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Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2

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ABSTRACT

To elucidate the mechanisms underlying the cholesterol lowering effects of PPARα agonists we investigated key regulators of cholesterol synthesis and uptake in rats and in the rat hepatoma cell line Fao after treatment with the PPARα agonists clofibrate and WY 14,643, respectively. In rat liver as well as in Fao cells, PPARα activation led to a decrease of transcriptionally active nuclear SREBP-2. mRNA concentrations of the key regulators of SREBP processing, Insig-1 in rat liver and Insig-1 and Insig-2a in Fao cells, were increased upon PPARα activation. Thus we suggest, that the observed reduction of the amount of nuclear SREBP-2 was due to an inhibition of the processing of the precursor protein. Both, in rat liver and in Fao cells, mRNA concentrations of the SREBP-2 target genes HMG-CoA reductase (EC1.1.1.34) and LDL receptor were reduced after treatment with the PPARα agonists. Furthermore, treatment of Fao cells with WY 14,643 reduced cholesterol synthesis. As a result, the amount of total cholesterol in liver, plasma and lipoproteins of clofibrate treated rats and in WY 14,643 treated Fao cells was decreased compared to control animals and cells, respectively. In conclusion, we could show a novel link between PPARα and cholesterol metabolism by demonstrating that PPARα activation lowers cholesterol concentration by reducing the abundance of nuclear SREBP-2.

1. Introduction

In animal cells, lipid homeostasis is maintained by a feedback mechanism that regulates the transcription of genes involved in lipid synthesis and uptake. Transcription factors that regulate the coordinated expression of these genes are the family of sterol regulatory element binding proteins (SREBPs) [1]. Three isoforms of SREBP are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis and their incorporation into triacylglycerols and phospholipids, SREBP-2 preferentially activates the low density lipoprotein (LDL) receptor gene and various genes required for cholesterol synthesis such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC1.1.1.34) [2]. SREBP-1a is an activator of both, the cholesterol and fatty acid biosynthetic pathway, but it is present in much lower amounts in liver than the other two forms [3]. SREBPs are

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Abbreviations: ACO, acyl-CoA oxidase; Cyp, cytochrome P450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Insig, insulin-induced gene; LDL, low density lipoprotein; PPAR, peroxisome proliferator-activated receptor; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element binding protein; VLDL, very low density lipoprotein

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synthesized as 120-kDa integral membrane proteins of the endoplasmic reticulum and form a complex with SREBP cleavage activating protein (SCAP). When sterol concentrations in cells are high, the SCAP/SREBP complex is retained in the ER. When cells are depleted of sterols, SCAP escorts SREBP to the Golgi for proteolytic processing. In the Golgi, sequential cleavages occur, releasing the mature N-terminal domain of SREBP that then translocates to the nucleus and activates transcription of sterol regulatory element-containing genes [2,4,5].

Retention of the SCAP/SREBP complex in the ER is mediated by sterol-dependent binding of the complex to one of two ER retention proteins designated insulin-induced gene (Insig)-1 and -2 [6,7]. Insig-1 and Insig-2 are integral membrane proteins that are expressed in most tissues with especially high expression in the liver [6,7]. Insig-1 differs from Insig-2 in its requirement of nuclear SREBPs for its expression providing a feedback mechanism for lipid homeostasis [7]. Furthermore, Insig-1 but not Insig-2 is able to cause ER retention of the SCAP/SREBP complex in the absence of sterols at high expression levels [7]. Unlike Insig-1, Insig-2a, the liver-specific isoform of Insig-2, is suppressed by insulin and induced by fasting [8]. Recently it has been shown that activation of the peroxisome proliferator-activated receptor (PPAR)-γ by rosiglitazone induced the expression of Insig-1 in white adipose tissue via a PPAR response element in the promoter region of Insig-1 [9].

PPAR are transcription factors belonging to the superfamily of nuclear receptors that can be activated by fatty acids and their metabolic derivatives. They are implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, cancer development as well as in the control of the inflammatory response [reviewed in 10–12]. There are three PPAR isotypes, PPARα, PPARβ/δ and PPARγ, all of which regulate the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-cis retinoic acid receptor after activation [13]. PPARα is highly expressed in tissues with high fatty acid oxidation, in which it controls a comprehensive set of genes that regulate most aspects of lipid catabolism [14]. Furthermore, the action of fibrates, a class of hypolipidemic drugs, on lipid metabolism is mediated by the activation of PPARα [15].

Both, natural and synthetic ligands of PPARα are known to lower the plasma and liver cholesterol concentrations in man and animals [16–19]. Mice in which the PPARα gene has been disrupted (PPARα-null (KO) mice) are hypercholesterolemic [20] and show a dysregulation in the hepatic expression of HMG-CoA reductase during the diurnal variation of cholesterogenesis [21]. Also other studies suggested an involvement of PPARα in the regulation of cholesterol synthesis; however, both, stimulatory and inhibitory effects of fibrates on hepatic HMG-CoA reductase and cholesterol synthesis in rats have been reported depending on fibrates and model used [22–24].

The objective of the present study was to further evaluate the mechanisms underlying the cholesterol lowering effects of PPARα activation in rat liver. Therefore we treated rats with the PPARα agonist clofibrate. Based on their central role in the regulation of the cholesterol metabolism, we focused on effects of PPARα activation on the gene expression and nuclear concentration of SREBP-2 and on the gene expression of Insig-1 and Insig-2a as key regulators of SREBP-2 activity. Furthermore, we analyzed the expression of SREBP-2 target genes HMG-CoA reductase and LDL receptor. To verify the obtained results, we used the Fao cell model which is commonly used to study the effects of PPARα agonists on hepatic lipid metabolism *in vitro* [25–27]. We examined the influence of WY 14,643, another PPARα agonist with high specificity [28], on Insigs, SREBP-2 and its target genes and on cholesterol synthesis in Fao cells.

2. Material and methods

2.1. Materials

WY 14,643, DMSO, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), TRIZOL™ reagent, SYBR® Green I, protease inhibitor mix and the anti-rabbit-IgG peroxidase conjugate antibody were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl 2-(4-chlorophenoxy)-2-methylpropionate (clofibrate) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Rat hepatoma Fao cell line was purchased from ECACC (Salisbury, UK). F-12 Nutrient Mixture (Ham), gentamycin and fetal calf serum (FCS) were obtained from Invitrogen (Karlsruhe, Germany). Reverse transcriptase was supplied by MBI Fermentas (St. Leon-Rot, Germany), and Taq polymerase by Promega (Mannheim, Germany). Bicinchoninic acid assay reagent was a product of Interchim (Montfucon, France). The nitrocellulose blotting membrane was from Pall (Pensacola, FL, USA), and the ECL-reagent kit from GE Healthcare (München, Germany). The anti-SREBP-2 antibody (rabbit polyclonal IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-β-Actin antibody (rabbit polyclonal IgG) was purchased from Abcam Ltd. (Cambridge, UK). Autoradiography film for Western blot analysis (Agfa Cronex) was from Roentgen Bender (Baden-Baden, Germany). Male Sprague–Dawley rats were supplied by Charles River (Sulzfeld, Germany). Radioactive [1,2-^{14}C] acetate (specific activity 110 mCi/mmol) was from Hartmann Analytic (Braunschweig, Germany), and TLC sheets (Si 60 aluminium sheets) were from VWR International (Darmstadt, Germany).

2.2. Cell culture

Fao rat hepatoma cells were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/ml gentamycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 24- or 6-well culture plates at a density of 2.1×10^5 and 1.05×10^6 cells, respectively, per well and used prior reaching confluence (usually 3 days after seeding). Experiments were carried out in low-serum medium (0.5% FCS) as commonly used for PPARα activation studies with several agonists since PPARα activation is more pronounced under these conditions [29–31]. Furthermore, expression of SREBP-2 target genes is upregulated compared to full-serum medium [32]. The cells were preincubated with low-serum medium for 16 h and then stimulated for 6 and 24 h with WY 14,643. WY 14,643 was

added to the low-serum medium from a stock solution in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability of Fao cells was not reduced by 24 h incubation with WY 14,643 up to a concentration of 100 μM as demonstrated by the MTT assay ([33]; data not shown).

2.3. Animals, diets and sample collection

Male Sprague–Dawley rats, with an average initial body weight of 366 g (±28; S.D.), were randomly assigned to two groups ($n = 8$) and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2 °C), relative humidity (50–60%) and light (12 h light/dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals were treated with 250 mg/kg of clofibrate in 1 ml sunflower oil or with an equal volume of the vehicle sunflower oil by gavage once a day 2 h after beginning of the light cycle. All rats were fed a commercial standard basal diet (''altromin 1324'', Altromin GmbH, Lage, Germany). To standardize food intake, the diets were fed daily in restricted amounts of 18 g per day. Water was available ad libitum from nipple drinkers during the whole experiment. At day 4 of treatment, animals received the last dose of clofibrate or vehicle alone and 9 g of the diet and were killed 4 h later by decapitation under light anaesthesia with diethyl ether. Rats were non-fasted before killing because food deprivation before killing leads to a significant down regulation of the genes involved in cholesterol metabolism to be considered in this study [34]. Blood was collected into heparinized polyethylene tubes. The liver was excised. Plasma was obtained by centrifugation of the blood (1100 × g, 10 min, 4 °C) and stored at −20 °C. Liver samples for RNA isolation and lipid extraction were snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. RT-PCR analysis

Total RNA was isolated from Fao cells after the incubation in 24-well plates and rat livers, respectively, by TRIZOL™ reagent according to the manufacturer's protocol. cDNA synthesis was carried out as described [31]. The mRNA expression of genes was measured by real-time detection PCR using SYBR® Green I. Real-time detection PCR was performed with 1.25 U Taq DNA polymerase, 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). For determination of mRNA concentration a threshold cycle (C_t) was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research, Australia). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described [35]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC1.2.1.12) was used for normalization.

2.5. Immunoblot analysis

Whole cell extracts of Fao cells were prepared by lysis in 20 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1% protease inhibitor mix, pH 7.5, after the incubation in six-well-plates. The protein content was determined by the bicinchoninic acid assay. Nuclear extracts of rat livers were prepared from fresh tissue samples (150 mg) according to Woo et al. [36] and equal amounts of proteins were pooled from four rats per treatment group. Thirty to fifty micrograms of Fao cell proteins and 100 μg of pooled rat liver nuclear extracts, respectively, were separated on 10% sodium dodecylsulfate acrylamide gel electrophoresis according to the method of Laemmli et al. [37] and electrotransferred to a nitrocellulose membrane. After blocking in 50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 7.5, containing 3% nonfat dry milk, bands corresponding to nuclear SREBP-2 (for Fao cell and rat liver samples) and β-Actin (for Fao cell samples, as a loading control) were visualized with

Table 1 – Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward and reverse primers	bp	Annealing temperature	NCBI GenBank
ACO	5′ CTTTCTTGCTTGCCTTCCTTCTCC 3′ 5′ GCCGTTTCACCGCCTCGTA 3′	415	60 °C	NM017340
Cyp4A1	5′ CAGAATGGAGAATGGGGACAGC 3′ 5′ TGAGAAGGGCAGGAATGAGTGG 3′	460	65 °C	NM175837
GAPDH	5′ GCATGGCCTTCCGTGTTCC 3′ 5′ GGGTGGTCCAGGGTTTCTTACTC 3′	337	60 °C	BC059110
HMG-CoA reductase	5′ AAGGGGCGTGCAAAGACAATC 3′ 5′ ATACGGCACGGAAAGAACCATAGT 3′	406	57 °C	BC064654
Insig-1	5′ ATTTGGCGTGGTCCTGGCTCTGG 3′ 5′ GCGTGGCTAGGAAGGCGATGGTG 3′	389	62 °C	NM022392
Insig-2a	5′ GACGGATGTGTTGAAGGATTTCT 3′ 5′ TGGACTGAAGCAGACCAATGTC 3′	83	59 °C	AY156086
LDL receptor	5′ AGAACTGCGGGGCCGAAGACAC 3′ 5′ AAACCGCTGGGACATAGGCACTCA 3′	490	65 °C	NM175762
SREBP-2	5′ ATCCGCCCACACTCACGCTCCTC 3′ 5′ GGCCGCATCCCTCGCACTG 3′	312	65 °C	BC101902

enhanced chemiluminescence reagents and exposed to autoradiography film. Films were analyzed with the Gel-Pro Analyzer software (Intas, Upland, CA, USA).

2.6. Determination of triacylglycerol and cholesterol concentrations in Fao cells, liver, plasma and lipoproteins

Lipid extraction from Fao cells after 24 h of incubation with 100 μM of WY 14,643 or vehicle alone and measurement of cellular and secreted triacylglycerols and cellular cholesterol was carried out as described [31]. Rat liver lipids were extracted using a mixture of *n*-hexane and isopropanol (3:2, v/v) [38]. Aliquots of the lipid extracts were dried and dissolved in a small volume of Triton X-100 [39]. Plasma lipoproteins were separated by stepwise ultracentrifugation (900,000 × *g*, 1.5 h, 4 °C; very low density lipoproteins (VLDL) + chylomicrons: $\delta < 1.006$ g/ml; LDL: $1.006 < \delta < 1.063$ g/ml; high density lipoproteins (HDL): $\delta > 1.063$ g/ml) using a Micro-Ultracentrifuge (Sorvall Products, Bad Homburg, Germany).

Concentrations of total cholesterol and triacylglycerols were determined using an enzymatic reagent kit (Ecoline S+, Merck, Darmstadt, Germany).

2.7. Determination of cholesterol synthesis

After a pre-incubation of 22 h at 37 °C, 5% CO_2 with the different concentrations of WY 14,643, 0.2 μCi [1,2-^{14}C]acetate (specific activity 110 mCi/mmol) were added in order to measure the newly synthesized cholesterol [40,41]. Cells were further incubated for 2 h at 37 °C, 5% CO_2. After incubation the cells were washed twice with cold PBS. The lipids were extracted twice with a mixture of hexane and isopropanol (3:2, v/v) [38]. After removing the solvents in a vacuum centrifugal evaporator the lipids were dissolved in 80 μl chloroform, 4 μL of which were applied to 10 × 20 cm^2 TLC using a TLC spotter PS01 (Desaga, Heidelberg, Germany). Plates were developed with a mixture of hexane, diethyl ether and acetic acid (80:20:3, v/v/v) [42]. Lipid-bound radioactivity was detected and quantified by autoradiography (Fuji imager system, Tina 2 software, Raytest, Straubenhart, Germany).

2.8. Statistical analysis

Treatment effects were analyzed by one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). In the cell culture experiments, means of the four treatments were compared by Fisher's multiple range test if the *F* value was significant ($P < 0.05$). Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Activation of PPARα in rat liver and Fao cells

We treated male Sprague–Dawley rats with the PPARα agonist clofibrate for 4 days. Animals were killed 4 h after the last dose of clofibrate and typical characteristics of PPARα activation were analyzed. Treatment with clofibrate led to an increase in the relative liver masses of the rats of about 22% compared to control animals ($P < 0.001$; Table 2). Rats treated with the PPARα agonist had higher mRNA concentrations of the PPARα downstream genes acyl-CoA oxidase (ACO; EC1.3.3.6) and cytochrome P450 (Cyp) 4A1 (EC1.14.15.3) of about 9- and 18-fold, respectively, in their livers compared to control animals ($P < 0.001$; Fig. 1). Furthermore, the triacylglycerol concentrations of the livers of rats treated with clofibrate were about 40% lower than those of control rats ($P < 0.05$; Table 2). The concentrations of triacylglycerols in plasma and VLDL + chylomicrons were also reduced about 59 and 81%, respectively, in clofibrate treated rats compared to control animals ($P < 0.001$).

Next, we incubated rat hepatoma Fao cells with increasing amounts of the PPARα agonist WY 14,643 for 6 and 24 h. Incubation of Fao cells with WY 14,643 for 6 and 24 h, respectively, led to a significant increase of the mRNA concentration of ACO and Cyp4A1 ($P < 0.05$; Fig. 2). Increase of ACO mRNA concentration by incubation of Fao cells with WY 14,643 was more pronounced after 6 h compared to 24 h treatment, whereas Cyp4A1 mRNA increased more after 24 h than after 6 h treatment, compared to control cells. Furthermore, we analyzed the influence of the PPARα agonist on the concentration of cellular and secreted triacylglycerols in Fao

Table 2 – Relative liver mass and triacylglycerol and cholesterol concentrations in liver, plasma and lipoproteins of rats treated with clofibrate compared to control rats

	Control	Clofibrate
Relative liver mass (g/kg body mass)	35.7 ± 2.7	45.7 ± 3.1**
Triacylglycerols		
Liver (μmol/g)	32.43 ± 7.26	19.75 ± 4.85*
Plasma (mM)	0.92 ± 0.19	0.38 ± 0.13**
VLDL + chylomicrons (mM)	0.74 ± 0.17	0.14 ± 0.03**
Total cholesterol		
Liver (μmol/g)	14.92 ± 2.50	12.85 ± 3.54*
Plasma (mM)	1.60 ± 0.34	0.49 ± 0.11**
VLDL + chylomicrons (mM)	0.09 ± 0.03	0.01 ± 0.00**
LDL (mM)	0.41 ± 0.12	0.12 ± 0.02**
HDL (mM)	0.92 ± 0.20	0.20 ± 0.05**

Values are means ± S.D. ($n = 8$). The asterisks indicate significant differences from control animals (*$P < 0.05$; **$P < 0.001$).

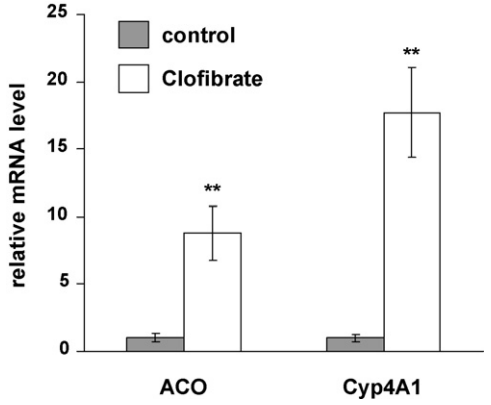

Fig. 1 – Effect of the PPARα agonist clofibrate on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from rat livers and relative mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 8$). **Significantly different from control animals, $P < 0.001$.

cells. The concentrations of cellular triacylglycerols of Fao cells incubated with WY 14,643 for 24 h was about 13% lower compared to control cells treated with vehicle alone [control: 41.3 ± 0.9 nmol mg cell protein^{-1}, 100 μM WY 14,643: 35.8 ± 4.4 nmol·mg cell protein^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$]. The amount of secreted triacylglycerols in VLDL was measured in the culture medium after incubation. Cells incubated with WY 14,643 for 24 h secreted about 24% less triacylglycerols than control cells [control: 411 ± 30 nmol·mg cell protein^{-1} 24 h^{-1}, 100 μM WY 14,643: 313 ± 41 nmol·mg cell protein^{-1} 24 h^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$].

3.2. *Influence of PPARα agonists on Insig-1, Insig-2a and SREBP-2 in rat liver and Fao cells*

To study the influence of PPARα activation on key regulators of cholesterol synthesis and uptake, we analyzed the mRNA concentrations of Insig-1, Insig-2a and SREBP-2. Furthermore, we determined the amount of the nuclear form of SREBP-2 by Western-blotting. In rats treated with clofibrate, the relative mRNA concentration of Insig-1 in the liver was about 80% higher than in control animals ($P < 0.05$; Fig. 3A), whereas the mRNA concentration of Insig-2a, the liver specific transcript of the Insig-2 gene, was about 80% lower in the liver of clofibrate treated rats than in control rats ($P < 0.001$; Fig. 3A). The concentration of SREBP-2 mRNA in the liver of rats treated with the PPARα agonist was about 40% lower than that of control rats ($P < 0.05$; Fig. 3A). To analyze the amount of nuclear SREBP-2, we isolated the nuclear fractions of livers of the rats. The relative protein level of the mature SREBP-2 in the livers of rats treated with clofibrate was about 70% lower than in control animals ($P < 0.05$; Fig. 3B and C).

Treatment of Fao cells with WY 14,643 for 6 h led to a significant and concentration dependent increase of Insig-1 mRNA concentration compared to control cells, and this effect was abolished after 24 h of treatment (Fig. 4A). At the highest concentration of WY 14,643 used, Insig-1 mRNA concentration was about 80% higher than that of control cells after 6 h of incubation ($P < 0.05$). Likewise, incubation of Fao cells with 100 μM WY 14,643 for 6 h increased the mRNA concentration of Insig-2a about 50% compared to control cells ($P < 0.05$). After 24 h of incubation, Insig-2a mRNA concentration in WY 14,643 treated cells still tended to be higher (about 20%, $P < 0.10$) than in control cells (Fig. 4A). SREBP-2 mRNA concentration was unchanged in cells incubated with WY 14,643 for 6 h, whereas it was about 20% lower after 24 h of treatment with all WY 14,643 concentrations used compared to control cells ($P < 0.05$; Fig. 4A). Western blot analysis of whole Fao cell lysates revealed that the relative protein concentration of the mature SREBP-2 after treatment of Fao cells with 100 μM of WY 14,643

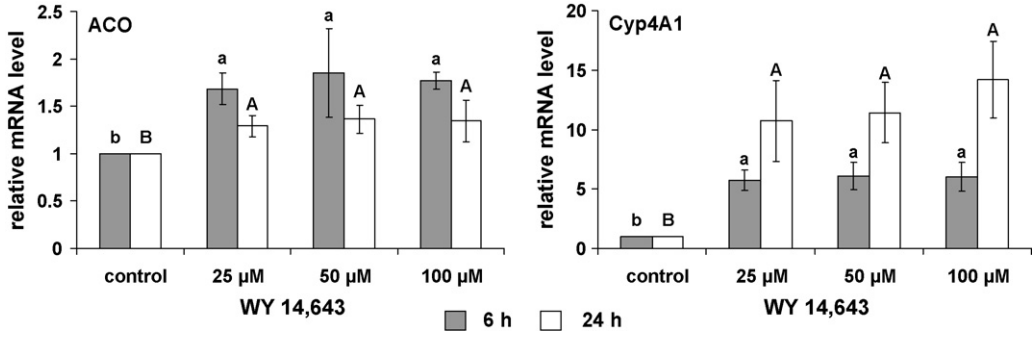

Fig. 2 – Effect of the PPARα agonist WY 14,643 on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and relative mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 3$). Bars without the same superscript letter are significantly different, $P < 0.05$. Small letters (a and b) denote differences in 6 h incubation, capital letters (A and B) denote differences in 24 h incubation.

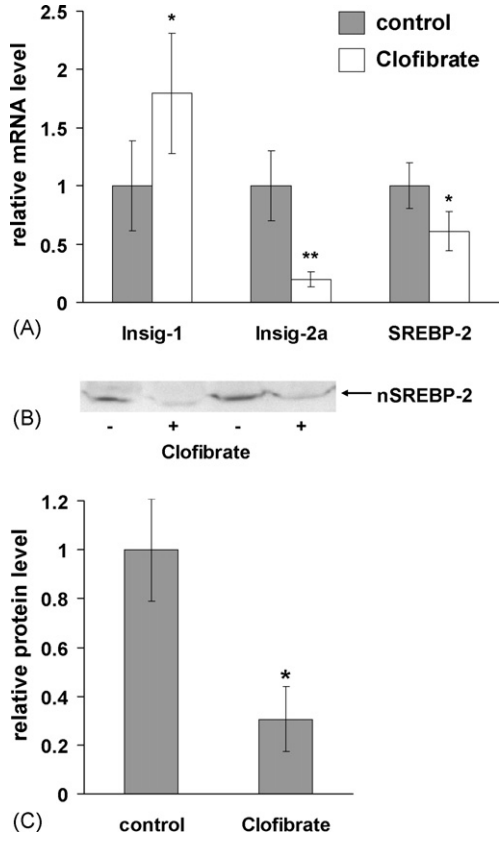

Fig. 3 – Effect of clofibrate treatment on Insig-1, Insig-2a and SREBP-2 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. (A) Total RNA was extracted from rat livers and Insig-1, Insig-2a and SREBP-2 mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 8$). (B) Liver nuclear extracts of four animals per group were pooled and 100 μg of the samples were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-2 antibodies. (C) Relative intensity of the bands in (B) was quantified by densitometry. The asterisks indicate significant differences from control animals ($^{*}P < 0.05$; $^{**}P < 0.001$).

for 6 h was not different from that of the control cells (Fig. 4B and C). After 24 h of treatment, mature SREBP-2 concentration was about 35% lower compared to untreated cells ($P < 0.05$).

3.3. *Influence of PPARα agonists on the mRNA concentration of HMG-CoA reductase and LDL receptor in rat liver and Fao cells*

We analyzed the effect of PPARα agonists on the expression of SREBP-2 target genes involved in cholesterol synthesis and uptake. In rats treated with clofibrate, the relative mRNA concentration of HMG-CoA reductase in the liver was about 40% lower than in control animals ($P < 0.05$; Fig. 5). Furthermore, treatment of rats with the PPARα agonist led to a reduction of LDL receptor mRNA concentration about 27% compared to control rats ($P < 0.05$; Fig. 5).

The level of HMG-CoA reductase mRNA in Fao cells after treatment with different concentrations of WY 14,643 for 6 h was not changed compared to control cells. Incubation of Fao cells with WY 14,643 for 24 h led to a reduction of HMG-CoA reductase mRNA concentration about 38, 35 and 24% at 25, 50 and 100 μM WY 14,643, respectively, compared to untreated cells ($P < 0.05$; Fig. 6). LDL receptor mRNA concentration was unchanged in cells incubated with WY 14,643 for 6 h compared to control cells. After 24 h treatment, LDL receptor mRNA concentration was about 30, 35 and 33% lower in Fao cells incubated with 25, 50 and 100 μM WY 14,643, respectively, than in untreated cells ($P < 0.05$; Fig. 6).

3.4. *Effect of PPARα agonists on cholesterol concentration in liver, plasma and lipoproteins of rats and on cholesterol concentration and synthesis in Fao cells*

PPARα activation in rats by clofibrate reduced the amount of total cholesterol in rat liver about 14% compared to control rats ($P < 0.05$; Table 2). Also, the cholesterol concentration of the plasma of clofibrate treated rats was about 69% lower than that of untreated animals ($P < 0.001$). The amount of total cholesterol in VLDL + chylomicrons, LDL and HDL was decreased about 89, 70 and 78%, respectively, by treatment of rats with the PPARα agonist ($P < 0.001$; Table 2).

In Fao cells treated with 100 μM WY 14,643 for 24 h, the concentration of cellular cholesterol was about 16% lower compared to control cells incubated with vehicle alone [control: 21.9 ± 1.5 nmol·mg cell protein^{-1}, 100 μM WY 14,643: 18.4 ± 3.1 nmol·mg cell protein^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$]. Furthermore, cholesterol synthesis was significantly lower about 27, 25 and 44% in Fao cells incubated with 25, 50 and 100 μM WY 14,643, respectively, for 24 h compared to control cells ($P < 0.05$; Fig. 7).

4. Discussion

The cholesterol concentration in mammalian cells is tightly controlled by a feedback mechanism involving Insigs, SCAP and SREBPs [43]. The aim of this study was to elucidate the mechanism by which PPARα ligands influence cholesterol synthesis and uptake in rat liver. For that, we first examined the effect of clofibrate treatment on the cholesterol metabolism of rats. Clofibrate is known to be a hypolipidemic drug and its plasma triacylglycerol and cholesterol lowering effects are well reported [18]. Treatment of rats with clofibrate for 4 days led to a strong PPARα activation as indicated by an upregulation of the PPARα target genes ACO and Cyp4A1 and increased relative liver weights which are due to the induced peroxisome proliferation [44,45]. Both, ACO and Cyp4A1 are typical PPARα downstream genes and are considered as marker genes for PPARα activation [46,47]. The increased expression of these genes involved in fatty acid β-oxidation is one of the mechanisms underlying the hypotriglyceridemic effect upon PPARα activation. Others are increased hydrolysis of plasma triglycerides, stimulation of cellular fatty acid uptake, decreased synthesis of fatty acids and triglycerides and

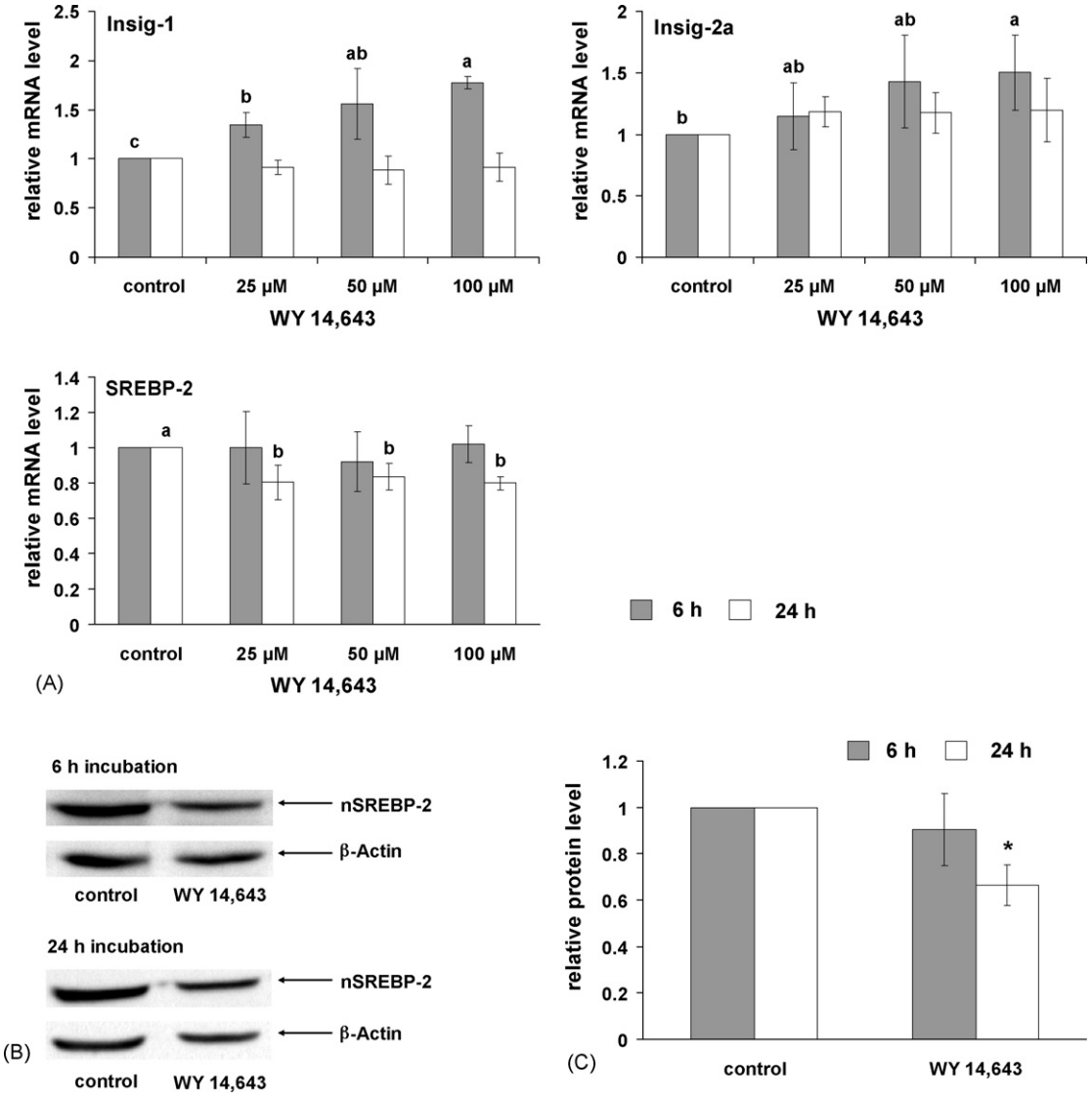

Fig. 4 – Effect of WY 14,643 on Insig-1, Insig-2a and SREBP-2 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. (A) Total RNA was extracted from cells and Insig-1, Insig-2a and SREBP-2 mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. (B) After cell lysis, equal amounts of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-2 and anti-β-Actin antibodies. Representative immunoblots after 6 and 24 h incubation of Fao cells with or without 100 μM WY 14,643 are shown. (C) Relative intensity of the bands in (B) was quantified by densitometry using β-Actin-specific band as loading control. Values are means ± S.D. ($n = 3$). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$. *Significantly different from control cells (*$P < 0.05$).

decreased production of VLDL [15]. Indeed, rats treated with the PPARα agonist had markedly reduced triacylglycerol levels in liver, plasma and VLDL + chylomicrons. Second, to verify the results obtained in the rat study we used an *in vitro* model and the PPARα agonist WY 14,643. Compared to clofibrate, WY 14,643 is a more potent PPARα agonist and exhibits a more strict PPAR subtype specificity [28,48] allowing us to assign the observed effects actually to an activation of PPARα. Incubation of the Fao cells with WY 14,643 for 6 and 24 h led to a strong activation of PPARα as indicated by several fold increased mRNA concentrations of ACO and Cyp4A1. Furthermore, treatment of cells with WY 14,643 largely reduced the concentration of intracellular and secreted triacylglycerols.

Next, we analyzed the effect of PPARα activation on key regulators of cholesterol synthesis and uptake. Both, in the liver of rats treated with clofibrate and in Fao cells treated with WY 14,643 for 24 h, the amount of the transcriptionally active form of SREBP-2 in the nucleus was reduced compared to

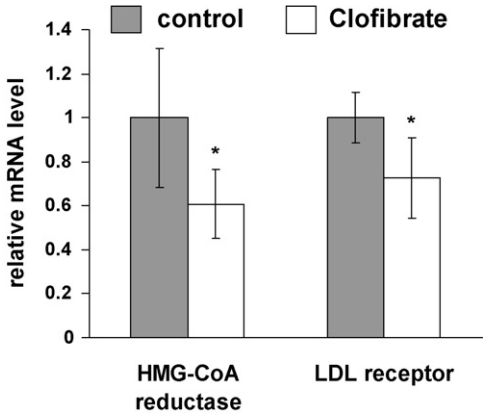

Fig. 5 – Effect of clofibrate treatment on the mRNA concentrations of HMG-CoA reductase and LDL receptor in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from rat livers and relative mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. (n = 8). *Significantly different from control animals, $P < 0.05$.

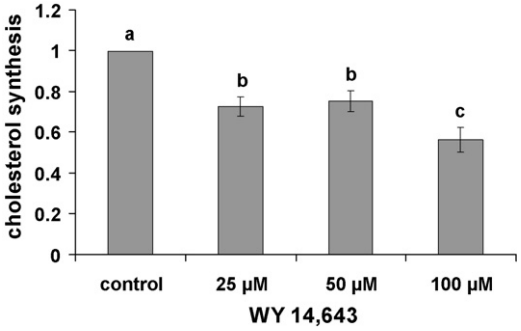

Fig. 7 – Effect of WY 14,643 on the relative cholesterol synthesis rate in Fao cells. Cells were pre-incubated for 22 h with different concentrations of WY 14,643 or with vehicle alone (control). Thereafter, cells were incubated for further 2 h with or without the indicated concentrations of WY 14,643 with addition of [1,2-^{14}C]acetate in order to measure the newly synthesized cholesterol. Cellular lipids were extracted with a mixture of hexane and isopropanol. Lipids were separated by thin-layer chromatography and lipid-bound radioactivity was detected and quantified by autoradiography. Values are means ± S.D. (n = 3). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$.

control animals and cells, respectively. This can be due on the one hand to a reduced transcription of the gene or reduced stability of the transcript resulting in lowered mRNA concentrations and reduced availability of the SREBP-2 precursor protein. However, also a reduced amount of nuclear SREBP-2 can lead to decreased mRNA concentrations of the SREBP-2 gene since SREBP-2 contains a sterol regulatory element in its enhancer/promoter region and the nuclear form can activate its own gene in an autoregulatory loop [49]. In the liver of clofibrate treated rats, the mRNA concentration of SREBP-2 was reduced compared to control rats. In Fao cells, after 6 h of incubation with WY 14,643 the mRNA concentration of SREBP-2 was unchanged, whereas it was significantly reduced after 24 h of incubation compared to control cells. This observation in Fao cells indicates, that a reduction of SREBP-2 mRNA did not precede the decrease of its nuclear form and thus there may be another reason for the observed reduction of nuclear SREBP-2 upon PPARα activation.

The integral membrane proteins Insig-1 and -2 have been previously identified as modulators of SREBP activity [6,7]. They anchor the SCAP/SREBP complex in the endoplasmic reticulum in the presence of sterols. Overexpression of Insig-1 in the liver of transgenic mice inhibited processing of SREBPs [50]. Inversely, reduction of both Insig mRNAs by RNA interference or by mutational inactivation led to an increase in nuclear SREBPs [51–53]. We could show that in the liver of

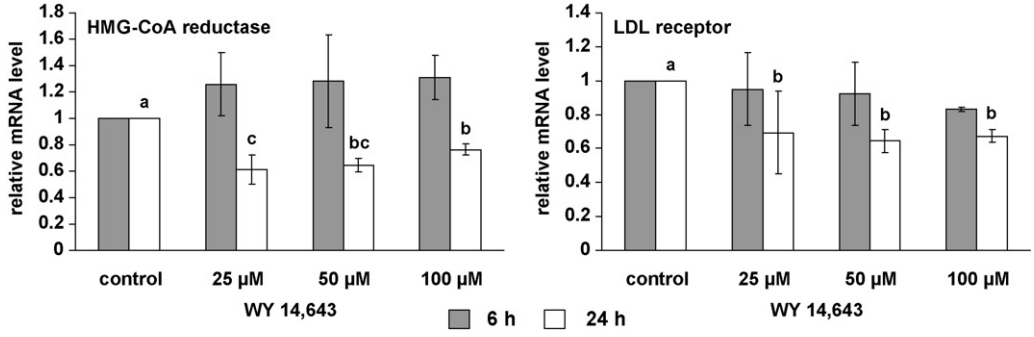

Fig. 6 – Effect of WY 14,643 on the mRNA concentration of HMG-CoA reductase and LDL receptor in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and relative mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. (n = 3). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$.

rats treated with clofibrate the mRNA concentration of Insig-1 was increased compared to control animals. Furthermore, incubation of Fao cells with WY 14,643 for 6 h led to a significant and dose-dependent increase of the mRNA concentrations of Insig-1 and, to a lesser extent, of Insig-2a, the liver-specific transcript of the Insig-2 gene. The induction of Insig-1 mRNA concentration in WY 14,643 treated Fao cells observed after 6 h of incubation was completely abolished after 24 h. We suggest that this is due to a decline in Insig-1 transcription caused by reduced nuclear SREBP-2. The transcription of Insig-1 requires nuclear SREBPs [7]. In contrast to Insig-1, the expression of Insig-2 is not dependent on nuclear SREBPs permitting feedback regulation of cholesterol synthesis over a wide range of sterol concentrations by the concerted action of both Insig-1 and Insig-2 [7]. The level of Insig-2a mRNA after 24 h of incubation of the Fao cells with WY 14,643 was not as high as after 6 h of incubation but tended to be still elevated over control.

Taken together these data suggest that the reduced amount of nuclear SREBP-2 upon PPARα activation in rat liver and Fao cells, respectively, may be rather due to increased expression of Insigs which retard SREBP-2 processing than to decreased transcription of the SREBP-2 gene. Recently it was demonstrated that Insig-1 is regulated by PPARγ in white adipose tissue of diabetic mice via a PPAR response element in its promoter region [9]. In Fao cells, the increase of Insig-1 and -2a mRNA concentrations was observed simultaneously with ACO and Cyp4A1 induction after short term incubation of the Fao cells with WY 14,643. Considering the existence of a PPAR response element in the Insig-1 promotor one could speculate, that the upregulation of Insig-1 and Insig-2a may be directly mediated by PPARα. Further experiments are required to prove this hypothesis.

In contrast to our results obtained with the Fao cell model, PPARα activation in rats markedly reduced the mRNA concentration of Insig-2a in the liver. The expression of Insig-2a, the liver-specific transcript of Insig-2, is specifically down-regulated by insulin [8]. It has been reported that activation of PPARα improved insulin sensitivity in different models of insulin resistance, probably by reducing lipid accumulation in tissues due to increased fatty acid oxidation and by down-regulation of a gene involved in insulin receptor signalling in hepatocytes [54–57]. Thus we suggest that improvement of insulin sensitivity in the liver of clofibrate treated rats may account for the down-regulation of Insig-2a. Nevertheless, the reduced expression of Insig-2a did not interfere with the inhibition of SREBP-2 processing in clofibrate treated rats, indicating that Insig-1 is more important than Insig-2a in the regulation of SREBP-2 activity. Yabe et al. [8] discussed a special role of Insig-2a in processing of SREBP-1c in the liver of mice allowing the SREBP-1c to exit the ER to stimulate fatty acid synthesis, even at elevated hepatic cholesterol concentrations.

The reduced abundance of transcriptionally active SREBP-2 in the nucleus upon PPARα activation in the livers of clofibrate treated rats and in Fao cells after 24 h of incubation with WY 14,643 was mirrored by lowered mRNA concentrations of two SREBP-2 target genes encoding proteins for cholesterol synthesis and uptake. In clofibrate treated rats as well as in Fao cells stimulated with WY 14,643 for 24 h, the mRNA concentrations of both, HMG-CoA reductase and LDL receptor were decreased compared to control animals and cells, respectively. Furthermore, cholesterol synthesis rate in Fao cells incubated with WY 14,643 for 24 h was decreased compared to control cells which is in agreement with the reduced mRNA concentration of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, upon PPARα activation.

In clofibrate treated rats, the reduced expression of genes involved in cholesterol synthesis and uptake was reflected by decreased concentrations of total cholesterol in the liver, plasma and lipoproteins. A similar decline in serum cholesterol levels associated with decreased HMG-CoA reductase activity in liver microsomes was observed in rats fed 0.3% clofibrate in the diet for 3–7 days [58]. Also in Fao cells, reduced cholesterol synthesis and uptake resulted in a decrease of the total cholesterol concentration after 24 h of incubation with WY 14,643.

Thus, our data show for the first time that PPARα activation lowers the cholesterol concentration in rat liver, plasma and lipoproteins and in Fao cells by reducing the amount of nuclear SREBP-2 thereby decreasing cholesterol synthesis and uptake. Further, our data indicate that this reduction of nuclear SREBP-2 is mediated by increased expression of Insigs (Fig. 8).

Several reports indicated an involvement of PPARα in the regulation of cholesterol synthesis in the liver. In wild-type mice, an antiparallel relationship exists between the expression of the PPARα gene and that of HMG-CoA reductase and LDL receptor genes; in PPARα-null (KO) mice, the diurnal variation of cholesterogenic gene expression was abolished [21]. These observations are in agreement with our study that shows that PPARα activation inhibits the expression of HMG-CoA reductase and LDL receptor by reducing the amount of nuclear SREBP-2.

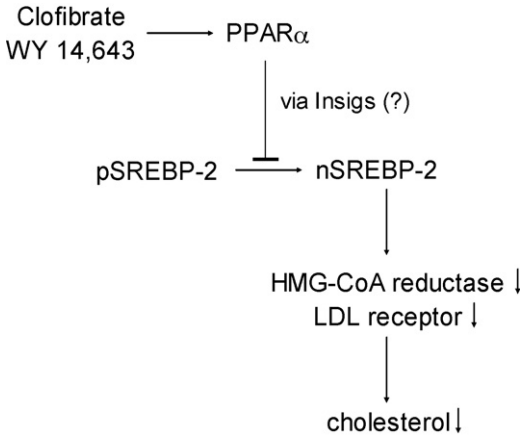

Fig. 8 – Schematic diagram of the proposed pathways leading to decreased cholesterol synthesis and concentration upon PPARα activation. PPARα activation by clofibrate or WY 14,643 reduces the amount of nuclear SREBP-2, probably via an upregulation of Insigs. In turn, this leads to a decreased expression of SREBP-2 target genes HMG-CoA reductase and LDL receptor implicated in cholesterol synthesis and uptake and finally to reduced cholesterol concentrations.

Also other studies supported our results on the mechanism underlying the cholesterol lowering effect of PPARα agonists by indicating that clofibrate inhibited HMG-CoA reductase activity and decreased cholesterol synthesis in rats and in cultured hepatocytes [22,24,58–60]. However, there are also few studies which are in contrast to our results. The PPARα agonists gemfibrozil and ciprofibrate upregulated cholesterol synthesis and HMG-CoA reductase activity or mRNA concentration in rats and in cultured hepatocytes [23,24,61]. WY 14,643 treatment of wild-type mice resulted in a decreased rate of cholesterol synthesis, whereas in PPARα-null (KO) mice cholesterol synthesis was unaffected by WY 14,643 treatment [62]. While this reduction of cholesterol synthesis by PPARα is consistent with our data, the authors found increased HMG-CoA reductase mRNA levels in wild-type mice but not PPARα-null (KO) mice treated with WY 14,643 [62]. The reasons for the conflicting results concerning the effects of PPARα agonists on cholesterol synthesis and HMG-CoA reductase are difficult to explain but may depend on experimental conditions, species and type of fibrate used. Furthermore, HMG-CoA reductase is regulated by a complex feedback mechanism including transcriptional, translational and posttranslational levels and the sterol-dependent ubiquitination and proteolytic degradation of the protein mediated by Insigs [63,64]. Thus, measurements of HMG-CoA reductase mRNA may not always reflect the actual activity of the enzyme.

In conclusion, data from the rat experiment and the *in vitro* study strongly suggest that PPARα activation lowers the cholesterol concentration by reducing the abundance of nuclear SREBP-2, probably via an upregulation of Insigs. This leads in turn to diminished expression of the SREBP-2 target genes HMG-CoA reductase and LDL receptor and reduced cholesterol synthesis and uptake. Thus, these date give important insights in the complex regulation of lipid homeostasis in liver cells by providing a novel link between PPARα and cholesterol metabolism. Moreover, these results may help to explain the cholesterol lowering effects of natural ligands of PPARα such as polyunsaturated fatty acids, conjugated linoleic acids and oxidized fatty acids in man and animals [16,17,65].

Acknowledgment

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REFERENCES

[1] Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci USA 1999;96:11041–8.
[2] Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 2002;109:1125–31.
[3] Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. J Clin Invest 1997;99:838–45.
[4] Espenshade PJ, Li WP, Yabe D. Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER. Proc Natl Acad Sci USA 2002;99:11694–9.
[5] Goldstein JL, Rawson RB, Brown MS. Mutant mammalian cells as tools to delineate the sterol regulatory element-binding protein pathway for feedback regulation of lipid synthesis. Arch Biochem Biophys 2002;397:139–48.
[6] Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, et al. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 2002;110:489–500.
[7] Yabe D, Brown MS, Goldstein JL. Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. Proc Natl Acad Sci USA 2002;99:12753–8.
[8] Yabe D, Komuro R, Liang G, Goldstein JL, Brown MS. Liver-specific mRNA for Insig-2 down-regulated by insulin: implications for fatty acid synthesis. Proc Natl Acad Sci USA 2003;100:3155–60.
[9] Kast-Woelbern HR, Dana SL, Cesario RM, Sun L, de Grandpre LY, Brooks ME, et al. Rosiglitazone induction of Insig-1 in white adipose tissue reveals a novel interplay of peroxisome proliferator-activated receptor gamma and sterol regulatory element-binding protein in the regulation of adipogenesis. J Biol Chem 2004;279:23908–15.
[10] Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev 1999;20:649–88.
[11] Duval C, Chinetti G, Trottein F, Fruchart JC, Staels B. The role of PPARs in atherosclerosis. Trends Mol Med 2002;8:422–30.
[12] Chinetti G, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. Inflamm Res 2000;49:497–505.
[13] Schoonjans K, Martin G, Staels B, Auwerx J. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol 1997;8:159–66.
[14] Mandard S, Müller M, Kersten S. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci 2004;61:393–416.
[15] Staels B, Dallongeville J, Auwerx J, Schoonjans K, Leitersdorf E, Fruchart JC. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088–93.
[16] Kris-Etherton PM, Yu S. Individual fatty acid effects on plasma lipids and lipoproteins: human studies. Am J Clin Nutr 1997;65:1628S–44S.
[17] Eder K, Suelzle A, Skufca P, Brandsch C, Hirche F. Effects of dietary thermoxidized fats on expression and activities of hepatic lipogenic enzymes in rats. Lipids 2003;38:31–8.
[18] Sirtori M, Montanari G, Gianfranceschi G, Malacrida MG, Battistin P, Morazzoni G, et al. Clofibrate and tiadenol treatment in hyperlipoproteinemias. A comparative trial of drugs affecting lipoprotein catabolism and biosynthesis. Atherosclerosis 1983;49:149–61.
[19] Elisaf M. Effects of fibrates on serum metabolic parameters. Curr Med Res Opin 2002;18:269–76.
[20] Peters JM, Hennuyer N, Staels B, Fruchart JC, Fievet C, Gonzalez FJ, et al. Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice. J Biol Chem 1997;272:27307–12.
[21] Patel DD, Knight BL, Wiggins D, Humphreys SM, Gibbons GF. Disturbances in the normal regulation of SREBP-sensitive genes in PPAR alpha-deficient mice. J Lipid Res 2001;42:328–37.

[22] Hashimoto F, Taira S, Hayashi H. Changes in isoprenoid lipid synthesis by gemfibrozil and clofibrate in rat hepatocytes. Biochem Pharmacol 2000;59:1203–10.
[23] Roglans N, Peris C, Verd JC, Alegret M, Vazquez M, Sanchez RM, et al. Increase in hepatic expression of SREBP-2 by gemfibrozil administration to rats. Biochem Pharmacol 2001;62:803–9.
[24] Shiota Y, Ikeda M, Hashimoto F, Hayashi H. Effects of peroxisome proliferators gemfibrozil and clofibrate on syntheses of dolichol and cholesterol in rat liver. J Biochem (Tokyo) 2003;134:197–202.
[25] Poirier H, Braissant O, Niot I, Wahli W, Besnard P. 9-cis-Retinoic acid enhances fatty acid-induced expression of the liver fatty acid-binding protein gene. FEBS Lett 1997;412:480–4.
[26] Vanden Heuvel JP, Kreder D, Belda B, Hannon DB, Nugent CA, Burns KA, et al. Comprehensive analysis of gene expression in rat and human hepatoma cells exposed to the peroxisome proliferator WY14,1643. Toxicol Appl Pharmacol 2003;188:185–98.
[27] Landrier JF, Thomas C, Grober J, Duez H, Percevault F, Souidi M, et al. Statin induction of liver fatty acid-binding protein (L-FABP) gene expression is peroxisome proliferator-activated receptor-alpha-dependent. J Biol Chem 2004;279:45512–8.
[28] Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, et al. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol 1997;11:779–91.
[29] Grau R, Punzon C, Fresno M, Iniguez MA. Peroxisome-proliferator-activated receptor alpha agonists inhibit cyclo-oxygenase 2 and vascular endothelial growth factor transcriptional activation in human colorectal carcinoma cells via inhibition of activator protein-1. Biochem J 2006;395:81–8.
[30] Rodriguez C, Cabrero A, Roglans N, Adzet T, Sanchez RM, Vazquez M, et al. Differential induction of stearoyl-CoA desaturase and acyl-CoA oxidase genes by fibrates in HepG2 cells. Biochem Pharmacol 2001;61:357–64.
[31] König B, Eder K. Differential action of 13-HPODE on PPARα downstream genes in rat Fao and human HepG2 hepatoma cell lines. J Nutr Biochem 2006;17:410–8.
[32] Yu-Poth S, Yin D, Kris-Etherton PM, Zhao G, Etherton TD. Long-chain polyunsaturated fatty acids upregulate LDL receptor protein expression in fibroblasts and HepG2 cells. J Nutr 2005;135:2541–5.
[33] Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[34] Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. Proc Natl Acad Sci USA 1998;95:5987–92.
[35] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;e29–45.
[36] Woo CWH, Siow YL, Pierce GN, Choy PC, Minuk GY, Mymin D, et al. Hyperhomocysteinemia induces hepatic cholesterol biosynthesis and lipid accumulation via activation of transcription factors. Am J Physiol Endocrinol Metab 2005;288:E1002–10.
[37] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[38] Hara A, Radin NS. Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 1978;90:420–6.
[39] De Hoff JL, Davidson JH, Kritchevsky V. An enzymatic assay for determining free and total cholesterol in tissues. Clin Chem 1978;24:433–5.
[40] Mehran M, Seidman E, Marchand R, Gurbindo C, Levy E. Tumor necrosis factor-alpha inhibits lipid and lipoprotein transport by Caco-2 cells. Am J Physiol 1995;269:G953–60.
[41] Lovati MR, Manzoni C, Gianazza E, Arnoldi A, Kurowska E, Carroll K, et al. Soy protein peptides regulate cholesterol homeostasis in Hep G2 cells. J Nutr 2000;130:2543–9.
[42] Levy E, Thibault L, Menard D. Intestinal lipids and lipoproteins in the human fetus: modulation by epidermal growth factor. J Lipid Res 1992;33:1607–17.
[43] McPherson R, Gauthier A. Molecular regulation of SREBP function: the Insig-SCAP connection and isoform-specific modulation of lipid synthesis. Biochem Cell Biol 2004;82:201–11.
[44] Hawkins JM, Jones WE, Bonner FW, Gibson GG. The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. Drug Metab Rev 1987;18:441–515.
[45] Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, et al. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol Cell Biol 1995;15:3012–22.
[46] Bentley P, Calder I, Elcombe C, Grasso P, Stringer D, Wiegand HJ. Hepatic peroxisome proliferation in rodents and its significance for humans. Food Chem Toxicol 1993;31:857–907.
[47] Aldridge TC, Tugwood JD, Green S. Identification and characterization of DNA elements implicated in the regulation of CYP4A1 transcription. Biochem J 1995;306:473–9.
[48] Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci USA 1997;94:4312–7.
[49] Amemiya-Kudo M, Shimano H, Hasty AH, Yahagi N, Yoshikawa T, Matsuzaka T, et al. Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. J Lipid Res 2002;43:1220–35.
[50] Engelking LJ, Kuriyama H, Hammer RE, Horton JD, Brown MS, Goldstein JL, et al. Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis. J Clin Invest 2004;113:1168–75.
[51] Adams CM, Reitz J, De Brabander JK, Feramisco JD, Li L, Brown MS, et al. Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. J Biol Chem 2004;279:52772–80.
[52] Sever N, Song BL, Yabe D, Goldstein JL, Brown MS, DeBose-Boyd RA. Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. J Biol Chem 2003;278:52479–90.
[53] Lee PC, Sever N, Debose-Boyd RA. Isolation of sterol-resistant Chinese hamster ovary cells with genetic deficiencies in both Insig-1 and Insig-2. J Biol Chem 2005;280:25242–9.
[54] Guerre-Millo M, Gervois P, Raspe E, Madsen L, Poulain P, Derudas B, et al. Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. J Biol Chem 2000;275:16638–42.
[55] Ye JM, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW. Peroxisome proliferator-activated receptor (PPAR)-alpha activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: comparison with PPAR-gamma activation. Diabetes 2001;50:411–7.

[56] Chou CJ, Haluzik M, Gregory C, Dietz KR, Vinson C, Gavrilova O, et al. WY14,643, a peroxisome proliferator-activated receptor alpha (PPARalpha) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic A-ZIP/F-1 mice. J Biol Chem 2002;277:24484–9.
[57] Frederiksen KS, Wulf EM, Wassermann K, Sauerberg P, Fleckner J. Identification of hepatic transcriptional changes in insulin-resistant rats treated with peroxisome proliferator activated receptor-alpha agonists. J Mol Endocrinol 2003;30:317–29.
[58] Berndt J, Gaumert R, Still J. Mode of action of the lipid-lowering agents, clofibrate and BM 15075, on cholesterol biosynthesis in rat liver. Atherosclerosis 1978;30:147–52.
[59] Hashimoto F, Taira S, Hayashi H. Comparison of the effects of gemfibrozil and clofibrate on peroxisomal enzymes and cholesterol synthesis of rat hepatocytes. Biol Pharm Bull 1998;21:1142–7.
[60] Hayashi H, Takahata S. Role of peroxisomal fatty acyl-CoA beta-oxidation in phospholipid biosynthesis. Arch Biochem Biophys 1991;284:326–31.
[61] Le Jossic-Corcos C, Duclos S, Ramirez LC, Zaghini I, Chevillard G, Martin P, et al. Effects of peroxisome proliferator-activated receptor alpha activation on pathways contributing to cholesterol homeostasis in rat hepatocytes. Biochim Biophys Acta 2004;1683:49–58.
[62] Knight BL, Hebbachi A, Hauton D, Brown AM, Wiggins D, Patel DD, et al. A role for PPARalpha in the control of SREBP activity and lipid synthesis in the liver. Biochem J 2005;389:413–21.
[63] Panda T, Devi VA. Regulation and degradation of HMGCo-A reductase. Appl Microbiol Biotechnol 2004;66:143–52.
[64] Sever N, Yang T, Brown MS, Goldstein JL, DeBose-Boyd RA. Accelerated degradation of HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. Mol Cell 2003;11:25–33.
[65] Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA, Huth PJ. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 1997;22:266–77.