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ORIGINAL ARTICLE

Role of Liver X Receptor, Insulin and Peroxisome Proliferator Activated Receptor α on in Vivo Desaturase Modulation of Unsaturated Fatty Acid Biosynthesis

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Abstract We examined the in vivo contribution of insulin, T090137 (T09), agonist of liver X receptor (LXR), fenofibrate, agonist of peroxisome proliferator activated receptor (PPAR-α) and sterol regulatory element binding protein-1c (SREBP-1c) on the unsaturated fatty acid synthesis controlled by Δ6 and Δ5 desaturases, compared with the effects on stearoyl-coenzyme A desaturase-1. When possible they were checked at three levels: messenger RNA (mRNA), desaturase protein and enzymatic activity. In control rats, only fenofibrate increased the insulinemia that was maintained by the simultaneous administration of T09, but this increase has no specific effect on desaturase activity. T09 enhanced SREBP-1 in control animals and the mRNAs and activity of the three desaturases in control and type-1 diabetic rats, demonstrating a LXR/SREBP-1-mediated activation independent of insulin. However, simultaneous administration of insulin and T09 to diabetic rats led to a several-fold increase of the mRNAs of the desaturases, suggesting a strong synergic effect between insulin and LXR/retinoic X receptor (RXR). Moreover, this demonstrates the existence of an interaction between unsaturated fatty acids and cholesterol metabolism performed by the insulin/SREBP-1c system and LXR/RXR. PPAR-α also increased the expression and activity of the three desaturases independently of the insulinemia since it was equivalently evoked in streptozotocin diabetic rats. Besides, PPAR-α increased the palmitoylcoenzyme A elongase, evidencing a dual regulation in the fatty acid biosynthesis at the level of desaturases and elongases. The simultaneous administration of fenofibrate and T09 did not show additive effects on the mRNA expression and activity of the desaturases. Therefore, the results indicate a necessary sophisticated interaction of all these factors to produce the physiological effects.

Keywords Δ9, Δ6 and Δ5 desaturases · Diabetes mellitus type 1 · Sterol regulatory element binding protein-1c · T091317 · Liver X receptor · Fenofibrate · Peroxisome proliferator activated receptor α

Abbreviations

CoA	Coenzyme A
LXR	Liver X receptor
mRNA	Messenger RNA
PPAR-α	Peroxisome proliferator activated receptor α
RXR	Retinoic X receptor
SCAP	SREBP cleavage activating protein
SCD-1	Stearoylcoenzyme A desaturase-1
SREBP-1c	Sterol regulatory element binding protein-1c
T09	T0901317

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Introduction

Unsaturated fatty acids, either essential or nonessential, play a relevant role in animal metabolism and physiology, and as lipid components they determine

the structure and properties of membranes. Polyunsaturated fatty acids accomplish very important physiological functions when converted to eicosanoids and docosanoids, and they are important brain components. They are agonists in the expression of several nuclear receptors that activate or deactivate enzymes and proteins, modulating cellular signaling involved in human health and diseases.

Unsaturated fatty acids are provided by the diet, but the endogenous biosynthesis is another important way they are provided. It provides palmitoleic and oleic acids by Δ9 desaturation of coenzyme A (CoA) thioesters of palmitic and stearic acids, respectively, whereas successive Δ6 and Δ5 desaturations evoked by the front-end Δ6 and Δ5 desaturases and elongation reactions of the CoA thioesters of the essential acids linoleic acid and α-linolenic acid are necessary to biosynthesize the highly polyunsaturated acids of the n-6 and n-3 families.

These enzymes are expressed mainly in liver, which is the major site of polyunsaturated fatty acid biosynthesis and provision to other tissues.

The desaturating enzymes modulate the unsaturated fatty acid biosynthesis, and their activity is regulated by diet components, hormones and other factors as well as by competition among the different acids [1]. However, we have demonstrated that many hormones, like corticoids, testosterone and 17-β-estradiol, upregulate Δ9 desaturase and downregulate Δ6 and Δ5 desaturases. In the case of experimental diabetes mellitus type 1, unlike diabetes type 2 characterized by insulin deficiency, they have been known since the 1960s to depress the biosynthesis of unsaturated fatty acids of the n-6, n-3 and n-9 families. This was evoked by decreasing the gene expression of the corresponding messenger RNAs (mRNAs) and enzymatic activities of Δ9, Δ6 and Δ5 desaturases [2–6] and that activity was recovered by insulin administration. Moreover, Waters and Ntambi [7] and Rimoldi et al. [8] have demonstrated that the insulin-dependent recovered expression of stearoyl-CoA desaturase-1 (SCD-1) (current name of one of the Δ9 desaturases) and Δ6 desaturase mRNAs is not a direct effect of insulin, but it is evoked by the previous biosynthesis of one or more proteins.

Since the studies by Shimomura et al. [9] and Matsuzaka et al. [10] and other authors, this protein has been considered to be mainly represented by the transcription factor sterol receptor element binding protein-1c (SREBP-1c) since insulin induces the transcription of the *srebp-1c* gene into the precursor SREBP-1c, which after a hydrolytic step is converted to the nuclear active form nSREBP-1c, which then reaches the nucleus and activates the expression of the three desaturases.

In addition, peroxisome proliferator activated receptor α (PPAR-α) (NR1-C1), which together with PPAR-β/PPAR-δ (NR1C2) and PPAR-γ1 and PPAR-γ2 (NR1C3) belongs to the nuclear receptor superfamily, is activated by xenobiotic agonists like fenofibrate and endogenous ligands like unsaturated fatty acids in general. Although the active PPAR-α is characterized by its catabolic effect on lipid metabolism, it has also been shown to enhance the activity of the fatty acid desaturases [11]. However, the effects are only evoked after heterodimerization with the retinoic X receptor (RXR).

srebp-1c, according to Repa et al. [12], is also a target gene of the nuclear liver X receptors (LXR) LXR-α (NR1-H3) and LXR-β (NR1-H2), which are activated by oxysterol and are considered as intracellular cholesterol sensors. Also, LXR-α, the predominant liver isoform, and RXR-α form a heterodimer and the SREBP-1c gene promoter revealed a RXR/LXR-DNA binding site that is essential for this regulation.

Moreover, polyunsaturated fatty acids suppress SREBP-1c promoter activity, and according to Yoshikawa et al. [13], this effect is evoked by inhibition of LXR binding to LXR response elements. To complete this picture, Chen et al. [14] have demonstrated a central role for LXR in insulin-mediated activation of SREBP-1c transcription and stimulation of fatty acid synthesis in liver. Therefore, LXR activates genes involved in the catabolism of cholesterol to bile acids, but it indirectly enhances lipogenic enzymes by LXR-mediated induction of SREBP-1-c.

This information mainly gathered through in vitro experiments led us to investigate the in vivo interactions among LXR, PPAR-α, SREBP-1c and insulin specifically in the modulation of hepatic Δ6 and Δ5 desaturases at three levels, the mRNA abundance, the amount of enzyme protein and the microsomal enzymatic activities, as well as the correlated fatty acid compositions, comparing them with the effects evoked on SCD-1.

The activation of LXR was achieved by administration of the exogenous agonist T091317 (T09) [15] and the activation of PPAR-α by fenofibrate. Both agonists were chosen because they are widely used in the activation of the aforementioned nuclear receptors. They were administered to streptozotocin diabetic and control rats. To assess the effect of these drugs on the nuclear receptors, peroxisomal acyl-CoA oxidase activity as well as SREBP-1 and nSREBP-1 were tested. In addition to the measurement of the desaturases, the effect on hepatic palmitoyl-CoA elongase was also investigated.

Experimental Procedure

Materials

[1-^{14}C]Stearic acid (58 mCi/mmol), 98% radiochemically pure, [1-^{14}C]α-linolenic acid (53 mCi/mmol), 97% radiochemically pure, and [1-^{14}C]eicosa-8,11,14-trienoic acid (52 mCi/mmol), 97% radiochemically pure, were purchased from Amersham Biosciences (Little Chalfont, UK), PerkinElmer (Boston, MA, USA) and New England Nuclear (Boston, MA, USA), respectively. Unlabeled free fatty acids were provided by Doosan Serdary Research Laboratories (Toronto, Canada).

Cofactors used for enzymatic analysis and fenofibrate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade solvents were purchased from Carlo Erba (Milan, Italy).

Rat complementary DNAs of SCD-1, Δ6 desaturase and Δ5 desaturase were kind gifts from Juris Ozols (Department of Biochemistry, University of Connecticut, Central Health, Farmington, CT, USA), Tsunehiro Aki (Department of Molecular Biotechnology, Hiroshima, Japan), and Reza Zolfaghari and Catharine Ross (Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA, USA), respectively. Restriction enzymes and other molecular biology reagents were obtained from Promega (Madison, WI, USA). LXR agonist T09 was a kind gift from Amgen (South San Francisco, CA, USA).

Animal Treatment

Male Wistar rats from our animal room, weighing 170–200 g, were used. Animal care followed international rules for experimentation with animals. They were fed on a complete commercial diet, containing 5.5% of lipids (Cargill, Buenos Aires, Argentina) and water ad libitum. The fatty acid composition of the food was 22.5 wt% 16:0, 1.1 wt% 16:1, 13.7 wt% 18:0, 25.9 wt% 18:1n-9, 2.5 wt% 18:1n-7, 30.7 wt% 18:2n-6 and 3.4 wt% 18:3n-3.

Diabetes type 1 was induced in nonfasted rats by intravenous injection of streptozotocin (70 mg/kg body weight) dissolved in 10 mM citrate buffer, pH 4.5, while control rats received only the vehicle. A week later, streptozotocin-treated rats were fasted for 12 h, and glycemia was determined by a commercial enzymatic method (Wiener Lab Test, Rosario, Argentina). Only animals with glycemia above 300 mg/dl were considered diabetic.

The rats were then separated into four control groups, three of which received the following by oral gavage: first group, 10 mg/kg weight/day T09 for 6 days; second group, 100 mg/kg weight/day fenofibrate for 9 days; third group, same doses of both drugs, fenofibrate for 9 days and T09 for the last 6 days.

The streptozotocin diabetic rats were separated into four groups, of which three received the following by oral gavage, where appropriate: first group, T09 for 6 days as before; the second group was injected with 5 U/kg weight/day glargine insulin for 6 days; the third group received simultaneously the insulin and T09 treatment for 6 days.

After the periods indicated, the animals were fasted overnight and killed by decapitation without anesthesia, at the same time, in the morning to avoid circadian rhythm effects.

Blood Parameters

Glycemia, insulinemia [16] and cholesterolemia (by a commercial enzymatic method, Wiener Lab Test, Rosario, Argentina) were measured.

Liver Organelle Fractionation

After killing the animals, livers were excised and homogenized in a solution (1:3 wt/vol) composed of 0.25 M sucrose, 0.15 M KCl, 9.1 mM EDTA, 1.41 mM *N*-acetyl cysteine, 5 mM $MgCl_2$ and 62 mM phosphate buffer (pH 7.4). Samples were centrifuged at 10,000*g* for 30 min and postmitochondrial supernatant was used for fatty acyl-CoA oxidase assay. Microsomes were separately by differential ultracentrifugation at 100,000*g* (Beckman Ultracentrifuge) as usual. Protein concentration was measured according to the procedure of Lowry et al. [17].

Lipid Analysis

Lipids were extracted from liver homogenate and microsomes according to the procedure of Folch et al. [18]. The samples were esterified with F_3B at 64°C for 3 h.

The fatty acid composition of microsome total lipids was determined by gas–liquid chromatography of their methyl esters. They were injected into an Omega Wax 250 (Supelco, Bellefonte, PA, USA) capillary column of 39 m, 0.25-mm inner diameter and 0.25 μm film. The temperature was programmed to obtain a linear increase of 3 °C/min from 175 to 230 °C. The chromatogram peaks were identified by comparison of their retention times with those of authentic standards.

Enzymatic Activity Assays

Acyl-CoA oxidase was assayed in the liver postmitochondrial supernatant by measuring palmitoyl-CoA dependent H_2O_2 production according to the spectrophotometric method of Small et al. [19].

The SCD-1 activity was estimated in hepatic microsomes using as a substrate 30 μM [1-^{14}C]stearic acid, the Δ6 desaturation using 40 μM [1-^{14}C]α-linolenic acid and the Δ5 desaturation using 40 μM [1-^{14}C]eicosa-8,11,14-trienoic acid. The acids were incubated with 2.5 mg of microsomal protein in a final volume of 1.5 ml at 36 °C for 15 min. The reaction mixture consisted of 0.25 M sucrose, 0.15 M KCl, 1.41 mM *N*-acetyl-L-cysteine, 40 mM NaF, 60 μM CoA (sodium salt), 1.3 mM ATP, 0.87 mM NADH, 5 mM $MgCl_2$ and 40 mM potassium phosphate buffer (pH 7.4). After incubation, free fatty acids were dissolved in methanol/water/acetic acid (85:15:0.2 by volume), and fractionated by reversed-phase high-performance liquid chromatography on an Econosil C18, 10-mm particle size, reversed-phase column (250 mm × 4.6 mm) (Alltech Associates, Deerfield, IL, USA), coupled to a guard column (10.4 mm filled with pellicular C18). The mobile phase consisted of methanol/water/acetic acid (90:10:0.2 by vol) at a flow rate of 1 ml/min. The Merck–Hitachi (Darmstadt, Germany) L-6200 solvent delivery system was used. The eluate from the colum was monitored by a UV spectrometer at 205 nm for fatty acid identification on the basis of retention times. The effluent was mixed with Ultima Flo-M scintillation cocktail (Packard Instruments, Downers Grove, IL, USA) at a 1:3 ratio, and the radioactivity was measured by passing the mixture through an online Radiomatic Instruments (Tampa, FL, USA) Flo-One-β radioactivity detector fitted with a 0.5-ml cell.

The assay for the microsomal chain elongation of palmitoyl-CoA was performed by measuring the incorporation of [2-^{14}C]malonyl-CoA into palmitoyl-CoA [20].

Measurement of mRNA of Desaturases

Total liver RNA of different animals tested was isolated with a Wizard RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Twenty micrograms of total RNA was size-fractionated on a 1% agarose/formaldehyde gel and then transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA, USA). SCD-1, Δ6 desaturase and Δ5 desaturase, and β-actin probes were prepared by incorporating [^{32}P]deoxycytidine 5′-triphosphate by random prime labeling. Northern blot hybridization analyses were performed as described by Sambrook et al. [21]. The radioactive signals for mRNAs of SCD-1, Δ6 desaturase and Δ5 desaturase were quantified using a Phosphorimager apparatus (Molecular Dynamics, Sunnyvale, CA, USA). They were normalized to mRNA for β-actin with all the mRNAs probed on the same gel. Northern blot analyses were performed using an unpaired *t* test.

Western Blot Analysis

Following the method performed by D'Andrea et al. [22], a polyclonal anti-rat Δ6 desaturase antibody was produced. The 131 N-terminal amino acid section of the enzyme was selected for the construction of the glutathione *S*-transferase/Δ6 desaturase fusion protein, using PCR amplification with the forward primer 5′-CAGTGGATCCATGGGGGAAGGGAGGTA-3′ and the reverse primer 5′-CTGCTCGAGTCACAG GTGGTT GGTTTTGAAAAGG-3′. The purified bacterial expressed fusion protein was used as an immunogen on rabbits.

Total microsomal protein samples (70 μg per lane) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was probed with the anti-Δ6 serum obtained (1:33,000 dilution) and then with horseradish peroxidase conjugated anti-rabbit (Sigma) (1:5,000 dilution), using PBS-T buffer [11.5 g anhydrous disodium hydrogen orthophosphate, 2.96 g sodium dihydrogen orthophosphate, 5.84 g sodium chloride per liter, pH 7.5, with 0.1% (v/v) Tween 20], containing 5 and 1% nonfat dried milk, for saturation and incubation with antibodies, respectively. Washes were carried out using PBS-T buffer. Finally, peroxidase activity was revealed using Pierce ECL Western blotting substrate (Pierce, Rockford, IL, USA) and quantified using Kodak Digital Science software (Kodak, Rochester, NY, USA).

For SREBP-1 measurements, cytoplasmic and nuclear protein extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA). They were subjected to Western blot analysis as indicated above, except that anti-SREBP-1 antibody, K-10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was used as the primary antibody (1:200 dilution).

Statistical Methods

Data are expressed as means ± standard deviation. Statistically significant differences among groups were

assessed by analysis of variance (Instat version 2.0, Graph Pad Software, San Diego, CA, USA). The Tukey–Kramer multiple-comparison test was used. Statistical significance was defined as $P < 0.05$.

Results

Effects of Fenofibrate and T09 on Acyl-CoA Oxidase and SREBP-1

To confirm that the administration of fenofibrate activated PPAR-α, we measured, as usual, the effect evoked on its specific hepatic target enzyme, the extramitochondrial acyl-CoA oxidase. Figure 1 shows clearly that fenofibrate evoked an eightfold increase of the enzyme activity, and that the addition of T09, a specific agonist of LXR, did not modify that increase. On the other hand, the activating effect of T09 administration on the LXR response was assessed by the induced expression of the LXR target gene *srebp-1* [15]. Figure 1b confirms that in our experimental conditions the administration of T09 to nondiabetic rats enhanced LXR activity as evidenced by a 7.3-fold increase of the hepatic 125-kD SREBP-1 precursor and a 3.8-fold increase of the nuclear active 68-kD n-SREBP-1. SREBP-2 was not measured because it is encoded by a different gene to that of SREBP-1, and it preferentially activates cholesterol synthesis [14].

Effects of T09 and Fenofibrate on Insulinemia and Cholesterolemia

Both in control rats, in which the level of insulin is normal, and in diabetic animals with undetectable insulinemia, T09 administration in the doses tested did not modify significantly glycemia, cholesterolemia or the corresponding insulinemia after 6 days of treatment (data not shown). On the other hand, fenofibrate administration to control animals for 9 days significantly increased insulinemia from 1.32 ± 0.61 to 2.36 ± 0.13 ng/ml ($P < 0.05$), confirming our previously published data [11], while it did not alter glycemia and cholesterolemia (data not shown). The simultaneous administration for the last 6 days of 10 mg/kg weight/day of T09 to the fenofibrate-treated control animals did not alter glycemia, while insulinemia increased (3.13 ± 0.47 ng/ml) ($P < 0.001$).

Fenofibrate enhancement of the circulating insulin level in normal rats is attributed to increased expression and activity of PPAR-α in view of the fact that using the test usually employed by other authors we found an increase of the target enzyme acyl-CoA oxidase (Fig. 1).

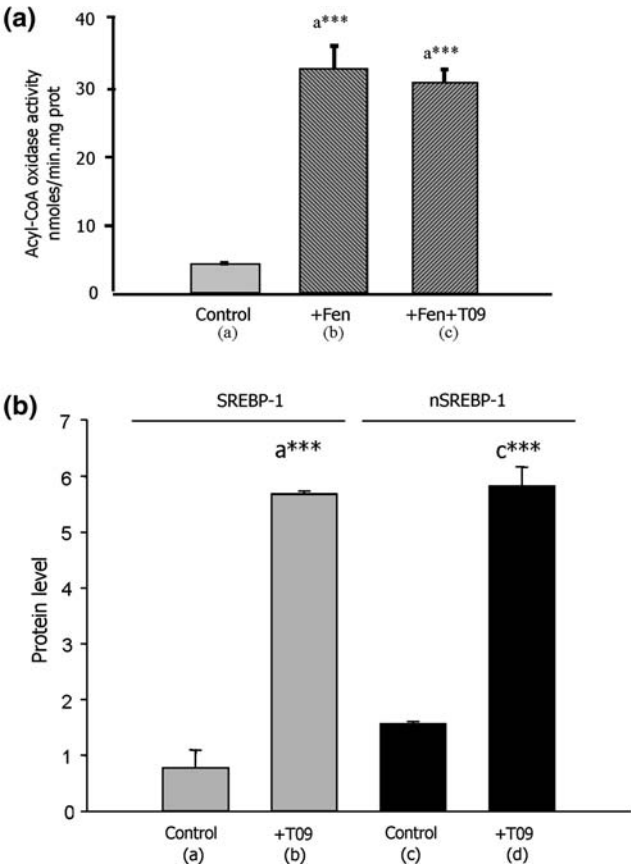

Fig. 1 Assessment of the efficacy of fenofibrate and T0901317 (*T09*) in in vivo activation of peroxisome proliferator activated receptor α (*PPAR-α*) and sterol regulatory element binding protein-1 (*SREBP-1*). **a** Effect of fenofibrate and T09 on extramitochondrial acylcoenzyme A oxidase activity in nondiabetic rats. Results expressed in nanomoles per minute per milligram of protein are the mean of four animals ± the standard deviation (*SD*). **b** Effect of T09 on SREBP-1 precursor and nuclear active nSREBP-1 forms. Cytoplasmic and nuclear protein extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents and were subjected to Western blot analyses. Results expressed in arbitrary units are the mean of four animals ± SD. Statistical differences were analyzed by analysis of variance (*ANOVA*). $P < 0.001$

On the other hand, the addition of T09 to the control rats treated with fenofibrate evoked a specific depression of cholesterolemia from 53.3 ± 6.6 mg/100 ml for the control rats, 46.5 ± 14.3 mg/100 ml for control rats treated with T09 and 60.9 ± 6.6 mg/100 ml for control rats treated with fenofibrate, to only 25.7 ± 5.7 mg/100 ml ($P < 0.001$) for control animals treated with fenofibrate and T09. In consequence, these results suggest an apparent interaction between PPAR-α and LXR in cholesterol-trafficking modulation.

Diabetic animals showed, as usual, significant increases of glycemia, no change of cholesterolemia and nearly undetectable insulinemia. T09 administration

did not significantly modify the levels of the aforementioned parameters as found in the diabetic animals and insulin increased insulinemia to about 0.20 mg/ml and normalized glycemia (data not shown).

Activating Effect of T09 and Fenofibrate on Fatty Acid Desaturase mRNA Levels

The effect of T09 and fenofibrate on control rats and that of T09 and insulin on the mRNAs levels of Δ6 and Δ5 desaturases and SCD-1 in diabetic animals are described in Fig. 2. It shows that not only the transcriptions of SCD-1 mRNA are modified but also that the mRNAs of Δ6 and Δ5 desaturases follow in general a similar pattern of changes.

Specifically Fig. 2 shows clearly that both in control and in diabetic rat livers, the mRNAs of SCD-1 and Δ6 and Δ5 desaturases were enhanced by the administration of LXR specific agonist T09, demonstrating that LXR activation evokes a similar insulin-independent increase of the three mRNAs. Therefore, these results support again, as found by Shulz et al. [15] and others, that LXR activates the *scd-1* liver expression, but now we add that this nuclear receptor also activates mRNA expression of Δ6 and Δ5 desaturases and the corresponding polyunsaturated fatty acid biosynthesis. Since we found that LXR-α agonist T09 not only enhanced the expression of the three desaturases, but also the hepatic cytosolic SREBP-1 precursor (125-kD spot) and its nuclear active form nSREBP-1 (68-kD spot) (Fig. 1), our results are also consistent with a SREBP-1c mediated LXR-α/RXR-α dependent desaturase activation in which the secreted insulin through activation of SREBP cleavage activating protein (SCAP) [23] would cleave SREBP-1c to its nuclear active form.

Moreover, a large increase of the mRNA levels of Δ5 and Δ6 desaturases and SCD-1 was evoked by the T09 administration to the diabetic animals simultaneously treated with insulin (Fig. 2). This indicates an extraordinary hyperactivation of the expression of these enzyme mRNAs owing to a cooperative effect of LXR-α and insulin. Although LXR-α activates the expression of the mRNA of the three enzymes, both in normal rats having a normal insulin cycle secretion and in diabetic animals with undetectable insulinemia, only when LXR-α along with insulin were administered to the diabetic rats was the unexpectedly high synergic effect evoked. This effect could be explained by a SREBP-1c confluent stimulation.

In control rats, the administration of fenofibrate, a PPAR-α agonist, evoked an increase of Δ5 desaturase and SCD-1 mRNAs (Fig. 2). A similar effect on Δ6

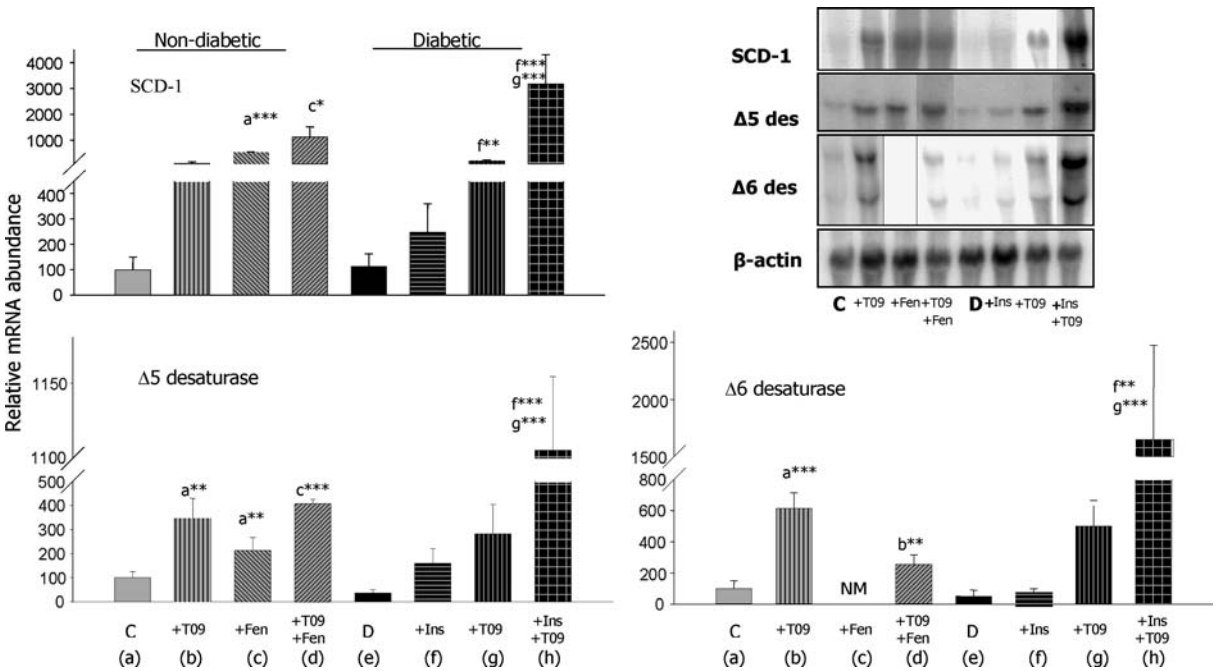

Fig. 2 Effects of T09 and fenofibrate (*Fen*) on control rats (*C*) and T09 and insulin (*Ins*) on diabetic rats (*D*) on the messenger RNAs (*mRNAs*) of stearoylcoenzyme A desaturase-1 (*SCD-1*), and Δ5 and Δ6 desaturases. Hepatic mRNA signals of desaturases as described in the text were quantified using a Phosphorimager apparatus and normalized to mRNA of β-actin. They are expressed in arbitrary units referred to control rats (100) as the mean of four animals ± SD. Statistical differences were analyzed by ANOVA in nondiabetic and diabetic animals, respectively. Different *superscript letters* indicate differences between this value and that in the group indicated by that letter. ***$P < 0.001$; **$P < 0.01$. *ND* not determined

desaturase mRNA, though not measured here, has been found by Montanaro and Brenner in an independent experiment (personal communication). In addition, Montanaro et al. [11] found an enhancement of Δ5 desaturase and SCD-1 mRNAs not only in nondiabetic but also in diabetic rat livers after forced feeding with fenofibrate for 9 days; therefore, they demonstrate that PPAR-α activation evoked an increase of the mRNAs of the three desaturases that is not related to the presence of insulin.

The simultaneous administration of T09 and fenofibrate to control rats also evoked an increase of mRNAs Δ6 desaturase, Δ5 desaturase and SCD-1. However, whereas the increases of Δ5 desaturase and SCD-1 were on the same order as for those rats administered T09 alone, the enhancement of Δ6 desaturase mRNA was smaller. Anyhow, this demonstrated the absence of an additive stimulating effect between LXR-α/RXR-α and PPAR-α/RXR-α in the transcription of mRNA of desaturases.

Activating Effect of T09 and Fenofibrate on Fatty Acid Desaturase Enzymatic Activities

Figure 3 displays the individual and combined effects of T09 and fenofibrate, as well as those induced by insulin, T09 and their combination on the three liver desaturase activities in control and diabetic rats, respectively.

In control rats the results show rather similar effects of T09 and fenofibrate on the SCD-1, Δ5 desaturase and Δ6 desaturase activities. On the other hand, they correlate fairly well with those changes evoked by the same factors on the mRNA expression of SCD-1, Δ5 desaturase and Δ6 desaturase in the same group of rats.

As in the case of these mRNAs, the three desaturating enzymes were activated by T09 both in control animals and in diabetic rats, confirming that LXR-α activation may evoke an insulin-independent increase of activities of hepatic SCD-1, Δ6 desaturase and Δ5 desaturase.

However, since the activations of SCD-1, Δ5 desaturase and Δ6 desaturase enzymes evoked in diabetic rats by T09 or T09 plus insulin as shown in Fig. 3 were quantitatively equivalent and no up-shooting was found by treatment with T09 plus insulin, we may deduce that the overexpression of the desaturase mRNAs did not produce enzyme-equivalent proteins synthesis and folding since this was not correlated by similar increases of the enzymatic activities.

The changes evoked by T09, fenofibrate and fenofibrate plus T09 treatment on the translation of the Δ6 desaturase in the control nondiabetic rats were also

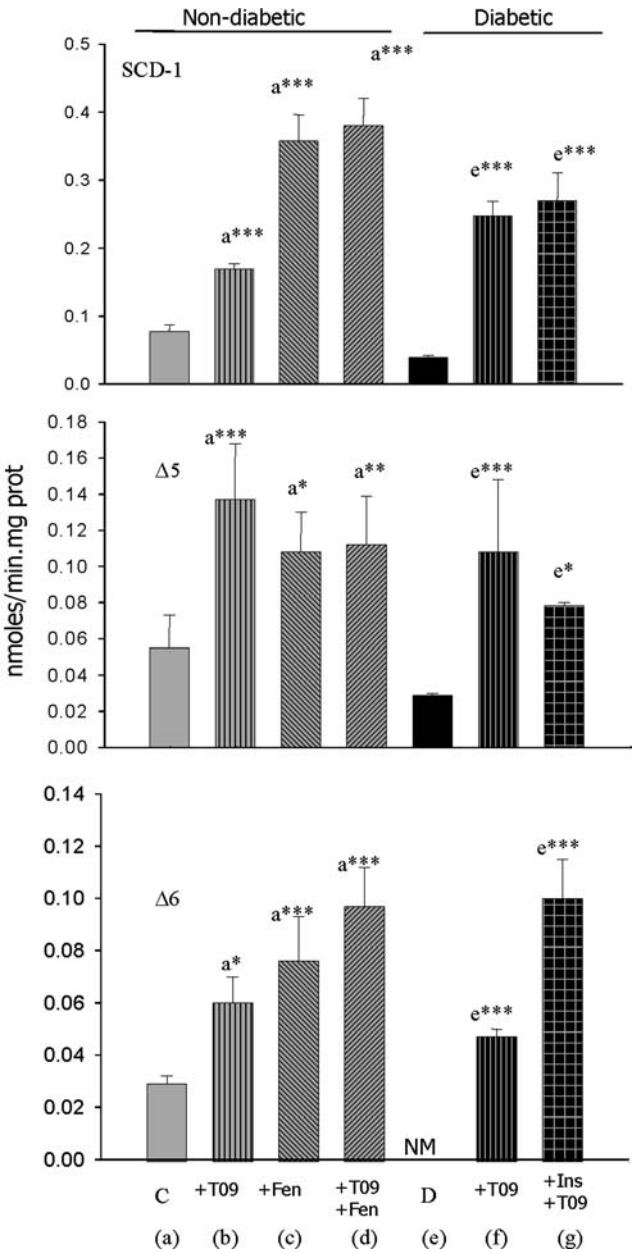

Fig. 3 Enzymatic activities of hepatic SCD-1 and Δ6 and Δ5 desaturases in nondiabetic and diabetic rats after T09 and fenofibrate treatment in vivo. Activities were measured as described in "Experimental Procedures" and are expressed in nanomoles per minute per milligram of protein and are the mean of four animals ± SD. Statistical differences were analyzed by ANOVA as described in the legend to Fig. 2. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. *NM* not measurable, *ND* not determined

monitored by measuring the amount of Δ6 desaturase protein by immunoblotting (Fig. 4). Data show that T09, fenofibrate and T09 plus fenofibrate increased the amount of Δ6 desaturase protein in control animals in correspondence to what happened with the mRNAs and with the enzymatic activity. In consequence, they

support the idea that the increased desaturase activity in these animals was due to an increase of the amount of enzyme and that PPAR-α competes with LXR-α in the activation of Δ6 desaturase.

Effect of T09 and Fenofibrate on Fatty Acid Elongation

Fatty acid elongation is another reaction that modifies the fatty acid composition of animal lipids by converting palmitoyl-CoA to stearoyl-CoA and also the polyunsaturated fatty acids of 18 carbons to long-chain polyunsaturated acids of 20, 22 and 24 carbons.

Figure 5 shows that whereas T09 administered alone to control rats only evoked a statistically nonsignificant increase of the microsomal hepatic palmitoyl-CoA elongase, fenofibrate alone or fenofibrate plus T09 increased fivefold and eightfold, respectively, the activity of the enzyme.

In the diabetic rats the elongase activity was low, but neither insulin nor T09 administration increased significantly the palmitoyl-CoA conversion to stearoyl-CoA: however, the simultaneous administration of T09 and insulin to diabetic rats increased around tenfold the activity of the reaction, showing again a synergic effect. Therefore, these results suggest that both LXR plus PPAR-α and LXR plus insulin evoke an important cooperative effect that upregulates palmitoyl-CoA elongase.

Effect of T09 and Fenofibrate on Microsomal Liver Lipid Fatty Acid Composition

Changes in the microsomal liver lipid fatty acid composition are monitored and widely used to detect the effect of changes in the food fatty acid composition as well as alterations evoked in the endogenous biosynthesis of fatty acids that may modify the biological status of an animal.

Table 1 shows the changes evoked by the aforementioned metabolic agents in microsomal fatty acid composition after only 9 days of treatment. Throughout this short period of time, the fatty acid composition of microsomal liver lipids was in general poorly modified by the different factors tested. However, early changes evoked by PPAR-α could be detected.

In control rats, though T09 increased the activity of all the desaturating enzymes, it did not evoke significant changes in the percentage of the acids, but on the other hand fenofibrate, which also modified the activity of the desaturases, not only doubled the percentages of palmitoleic acid and oleic acid and increased arachidonic acid, but decreased by half the proportion of the biologically important docosahexaenoic acid (22:6n-3)

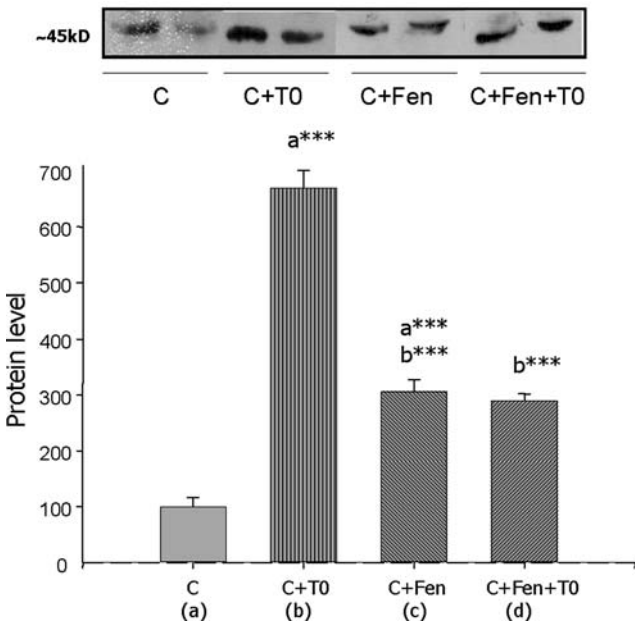

Fig. 4 Western blot analysis of Δ6 desaturase of nondiabetic rats (*C*) treated with T09 and fenofibrate (*Fen*). A polyclonal anti-rat Δ6 desaturase antibody was produced and total microsomal protein samples (70 μg per lane) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted on a Hybond-ECL nitrocellulose membrane, and the 45-kD spots were quantified as described in "Experimental Procedures." Results expressed in arbitrary units referred to nondiabetic rats (100) are the mean of three animals ± SD. Statistical differences were analyzed by ANOVA. $^{**}P < 0.01$

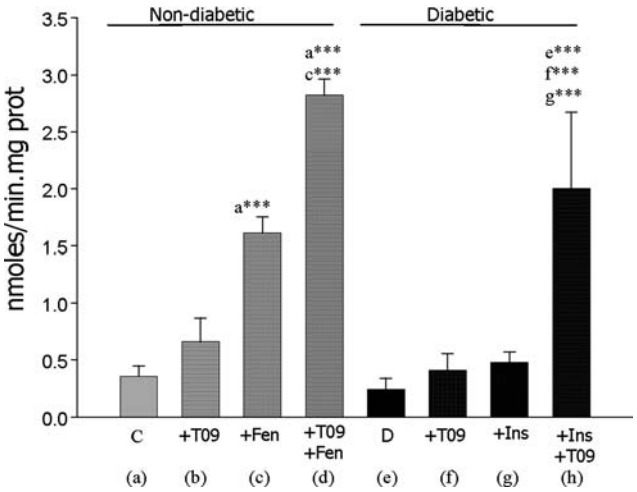

Fig. 5 Effect of T09, fenofibrate and insulin in vivo on hepatic palmitoylcoenzyme A elongase activity of nondiabetic and diabetic rats. Results expressed in nanomoles per minute per milligram of protein are the mean of four animals ± SD. Experimental conditions, abbreviations and statistical differences analyzed by ANOVA are as described in the legend to Fig. 2. $^{***}P < 0.001$

Table 1 Fatty acid composition of liver microsomal lipids (weight percent)

Fatty acid	C	C + T09	C + Fen	C + Fen +T09	D	D + T09	D + Ins	D + Ins + T09
16:0	17.42 ± 1.66	20.75 ± 4.38	25.34 ± 0.56^{a***}	19.87 ± 1.02^{c***}	17.62 ± 0.59	19.77 ± 2.59	19.59 ± 2.82	17.94 ± 3.15
16:1	0.32 ± 0.10	0.58 ± 0.07	0.61 ± 0.10^{a***}	0.74 ± 0.05^{a***}	0.32 ± 0.08	0.46 ± 0.30	0.17 ± 0.13	1.10 ± 0.16^{e***f***g***}
18:0	28.97 ± 2.19	28.77 ± 6.65	23.14 ± 0.86^{a***}	27.78 ± 0.71^{c***}	29.83 ± 1.78	26.80 ± 2.18	31.48 ± 5.86	23.38 ± 1.23
18:1n-9	4.40 ± 0.07	6.06 ± 0.95	8.78 ± 1.30^{a***}	10.88 ± 0.54^{a***}	4.75 ± 0.54	6.02 ± 0.92	4.57 ± 0.85	12.65 ± 2.51^{f***g***}
18:1n-7	1.15 ± 0.09	1.44 ± 0.23	0.85 ± 0.02^{a***}	0.77 ± 0.09^{a***}	0.90 ± 0.07	1.22 ± 0.17	0.90 ± 0.14	1.82 ± 0.17
18:2 n-6	11.03 ± 0.88	9.09 ± 2.13	9.30 ± 0.93^{a*}	7.68 ± 0.69^{c*a**}	12.21 ± 0.96	9.33 ± 0.79	11.69 ± 2.20	11.34 ± 1.69
18:3n-3	0.58 ± 0.10	0.49 ± 0.18	0.51 ± 0.09	0.44 ± 0.16	0.39 ± 0.19	0.46 ± 0.16	0.52 ± 0.20	0.36 ± 0.18
20:3n-9	0.20 ± 0.02	0.17 ± 0.05	0.29 ± 0.08	0.27 ± 0.05	0.14 ± 0.01	0.15 ± 0.02	0.17 ± 0.09	0.25 ± 0.04
20:3n-6	0.35 ± 0.05	0.67 ± 0.02	1.71 ± 0.35^{a***}	1.90 ± 0.12^{a***}	0.76 ± 0.10	0.57 ± 0.03	0.74 ± 0.23	1.06 ± 0.27
20:4n-6	28.81 ± 2.61	24.33 ± 6.62	26.21 ± 1.84^{a*}	26.53 ± 0.69^{a*}	23.89 ± 1.30	23.87 ± 2.47	22.33 ± 2.75	20.85 ± 0.84
20:5n-3	0.51 ± 0.04	0.44 ± 0.13	0.15 ± 0.03^{a***}	0.14 ± 0.01^{a***}	0.56 ± 0.04	0.58 ± 0.07	0.51 ± 0.06	0.76 ± 0.15
22:4n-6	0.47 ± 0.10	0.38 ± 0.08	0.06 ± 0.11	0.09 ± 0.11	1.02 ± 0.38^{a**}	1.46 ± 0.64^{b**}	0.80 ± 0.21	1.28 ± 0.18
22:5n-3	0.72 ± 0.08	0.68 ± 0.17	0.27 ±0.06^{a***}	0.21 ± 0.04^{a***}	0.59 ± 0.07	0.60 ± 0.07	0.58 ± 0.07	0.76 ± 0.14
22:6n-3	5.08 ± 0.45	6.17 ± 1.13	2.80 ± 0.63^{a***}	2.71 ± 0.24^{a***}	7.03 ± 0.88^{a*}	8.72 ± 1.52^{b*}	5.98 ± 1.09^{f*}	6.47 ± 0.81
Total fatty acid (mg fatty acid/mg protein)	0.27 ± 0.05	0.30 ± 0.02	0.32 ± 0.03	0.30 ± 0.02	0.33 ± 0.04	0.39 ± 0.07	0.29 ± 0.02	0.29 ± 0.03

Results are the mean of four animals ± the standard deviation. Statistical differences were analyzed by analysis of variance as described in the legend to Fig. 2

C control rats, *T09* T0901317, *Fen* fenofibrate, *D* diabetic rats, *Ins* insulin

$^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$

and of the other n-3 polyunsaturated acids of 20 and 22 carbons. Also, it enhanced palmitic acid and depressed stearic acid.

The addition of T09 to fenofibrate did not much alter the effect of fenofibrate alone, and a similar increase of the percentages of palmitoleic acid and oleic acid, an equivalent depression of 22:6n-3 as well as the other changes were found. Therefore, it seems that in normal rats PPAR-α/RXR-α activation through the fenofibrate treatment was more effective than LXR-α/RXR-α activation through the T09 administration to evoke earlier changes in the fatty acid composition of liver lipids.

On the other hand, during the short period of diabetes tested, the only important change found was the increase of 22:6n-3, and insulin injection did not modify it significantly. This delayed action of diabetes and insulin agrees with their recognized time-dependent effect in the alteration of the fatty acid composition of different animal tissues [24]. T09 administration to diabetic animals did not modify the fatty acid composition, and the increase of 22:6n-3 was maintained. Besides, insulin and T09 when simultaneously administered to diabetic rats promoted a very significant increase of the monoenoic acids, palmitoleic and oleic, but not of other acids. Therefore, SCD-1 showed a stronger and more effective response than that of Δ5 and Δ6 desaturases to these combined effectors.

Discussion

Since Burr and Burr demonstrated the essentiality of linoleic and α-linolenic acids, it has been gradually shown that their biological effects are mainly produced through their conversion to the high polyunsaturated acids of 20 and 22 carbons of the n-6 and n-3 families and their derivatives, respectively. This conversion is mainly modulated by the Δ6 and Δ5 desaturases [1] that, therefore, play a key role in maintaining the healthy status of individuals. Their activity is altered in several diseases and it has been studied in depth in diabetes, in relation to inflammatory effects, oxidative stress, cholesterol metabolism, atherogenesis and, in general, cardiovascular diseases. However we have proved [1, 25] that the two main types of diabetes, type 1, evoked by insulin deficiency, and type 2, which shows normoglycemia or hyperglycemia, modify the activity of SCD-1 and Δ6 and Δ5 desaturases in opposite directions. In diabetes type 1, the three desaturases are downregulated, whereas in diabetes type 2, they are generally upregulated.

The downregulation evoked by diabetes type 1 is corrected by insulin, but we showed in the present

study that SREBP-1c, LXR and PPAR-α are also involved in this reactivation.

Firstly, we found that in control rats in which the cycle of blood insulin follows its normal rhythm of synthesis and degradation fenofibrate evoked an increase of insulinemia that exceeded the normal values. This effect was undoubtedly due to an activation of the PPAR-α/RXR heterodimer as usually tested by the enhancement of the peroxisomal specific target enzyme acyl-CoA oxidase; however, this hyperinsulinemia did not decrease the glycemia normal values, suggesting poor efficiency or the presence of compensatory metabolic effects.

The increasing fenofibrate effect on insulinemia in normal rats has already been shown and discussed in our previous publication [11] where it was found not to be evoked in insulin-treated diabetic rats. Therefore, the results suggest that PPAR-α stimulates pancreas insulin secretion and not decreased insulin degradation. However, while it is known [26] that PPARs are expressed in pancreatic β cells and many authors conclude that PPAR-α modulates insulin secretion of islets [27–29], there is no consensus on the mechanisms of action. In that respect, Tordzman et al. [30] have found by incubation of INS-1 cells, a rat insulinoma cell line, with clofibric acid for 48 h that this PPAR-α agonist decreased basal and glucose-stimulated insulin secretion. This effect would be evoked through a PPAR-α stimulated fatty acid oxidation that impairs β-cell function. However, Ravnskjaer et al. [29] proved that the expression of PPAR-α/RXR-α indeed potentiates glucose-stimulated insulin secretion in rat islets and INS-1E cells without affecting the mitochondrial membrane potential.

T09, which activates LXR-α, when administered to normal rats has no effect on insulinemia and glycemia, and it did not modify the hyperinsulinemia induced by fenofibrate, showing there was no interaction.

Notwithstanding this, Anderson et al. [31] and Ide et al. [32] found overlapping transcriptional programs regulated by PPAR-α, RXR and LXR-α in rodent liver and cross-talk between PPAR-α and LXR-α in nutritional regulation of fatty acid metabolism. These results suggested to the authors that LXR-α ligands evoked a reduction in the PPAR-α/RXR formation by competition of new LXR-α with PPAR-α for RXR in heterodimer formation, and therefore they decreased PPAR-α/RXR-α effects. This expected result, however, could not be detected in our experiment when administering T09 to these animals with increased fenofibrate-dependent insulinemia, suggesting an apparent lack of interaction in the case of pancreas insulin secretion.

The ineffectiveness of T09 to depress the normoglycemia in nondiabetic rats agrees with the results of Cao et al. [33] that showed that 1–100 mg/kg weight per day dosages of T09 were unable to evoke hypoglycemia. However, while we found that 10 mg/kg weight of T09 did not decrease hyperglycemia in our diabetic type 1 rats, they found significant decreases in hyperglycemia in obese insulin-resistant female Zucker (fa/fa) rats with doses from 3.0 to 30 mg/kg weight. That decrease was attributed by the authors to an increased insulin sensitivity and reduced gluconeogenesis derived from a depressed phosphoenolpiruvate carboxykinase. This apparent discrepancy might be due to the model of the diabetic animal studied. We used streptozotocin, insulin-deficient diabetic type 1 rats, whereas they used insulin-resistant diabetic type 2 animals [25].

However, the stimulation of LXR-α by T09 required the corresponding stimulation of PPAR-α by fenofibrate to depress the cholesterolemia that was decreased neither by LXR-α nor by PPAR-α separately. This cooperative effect between the two nuclear receptors on the depression of blood cholesterol level is due to the modulation of integrating factors of the PPAR/LXR cholesterol oxidation and efflux pathways [31, 34–36]. This suggests the convergence of the homeostatic mechanisms for cholesterol and fatty acid metabolism. In this respect, we demonstrated some time ago [37] an interaction of cholesterol with unsaturated fatty acid biosynthesis. It was shown that 1% of cholesterol in the diet for 21 days progressively activated hepatic SCD-1 mRNA transcription and activity, but it depressed enzymatic activity of Δ6 and Δ5 desaturases, modifying correlatively the fatty acid composition of liver lipids.

The present study of the effect of the mentioned nuclear receptors on liver fatty acid desaturase modulation showed, in the first place (Figs. 2, 3), that T09 administration by increasing LXR-α expression enhanced mRNA expression of hepatic SCD-1 and Δ6 and Δ5 desaturases as well as the activities of the same enzymes not only in control but also in diabetic rats with undetectable insulinemia.

The fact that LXR-α activation enhances the activity of SCD-1 has been already demonstrated in different ways in the last years by several authors [15, 38, 39], but in addition we prove here that LXR also activates Δ6 and Δ5 desaturases.

Moreover, Fig. 2 demonstrates a marked interaction between LXR-α and insulin in the modulation of the expression of fatty acid desaturases. In the first place, as indicated, LXR-α does not require the absolute assistance of insulin to evoke an enhancement of the desaturase expression, in view of the fact that in both normal and diabetic rats T09 administration increased

the mRNA expression of Δ6 and Δ5 desaturases and SCD-1 (Fig. 2). However, what is really remarkable, indicating an important insulin–LXR-α interaction, is that the activation of the LXR-α receptor not only increased per se the mRNA expression of Δ6 and Δ5 desaturases and SCD-1 in diabetic rats, but also the injection of insulin into the diabetic animals treated with T09 increased the expression strongly.

LXR-α not only enhanced the activity of the desaturases but also both SREBP-1 and nSREBP-1, and since Chen et al. [14] have already demonstrated the central role for LXR-α in the insulin activation of SREBP-1c transcription, and SREBP-1c per se has a pivotal role in expression and activity of desaturases [9, 10], the results obtained are easily explained by a collaborative effect of LXR-α and insulin in SREBP-1c activation that evokes the hyperexpression of the desaturases. The contribution of LXR-α/RXR-α is evoked through the induction of SREBP-1c gene expression as well as by an additional contribution of insulin through SCAP-dependent cleavage of the precursor SREBP-1c to the mature nuclear form nSREBP-1c [23].

This hyperexpression of the desaturase mRNAs by the joint action of LXR-α and insulin on the diabetic rats was, however, not correlated by a quantitatively similar increase of the desaturase enzymatic activities (Fig. 3), and therefore was not quantitatively transmitted to the ribosomal synthesis of desaturases.

PPAR-α activation by fibrates evokes also an increase of mRNAs and enzymatic activities of SCD-1 and Δ5 and Δ6 desaturases in control and diabetic rats as proved in this experiment and by Montanaro et al. [11]. In addition, Tang et al. [40] demonstrated that in rats,another PPAR-α activator, WY 14643, enhanced the transcription of hepatic Δ6 desaturase by more than 500%. Moreover, they found that an imperfect direct repeat DR-1 element located at –385/–373 of the human Δ6 desaturase gene possesses the ability to bind the heterodimer PPAR-α/RXR-α and subsequently to function as a PPAR-α-RE. The activation of Δ6 desaturase mRNA expression by agonists of PPAR-α was also demonstrated by Matsuzaka et al. [10] and He et al. [41]. This fact that PPAR-α activation through fenofibrate administration activates both normal and diabetic rat [11] expression and activity of the desaturases demonstrates that the fenofibrate-dependent desaturase increase found in normal rats was not due to insulin in spite of the fact that insulinemia was enhanced by fenofibrate, but it was due to the genuine action of insulin-independent PPAR-α.

The simultaneous administration of T09 to fenofibrate-treated control rats did not further enhance significantly the activation of mRNA expression and enzymatic activities of Δ5 and Δ6 desaturases and SCD-1 (Figs. 2, 3), and even the presence of fenofibrate reduced the enhancement of Δ6 desaturase mRNA evoked by T09. Besides, Fig. 4 depicts a fenofibrate- and T09-dependent enhancement of the Δ6 desaturase protein that follows a parallel pattern to the mRNA changes, but not the enzymatic activity. Therefore, we did not detect the existence, in control rats, of an additive effect of LXR-α and PPAR-α effects leading to an activation of unsaturated fatty acid biosynthesis through desaturase regulation.

The elongation of fatty acyl-CoAs is now considered another putative step in the control of polyunsaturated fatty acid biosynthesis [42]. PPAR-α as shown in Fig. 5 activates palmitoyl-CoA elongase in control rats and a simultaneous activation of LXR-α evokes a synergic effect. In that respect, according to Inagaki et al. [43] from two elongase genes identified in rats, rELO1 and rELO2, the rELO1 gene, a homologue of human ELOVL5, was shown to elongate monosaturated and polyunsaturated fatty acids of 16–20 carbons; however, its expression was little modified by fasting or rat refeeding. On the other hand, the rELO2 gene expression in hepatocytes, which is responsible for palmitic acid elongation, was activated by fasting and refeeding cycles of the rats [42, 43]. Besides, a similar long chain fatty acid elongase that elongates saturated and monounsaturated acids was found to be activated in mouse liver by nSREBP-1c overexpression and T09 administration [44]. Therefore, a cross-talk exists between LXR-α and PPAR-α and LXR-α and insulin in elongase regulation. The mechanism and consequences of this activation are an interesting field for further investigation.

The translation of insulin, PPAR-α, LXR and SREBP activation of desaturase mRNA expression to the desaturases and their effects on liver fatty acid composition after a short treatment are depicted in Table 1. During this short period of time, the depressing effect of diabetes on the desaturase enzymatic activity was not found to be transferred to a statistically significant reduction of the unsaturated fatty acids. However, the activation of LXR-α by T09 and the simultaneous insulin administration to the diabetic rats, which evoked the highest desaturase expression, enhanced specifically palmitoleic and oleic acids synthesized by SCD-1, but not the Δ6 and Δ5 desaturase-dependent polyunsaturated acids. Therefore, that result is probably the consequence of a strong effect of LXR-α and insulin interaction on a SREBP-1c dependent activation specific for the SCD-1 desaturase reaction since it could not be detected after T09 administration to control and diabetic rats.

PPAR-α, in contrast, enhanced palmitic acid and depressed the amount of stearic acid, as already shown by Montanaro et al. [11], though it activated palmitoyl-CoA elongase (Fig. 5) and increased palmitoleic and oleic acids in correlation with an increase of SCD-1 activity (Fig. 3) [11, 45]. The increase of palmitic acid evoked by fenofibrate administration is difficult to understand, as attributed to an increased synthesis as already discussed by Montanaro et al. [11], in view of the antidyslipidemic, catabolic effect generally attributed to PPAR-α that would lead, as pointed out by Desvergne and Wahli [46], to the simultaneous activation of both anabolic and catabolic effects producing a futile cycle. However, in spite of the fact that the PPAR-α mediated activation of the malic enzyme gene generating the NADPH required for fatty acid synthesis might lead to lipid increases, on the other hand the coordinate genetic activation of acyl-CoA synthase, carnitine palmitoyl transferase I, medium-chain acyl-CoA dehydrogenase and liver fatty acid binding protein would evoke an increase of mitochondrial fatty acid oxidation [46], and we consider that the increase of palmitic acid in liver phospholipids does not necessarily indicate an enhanced fatty acid biosynthesis antagonistic to the catabolic effect. We suggest that a possible PPAR-α dependent activation of a specific acyl-CoA synthase in coordination with an acyl-CoA transferase could lead to a diversion of palmitic acid to interesterify membrane phospholipids without antagonizing the catabolic pathway.

PPAR-α activation depressed the highly polyunsaturated acids of the n-3 family, 20:5n-3, 22:5n-3 and 22:6n-3, as usually observed in some treatments in spite of the increase of desaturase activity [1, 11]; therefore, it would depress the anti-inflammatory, antioxidant and anti-atherogenic effects attributed to these acids as well as the beneficial effects in brain cognitive behavioral development and glucose uptake.

Conclusions

As summarized in Fig. 6, we have been able to demonstrate the contribution of LXR-α, insulin, SREBP-1c and

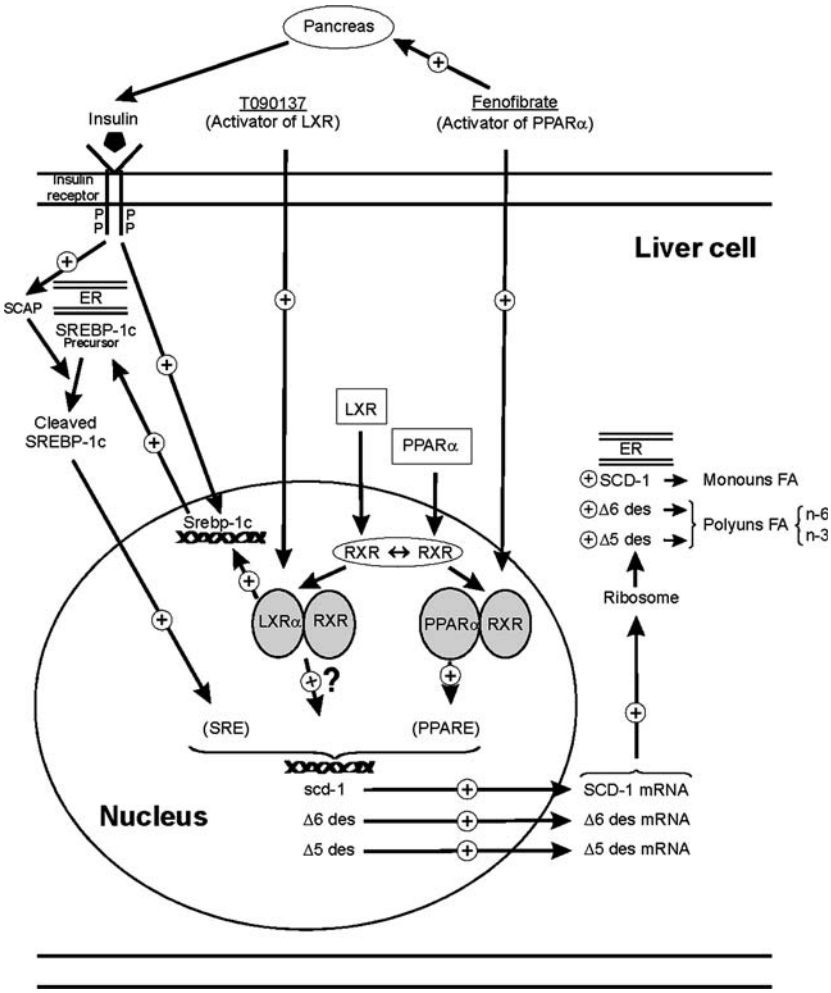

Fig. 6 LXR-α, PPAR-α, SREBP-1c and insulin interactions on modulation of liver fatty acid desaturases and unsaturated fatty acid biosynthesis

PPAR-α in vivo interaction in the sophisticated modulation of SCD-1 and Δ6 and Δ5 hepatic desaturases. Briefly, LXR-α activation increases Srebp-1c expression. Also insulin increases srebp-1c expression in addition to SREBP-1c hydrolysis to the nuclear active form. These increases enhance the mRNAs of SCD-1 and also of the Δ6 and Δ5 desaturases. LXR-α and insulin interact evoking a large cooperative enhancement of mRNAs of the desaturases. PPAR-α also activates the expression of the desaturases, but independently of the contribution of SREBP-1c and insulin. LXR-α and PPAR-α interact through the coparticipation of RXR-α in the formation of the corresponding heterodimers LXR-α/RXR-α and PPAR-α/RXR-α in the activation of mRNAs of the desaturases and the enzymatic activities reach levels not higher than those evoked by LXR-α. However, the level of expression and the enzymatic activities reached require some time to concomitantly modify the fatty acid composition of tissue lipids. Therefore, the biosynthesis of unsaturated fatty acids is modulated in vivo by the interplay of all these factors that may modify the specific effects of insulin and are very important in human physiology. They must be considered for an appropriate treatment of diabetes and cardiovascular diseases.

Anyhow, it is necessary to remark that in normal and even abnormal physiological conditions, in vivo, the real modulators of these factors are the unsaturated fatty acids and derivatives which also control their own biosynthesis and are supplied in foods [47]. The structure of the unsaturated fatty acids determines also their activity [48, 49], and they compete in their own biosynthesis and even evoke retroinhibitions as demonstrated a long time ago [50].

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