95.

[4] Freeman DA. Cyclic-AMP mediated modification of cholesterol traffic in Leydig tumor cells. J Biol Chem 1987;262:13061–8.

[5] Freeman DA, Ascoli M. Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig tumor cells. J Biol Chem 1982;257:14231–8.

[6] Nagy L, Freeman DA. Effect of cholesterol transport inhibitors on steroidogenesis and cholesterol transport in cultured Leydig tumor cells. Endocrinology 1990;126:2267–76.

[7] Freeman DA, Ontko JA. Accumulation and mobilization of triglycerides and cholesteryl esters in Leydig tumor cells. J Lipid Res 1992; 33:1139–46.

[8] Van Harkin DR, Dixon CW, Heinberg M. Hepatic lipid metabolism in experimental diabetes. J Biol Chem 1969;244:2278–86.

[9] Lange Y, Ramos BV. Analysis of the distribution of cholesterol in the intact cell. J Biol Chem 1983;258:15130–4.

[10] Wang X, Briggs MR, Hua X, Yokoyama C, Goldstein JL, Brown MS. Nuclear protein that binds sterol regulatory elements of low density lipoprotein receptor promoter π purification and characterization. J Biol Chem 1993;268:14497–504.

[11] Freeman DA, Ascoli M. The low-density lipoprotein pathway of cultured Leydig tumor cells: utilization of low-density lipoprotein cholesterol for steroidogenesis. Biochem Biophys Acta 1983;754: 72–81.

[12] Bates SR, Jeff CM, Miller JE. Prevention of the hyperlipidemic serum of LDL induced cellular cholesterol ester accumulation by 22-hydroxycholesterol and its analogue. Biochem Biophys Acta 1983;753: 281–93.

[13] Tabas I, Rosoft WJ, Boykow GC. Acyl coenzyme A: cholesterol acyl transferase in macrophages utilizes a cellular pool of cholesterol oxidase-accessible cholesterol as substrate. J Biol Chem 1988;263: 1266–72.

[14] Slotte JR, Bierman EL. Depletion of plasma membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular pools in cultured fibroblasts. Biochem J 1988; 250:653–8.

[15] Nagy L, Freeman DA. Cholesterol movement between the plasma membrane and cholesteryl ester droplet of cultured Leydig tumor cells. Biochem J 1990;271:809–14.

[16] Gasic S, Bodenburg Y, Nagamani M, et al. Troglitazone inhibits progesterone production in porcine granulosa cells. Endocrinology 1998;139:4962–6.

[17] Nagy L, Tontonoz P, Alvarez JGA, et al. Oxidized LDL regulates macrophage/gene expression through liquid activation of PPARγ. Cell 1998;93:229–40.

[18] Tontonoz P, Nagy L, Alvarez JGA, et al. PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 1998; 93:241–52.

[19] Yechiel E, Barenholz Y. Relationships between membrane lipid composition and biological properties of rat myocytes. J Biol Chem 1985;260:9123–31.

[20] Friedlander G, Shohedi M, LeGriemellec C, Amiel C. Increase in membrane fluidity and openings of tight junctions have similar effects on sodium-coupled uptakes in renal epithelial cells. J Biol Chem 1988;263:11183–8.