Gene transcription of the retinoid X receptor α (RXRα) is regulated by fatty acids and hormones in rat hepatic cells

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Abstract This work describes the molecular mechanisms of fatty acid and hormonal modulations of the retinoid X receptor α (RXRα) in rat liver cells. We examined the effects of different fatty acids (myristic, stearic, oleic, linolenic, and arachidonic acids, EPA, and the peroxisomal proliferator TTA) and several hormones (the glucocorticoid analogue dexamethasone, insulin, and retinoic acid) on the RXRα mRNA and protein levels in rat hepatoma cells and cultured hepatocytes. The fatty acids induced the RXRα gene expression resulting in up to 3-fold induction. Dexamethasone alone induced the mRNA level and, in combination with fatty acids, an additive or synergistic effect was observed. The dexamethasone-increased mRNA level was obliterated by insulin. The same pattern of regulation of the protein level was observed when determined in cultured hepatocytes, but the induced protein level showed a lower magnitude of stimulation than the mRNA level. This could indicate a post-transcriptional modulation of the RXRα gene expression. Time course studies showed a maximal induction of mRNA and protein levels after 18 h and 48 h, respectively. Our results uniformly show that the RXRα gene expression is under distinct regulation by fatty acids and hormones which suggests a coupling with the lipid metabolizing system and the hormonal signaling pathway.—**Steineger, H. H., B. M. Arntsen, Ø. Spydevold, and H. N. Sørensen.** Gene transcription of the retinoid X receptor α (RXRα) is regulated by fatty acids and hormones in rat hepatic cells. *J. Lipid Res.* 1998. **39:** 744–754.

Supplementary key words dexamethasone • insulin • mRNA level • protein level • regulation

Liver peroxisomal genes may be induced by a variety of peroxisomal proliferators as well as by natural factors such as high-fat diets (1–6). Transcriptional activation by peroxisomal proliferators and fatty acids is thought to be mediated by the peroxisomal proliferator activated receptor (PPAR) (7), which regulates the expression of several target genes, crucial for lipid metabolism (8), by binding to specific PPAR response elements (PPREs) (9–13). PPARs belong to the thyroid hormone and retinoic acid receptor family within the nuclear hormone receptor superfamily.

It has been shown that PPARs bind synergistically to PPREs in vitro through cooperative interactions with the retinoid X receptor (RXRs) (14). The PPARs, the retinoic acid receptors (RARs), the vitamin D_3 receptor (VDR), and the thyroid hormone receptor (TR) all preferentially bind to their hormone response elements in vitro as heterodimers complexed by retinoid X receptor (RXR) (reviewed in refs. 15–18). There are two classes of retinoid receptors (RARs and RXRs) that bind all-*trans* retinoic acid (RA) and/or 9-*cis* RA, respectively. They act as sequence specific transcription factors for a variety of genes (reviewed in ref. 19). The RARs consist of three subtypes, referred to as RARα, RARβ, and RARγ (20–25). RXRs are the second class of retinoid receptors and consist of RXRα, RXRβ, and RXRγ (15, 26–29). Gene knock-out studies in mice (30–33) have confirmed the function of the RXR heterodimer suggesting that RXRs appear to be essential regulators of several signaling pathways.

We have previously shown that normal fatty acids and the peroxisomal proliferator, tetradecylthioacetic acid (TTA), separately and especially in combination with dexamethasone have a strong stimulatory effect on peroxisomal β-oxidation enzymes as well as PPARα in hepatoma cells and hepatocytes in culture (34–38), and that these actions could be obliterated by insulin (36, 37). Studies conducted by Lemberger et al. (39, 40) have also shown that the gene transcription of PPARα is regulated by glucocorticoids, both as administration to cell cultures (39) and as a response to elevated levels of glucocorticoid in due to circadian

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Abbreviations: PPAR, peroxisomal proliferator activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; acyl-CoA oxidase, [E.C. 1.3.99.3].

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rhythms or due to stress in rats (40). Given what is believed to be the essential regulatory role of RXRs and the fact that RXRα is the heterodimer to PPARα, we found it interesting to investigate whether the RXRα gene transcription also was regulated by these key metabolic hormones and particularly whether the transcription of RXRα was regulated by fatty acids.

So far, little or no information is available regarding fatty acid and hormonal regulation of the RXRα gene. We examined the regulation of dexamethasone, insulin, retinoic acid, and fatty acids on RXRα gene expression in rat hepatoma cells and hepatocytes to get more details about the possible physiological role of RXRα in fatty acid metabolism. The present observations represent new information regarding the regulation of the RXRα gene and they uniformly show that the RXRα gene expression is under distinct regulation by fatty acids, dexamethasone, insulin, and retinoic acid, which strongly suggests a coupling with the lipid metabolizing system and the hormonal signaling pathway.

MATERIALS AND METHODS

Materials

Horse serum was from Sera Lab (Crawley Dow, Sussex, England). Ham's F-10 medium, calf serum, anti PPLO, fungizone, penicillin, and streptomycin were from Gibco (Grand Island, NY). TTA (tetradecylthioacetic acid) was synthesized as previously described (41). Guanidinium isothiocyanate was obtained from Merck (Hohenbrunn, München, Germany). Multiprime DNA labeling systems and the ECL Western Blotting Kit were purchased from Amersham (Buckinghamshire, UK). Bio-Trans nylon filters were from ICN (Irvine, CA) and Immunobilon transfer membrane was purchased from Millipore (Milford, MA). cDNA-probe for the human ribosomal protein L27 with ATCC no. 107385 was purchased from ATCC (Rockville, MD). Polyclonal antibodies against RXRα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

Animals

Male Wistar rats of approximately 250 g were maintained in cages at 23°C in rooms with lights on from 8.00–20.00 h. All rats had free access to water and a standard commercial, low-fat diet (2.9%, w/w).

Cell culture/preparation of hepatocytes/cell treatment

Cell culture. The establishment, cloning, and cell propagation of Morris hepatoma 7800C1 cells has been described previously (42). The cells were cultivated as monolayers in 140×20 mm culture dishes (Greiner). The cells were plated at 2–4×10^5 cells per dish from cultures in plateau phase of growth, and were grown in F-10 medium with 10% horse serum, 3% fetal calf serum and incubated in a humidified atmosphere of 5% CO_2 and 95% O_2. The growth medium was supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), fungizone (2.5 μg/ml), and anti PPLO (50 μl/ml). Medium and additions were renewed every 48 h and always 24 h before harvesting the cells.

Preparation of hepatocytes. Hepatocytes from male Wistar rats were isolated by the method of Berry and Friend (43) with the modifications described by Seglen (44). The culture conditions were as described previously (45) and the cell treatment during the experimental period was the same as for hepatoma cells.

Cell treatment. TTA (50 μm), myristic acid (1 mm), stearic acid (1 mm), linolenic acid (1 mm), oleic acid (1 mm), arachidonic acid (0.3 mm), and eicosapentaenoic acid (EPA) (0.3 mm) were added to the cell cultures. The fatty acids were added as a 4 mm stock solution dissolved in 6% fatty acid-free BSA (1 mm). Dexamethasone (0.25 μm), insulin (0.4 μm), and retinoic acid (0.1 μm) were added to cell cultures alone or in combination with fatty acids during the experimental period from 4 h up to 7 days. Fifty μm concentration of TTA was used as that concentration resulted in submaximal induction of the peroxisomal β-oxidation enzymes (41). Medium and additions were renewed every second day and always 24 h before the experiment was terminated. The concentration of fatty acids was based on dose–response experiments where three fatty acids (myristic acid, oleic acid, and linolenic acid) resulted in a submaximal induction of peroxisomal acyl-CoA oxidase in rat hepatoma cells, as described earlier (38) and on dose–response experiments where linolenic acid resulted in a submaximal induction of RXRα mRNA level (see Fig. 2). The concentration of arachidonic acid and EPA was chosen based on studies by Tollet et al. (46), where dose–response studies were performed in cultured hepatocytes with 0.3 mm arachidonic acid resulting in maximal induction of cytochrome P4504A1 mRNA levels. The concentration of all-*trans* retinoic acid was based on studies by Mangelsdorf et al. (47), where dose–response studies were performed giving maximal induction of RXRα in a transactivation study. Dexamethasone was used at a final concentration of 0.25 μm, that resulted in maximal binding to glucocorticoid receptors in Morris 7800 rat hepatoma cells (34). Maximal effects on peroxisomal acyl-CoA oxidase of insulin were obtained when the concentration was equal or above 0.25 μm (35).

Northern blot analysis of mRNA

Total RNA from 7800C1 hepatoma cells and hepatocytes was extracted by the guanidinium thiocyanate method (48) as described earlier (36). Total RNA (20 μg) was denatured in 50% formamide and 6% formaldehyde by heating for 15 min at 50°C, followed by 15 min on ice, then resolved on a 1.5% agarose gel containing 6.7% formaldehyde and 20 mm sodium phosphate, pH 7.0. The RNA was visually controlled by staining with ethidium bromide, then transferred to a Bio-Trans nylon filter using 20 × SSC (1 × SSC:150 mm NaCl, 15 mm trisodium citrate, pH 7.0) and a capillary-blotting technique (49). The filter was baked for 1 h at 80°C. The membrane was then prehybridized for at least 3 h in a solution containing 5 × Denhardt, 5 × SSC, 50 mm sodium phosphate, pH 6.5, and 0.1% SDS, pH 7.2. To decrease non-specific binding, 50% deionized formamide and herring DNA were used. The filters were hybridized overnight with [α-^{32}P] dCTP-labeled probes (10^6 cpm/ml hybridization solution) at 42°C. After hybridization, the filters were washed four times in 2 × SSC with 0.1% SDS at room temperature for 5 min and 0.1 × SSC with 0.1% SDS at 45–50°C for 15 min. Autoradiography was performed using Amersham hyperfilm (MP RPN 1675) with intensifying screens at −70°C for varying lengths of time where the autoradiograms that were used for semi-quantitative scanning varied from 1 to 5 days. The sizes of the mRNA transcripts were calculated on the basis of 18S and 28S rRNA which were visualized by ethidium bromide.

cDNAs used as probes

cDNA for mouse RXRα, RXRβ, and RXRγ (15), cDNA for human RARα (21), and cDNA for the human ribosomal protein L27 purchased from ATCC were used. The [α-^{32}P]dCTP-labeled cDNA probes were prepared using a standard multiprime DNA-labeling kit (Amersham, RPN 1601 Y). Specific activities of 2–6 × 10^8 cpm/μg DNA were obtained.

Scanning

Semiquantitative results were obtained from scanning of autoradiograms using a XRS 3sc scanner and the Bio Image System from Millipore Corporation, showing linear increments within the working range used (5–30 μg RNA).

Rehybridization of filters

After autoradiography, the RNA filters were washed using 50% formamide and 10 mm sodium phosphate, pH 6.5, for 1 h at 65°C and reprobed. In general, filters treated this way revealed no signs of diminished quality, even after reprobing the filters five or six times.

Preparation of soluble cell fraction/Western blotting

The medium was removed and the cells were scraped into ice-cold 150 mm KCl and 20 mm HEPES, pH 7.4, and pelleted (700 *g*, 5 min, 4°C). The cell pellet was washed twice with the same buffer, resuspended in 1 ml of the buffer supplied with 0.2% Triton, and homogenized (Sonicator™ cell disrupter, model W-10). The homogenate was centrifuged in an Eppendorf centrifuge (max rate, 30 min, 4°C) to obtain the soluble cell fraction that represents total cell protein. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (36). Proteins were electrophoretically transferred from gel to Immunobilon transfer membranes with constant current (100 mA) for about 1 h in Bio-Rad Transblot apparatus. After transfer, the filter was incubated for 18 h with PBS including 5% fat-free dry milk powder at 4°C, and subsequently incubated for 1 h at room temperature in PBS containing 1% dry milk powder with antiserum against RXRα (1/1000). After being washed with PBS containing 0.1% Tween 20, the filter was incubated with detection reagents from the ECL-kit (Amersham) prior to autoradiography.

Statistics

For statistical analysis of the mRNA and protein level results, the mean control values were set equal to unity and variation within the group was calculated accordingly. Corresponding relative values (mean ± SEM) were calculated for the experimental groups.

RESULTS

Regulation of RXRα gene expression by fatty acids, dexamethasone and insulin in hepatoma cells and hepatocytes in culture

Measurements of the steady-state RXRα mRNA level in rat hepatoma cells and hepatocytes in culture were conducted using the semiquantitative Northern blot analysis with [^{32}P]α-dCTP-labeled RXRα cDNA as probe. Several fatty acids (1 mm or 0.3 mm), myristic acid, oleic acid, linolenic acid, arachidonic acid, and the synthetic thia fatty acid TTA (50 μm), the glucocorticoid analogue dexamethasone (0.25 μm), and insulin (0.40 μm) were used to study the effects of the RXRα gene expression. Treatment of the hepatocytes with the fatty acids for 72 h resulted in a slight increase from 2.0- to 7.0-fold induction (for myristic acid, oleic acid, linolenic acid) of the RXRα mRNA level (**Table 1**). Arachidonic acid and the synthetic fatty acid analogue, TTA, gave the strongest stimulatory effect (2.8- and 3.2-fold, respectively) in rat hepatoma cells (Table 1 and

TABLE 1. Effects of different fatty acids in combination with dexamethasone on RXRα mRNA expression
in 7800C1 hepatoma cells and cultured hepatocytes

| | Relative mRNA Values | | |
| | Hepatocytes | | Hepatoma Cells |
Treatment	24 h	72 h	72 h
Control	1.0	1.0	1.0
Dexamethasone	6.43 ± 2.21	6.76 ± 1.66	3.46 ± 0.45
Insulin	0.99 ± 0.09	1.00 ± 0.16	1.15 ± 0.32
Dexamethasone + insulin	1.33 ± 0.06	2.25 ± 0.31	2.43 ± 0.75
Myristic acid (C 14:0)	1.97 ± 0.45	1.03 ± 0.12	1.55 ± 0.50
Myristic acid + dexamethasone	3.64 ± 0.90	3.34 ± 0.36	6.37 ± 0.91
Myristic acid + insulin	nd	0.65 ± 0.33	1.46 ± 0.30
Stearic acid	nd	nd	2.08 ± 0.30
Stearic acid + dexamethasone	nd	nd	3.58 ± 0.80
Oleic acid	4.53 ± 0.47	4.22 ± 0.69	1.74 ± 0.09
Oleic acid + dexamethasone	12.46 ± 5.57	14.33 ± 4.78	3.90 ± 0.32
Linolenic acid	6.99 ± 1.37	4.85 ± 1.29	1.84 ± 0.40
Linolenic acid + dexamethasone	14.48 ± 1.10	10.44 ± 1.71	5.16 ± 0.92
Arachidonic acid	nd	nd	2.76 ± 0.52
Arachidonic acid + dexamethasone	nd	nd	10.97 ± 1.96
TTA	2.26 ± 1.53	2.91 ± 0.72	3.17 ± 0.66
TTA + dexamethasone	8.17 ± 0.93	15.69 ± 3.27	9.09 ± 2.06

Alterations in mRNA levels in cultured hepatocytes and 7800C1 Morris hepatoma cells with different fatty acids. The fatty acids were added for 24 or 72 h alone or in combination with 0.25 μm dexamethasone or insulin (0.4 μm). The fatty acids used were myristic acid (1 mm), stearic acid (1 mm), oleic acid (1 mm), linolenic acid (1 mm), arachidonic acid (1 mm), and tetradecylthioacetic acid (TTA) (50 μm) in combination with 0.25 μm dexamethasone. There were no less than three experiments in each group with two duplicates in each experiment (n = 4) and the densities of the hybridization signals are presented as relative to the control level for each experiment. The values are given as mean ± SEM; nd, not determined.

Fig. 1). In comparison, the fatty acids resulted in an overall stronger induction of the RXRα mRNA level in cultured hepatocytes than in hepatoma cells (Table 1). We investigated the effects of fatty acids, alone or in combination with dexamethasone, after 24 h and 72 h in hepatocytes (Table 1), where the strongest inductions of the RXRα mRNA level by fatty acids were observed after 24 h. The inductive effect of myristic acid on hepatocytes in culture was transient and was not observed after 72 h of treatment (Table 1). These results show a lack of fatty acid chain specificity regarding the regulatory effect on RXRα mRNA level by the different fatty acids.

Dose–response analysis of RXRα mRNA steady-state level after treatment of linolenic acid in cultured hepatocytes was conducted. The hepatocytes were treated for 24 h with different concentrations of linolenic acid; 0.15, 0.30, 0.50, and 1.0 mm (Fig. 2). When the hepatocytes were treated with 0.15, 0.30, 0.50 mm of linolenic acid, no significant regulation of RXRα mRNA levels were observed, whereas treatment with 1 mm linolenic acid resulted in an approx. 5.0-fold induction.

When hepatocytes were treated with dexamethasone, the RXRα mRNA level increased to approximately 6.8-fold after 72 h compared to control cells (Table 1). Similar hormonal regulation of the RXRα mRNA level

was observed in 7800C1 hepatoma cells (approx. 3.5-fold) after 72 h (Table 1). The combined treatment with dexamethasone and fatty acids resulted in either additive effects or synergistic effects (except for myristic acid) when treating the cultured hepatocytes) depending on the fatty acid combined. Treatment with myristic acid in combination with dexamethasone resulted in approximately 3-fold induction both after 24 and 72 h; linolenic acid in combination with dexamethasone after 72 h resulted in 10.4-fold induction; and the combined treatment with TTA after 24 h resulted in 8.2-fold induction (Table 1). When the hepatocytes were treated for 24 or 72 h with dexamethasone in combination with oleic acid, linolenic acid (for 24 h) and TTA (for 72 h), a synergistic effect was observed resulting in an induction up to 15.7-fold for TTA in combination with dexamethasone after 72 h (Table 1).

Little or no changes could be observed on the RXRα mRNA levels when hepatoma cells or cultured hepatocytes were treated with insulin alone. However, insulin effectively antagonized the stimulatory effect of dexamethasone (Table 1). To further examine the effects of insulin in combination with dexamethasone on RXRα mRNA regulation, time–kinetic and dose–response studies were conducted. Time–kinetic studies were conducted with 0.4 μm insulin in combination with 0.25
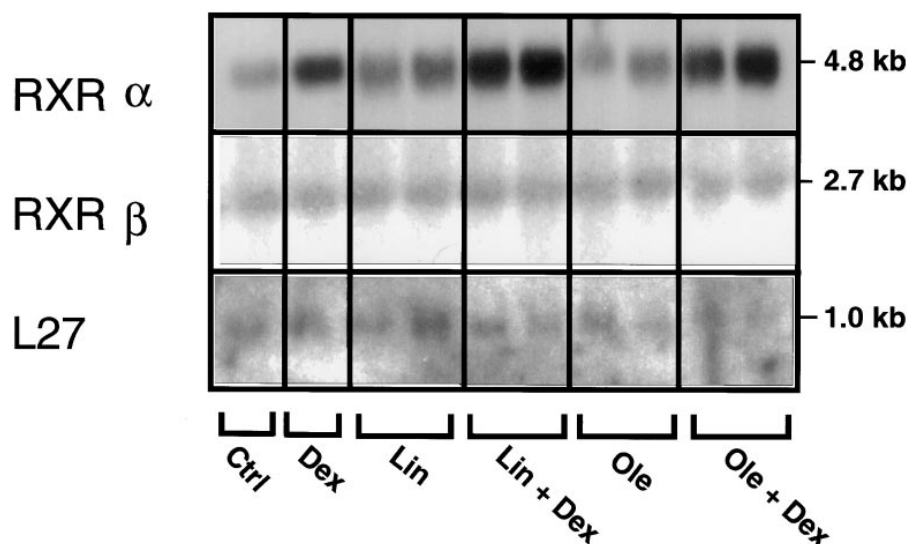
Fig. 1. Expression of RXRα, RXRβ, and L27 mRNAs in 7800C1 Morris hepatoma cells after treatment with linolenic acid and oleic acid in combination with dexamethasone. The mRNA levels of RXRα, RXRβ, and the ribosomal protein L27 were measured after Northern blot analysis of total RNA. The RNA was isolated from untreated hepatoma cells (Ctrl), and hepatoma cells treated for 72 h with 0.25 μm dexamethasone (Dex) and 1 mm linolenic acid, linolenic acid + dexamethasone (Lin + Dex), 1 mm oleic acid (Ole), oleic acid + dexamethasone (Ole + Dex). All treatments were carried out in duplicate. Of eight similar experiments (with varying treatments), the results of one experiment are shown. 18S and 28S rRNA were used as size markers to determine the size of the mRNA transcripts. Results of semiquantitative scanning of the autoradiograms are included in Table 1.

μm dexamethasone added to cultured hepatocytes and mRNA was extracted after 4, 12, 24, and 48 h. Insulin obliterated the stimulatory effect of dexamethasone al-
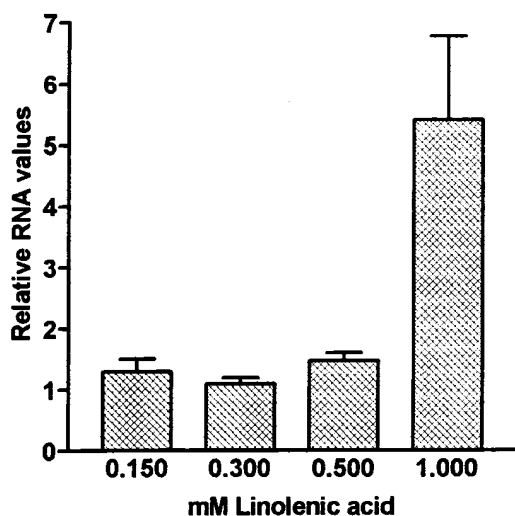
Fig. 2. Dose–response of linolenic acid in cultured hepatocytes. The effects of linolenic acid on RXRα mRNA levels were determined in cultured hepatocytes after 24 h by the Northern blotting technique as described in Materials and Methods. The treatment of the hepatocytes was with linolenic acid in different concentrations and the concentrations were from 0.15 to 1.0 mm. The values are presented relative to the control (C = 1) and given as mean ± SEM.

most completely during the whole experimental period from 4 to 24 h (data not shown). In the dose–response studies performed, cultured hepatocytes were treated with varying amounts of insulin (10 nm, 50 nm, 100 nm, and 400 nm) in combination with 0.25 μm of dexamethasone for 24 h. An inhibitory effect of insulin was observed when the concentration of insulin was equal to or greater than 50 nm (data not shown).

No significant alteration of the ribosomal protein L27 (Fig. 1), RXRβ (Fig. 1), RXRγ, and the related RARα mRNA level could be observed after the same treatments with fatty acids alone or in combination with dexamethasone or insulin (data not shown). The changes in the mRNA level of RXRα in both hepatoma cells and cultured hepatocytes, therefore, did not reflect nonspecific alterations.

Effects of retinoic acid alone and in combination with fatty acids or dexamethasone on RXRα mRNA levels in hepatocytes in culture

Cultured hepatocytes were treated for 24 h with all-*trans* retinoic acid (0.1 μm) and dexamethasone (0.25 μm) in combination with arachidonic acid (0.3 mm) and the ω–3 fatty acid EPA (0.3 mm). When the hepatocytes were treated with retinoic acid alone, a 6.5-fold induction of the RXRα mRNA level was observed (**Table 2**). No further induction of the gene expression could be

TABLE 2. Effects of retinoic acid in combination with dexamethasone, arachidonic acid, and eicosapentanoic acid (EPA) on RXRα mRNA expression in cultured hepatocytes

Treatment	Relative mRNA Values
Dexamethasone	9.40 ± 2.14
Retinoic acid	6.48 ± 0.91
Retinoic acid + dexamethasone	5.48 ± 1.64
Retinoic acid + arachidonic acid	3.86 ± 1.49
Retinoic acid + EPA	4.93 ± 0.72
Retinoic acid + EPA + dexamethasone	6.78 ± 0.88

Alterations in RXRα steady-state mRNA levels in cultured hepatocytes after treatment for 24 h. The hepatocytes were treated with 0.1 μm all-*trans* retinoic acid alone or in combination with 0.25 μm dexamethasone and the fatty acids arachidonic acid (0.3 mm) and eicosapentanoic acid (EPA) (0.3 mm). There were no less than two experiments in each group with two duplicates in each experiment (n = 4) and the densities of the hybridization signals are presented as relative to the control level for each experiment. The values are given as mean ± SEM.

TABLE 3. Effects of different fatty acids in combination with dexamethasone on RXRα protein levels in cultured hepatocytes

| Treatment	Relative Protein Levels
Control	1.0
Dexamethasone	1.63 ± 0.22
Myristic acid	1.69 ± 0.49
Myristic acid + dexamethasone	2.67 ± 0.06
Linolenic acid	1.80 ± 0.03
Linolenic acid + dexamethasone	6.20 ± 0.56
Oleic acid	2.39 ± 0.50
Oleic acid + dexamethasone	6.88 ± 0.27

Alterations in RXRα protein levels in cultured hepatocytes estimated with Western blotting. The hepatocytes were treated with different fatty acids (1 mm) alone or in combination with 0.25 μm dexamethasone for 72 h. The fatty acids used were myristic acid, oleic acid, and linolenic acid. There were no less than two experiments in each group with two duplicates in each experiment (n = 4) and the densities of the hybridization signals are presented as relative to the control level for each experiment. The values are given as mean ± SEM.

observed by combining retinoic acid with dexamethasone and EPA. A slight reduction of the induced RXRα gene expression by retinoic acid could be observed when combined with arachidonic acid (Table 2).

Western blot analysis of RXRα during treatment with different fatty acids, alone or in combination with dexamethasone in hepatocytes

Semiquantitative immunoblot analyses of RXRα after SDS-polyacrylamide gel electrophoresis were carried out using total cell proteins of cultured hepatocytes treated for 3 days as described in Materials and Methods (**Table 3** and **Fig. 3**). The overall results from the protein analysis supported the RXRα mRNA induction pattern where fatty acids alone resulted in a moderate increase of the RXRα protein level after treatment with 1 mm myristic acid (1.7-fold induction), oleic acid (2.4-fold induction), and linolenic acid (1.8-fold induction) (Table 3). Additive effects were observed in the RXRα protein level when dexamethasone was combined with myristic acid (2.7-fold induction) and a synergistic effect was observed when linolenic acid was combined with dexamethasone (6.2-fold induction) (Table 3). However, the stimulation of RXRα protein levels by fatty acids or/and dexamethasone did not result in the same degree of induction compared to the mRNA levels. The strongest effect on the RXRα protein level was observed for linolenic acid in combination with dexamethasone, resulting in a 6.2-fold induction compared to 14.8-fold induction of the mRNA level (Tables 1 and 3).

Figure 3 shows cultured hepatocytes treated with myristic acid alone or in combination with dexamethasone as an illustration of the inductive effects on RXRα protein levels that were observed.

Time–response analysis of RXRα mRNA and protein levels in 7800C1 Morris hepatoma cells and cultured hepatocytes

We conducted time–kinetic studies in cultured hepatocytes and examined both the mRNA and protein level after treatment of linolenic acid alone or in combination dexamethasone, as linolenic acid was the fatty acid with the greatest inductive effect on PPARα mRNA level (38). **Figures 4A** and **4B** show the relative changes in RXRα mRNA and protein levels as a function of time in the cultured hepatocytes, as determined by light scanning of Northern and Western blots, respectively. Both dexamethasone and the combined treatment with linolenic acid resulted in the strongest induction of the RXRα mRNA level after 18 h (approximately 12-fold), while maximal induction after treatment with linolenic acid alone was 10.3-fold (Fig. 4A). A steady decline of the RXRα mRNA level was observed for all treatments after 18 h (Fig. 4A).

Next, we analyzed the time–kinetics of the RXRα protein level in cultured hepatocytes after treatment with linolenic acid alone or in combination with dexamethasone (Fig. 4B). Linolenic acid induced the protein level maximally after 24 h (7.6-fold), while dexamethasone alone or in combination with linolenic acid resulted in the strongest induction of the protein level after 48 h, giving rise to a 10.1- and 19.9-fold induction, respectively. The RXRα protein levels declined after 48 h for treatments with dexamethasone, alone or in combination with linolenic acid, while treatment with linolenic acid alone resulted in a RXRα protein level that was fairly stable from 24 to 72 h (Fig. 4B).

In addition to time–kinetic studies in cultured hepatocytes, we performed a time–response analysis of the
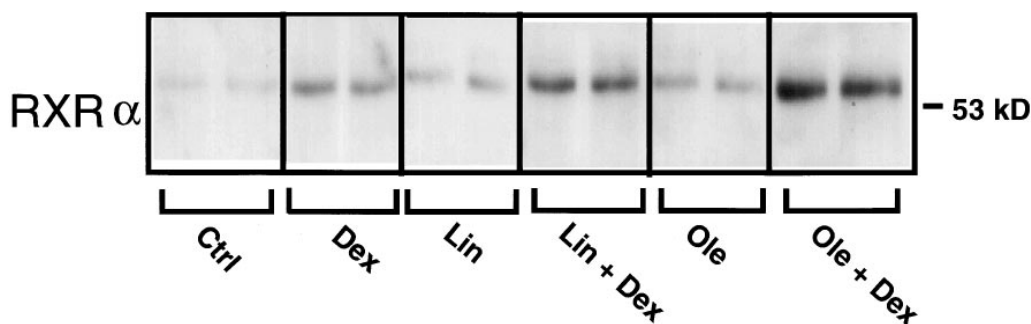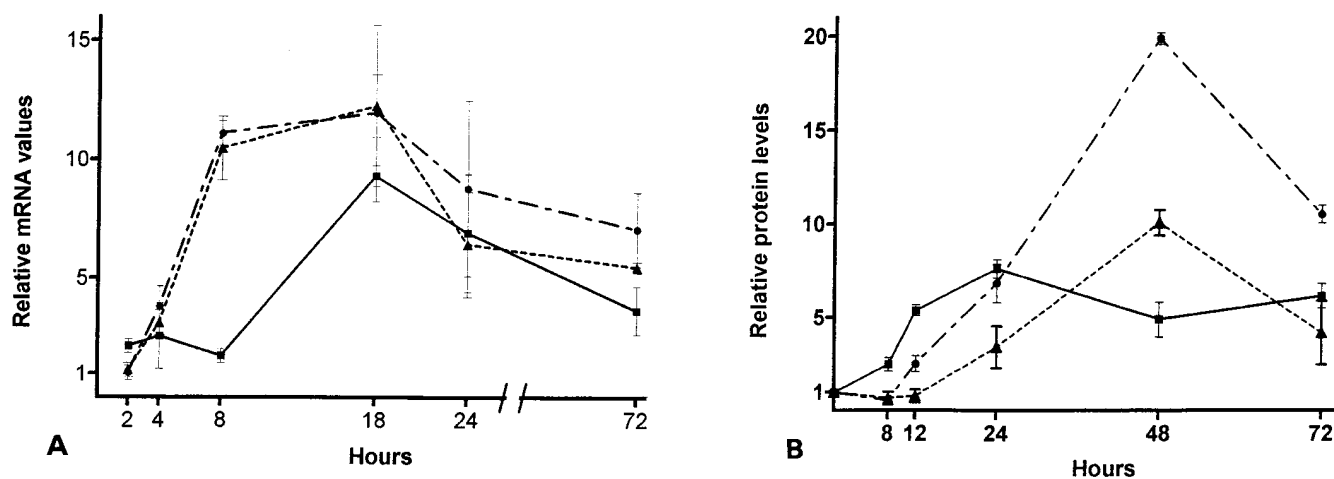
Fig. 3. Induction of RXRα protein levels by dexamethasone, alone or in combination with linolenic and oleic acid in cultured hepatocytes. RXRα protein levels from cultured hepatocytes were determined by Western blotting technique as described in Materials and Methods. The total protein extracts were from untreated cells (Ctrl), or cells treated for 72 h with 0.25 μm dexamethasone (Dex), 1 mm linolenic acid (Lin), linolenic acid combined with dexamethasone (Lin + Dex), 1 mm oleic acid (Ole), and oleic acid combined with dexamethasone (Ole + Dex). All treatments were carried out in duplicate. Of six similar experiments (with varying treatments), the results of one experiment are shown. Protein size markers were used to determine the size of the RXRα protein. Results of semiquantitative scanning of the autoradiograms are included in Table 3.

mRNA steady-state level of RXRα in 7800C1 hepatoma cells by stimulating the cells with the thia-substituted fatty acid analogue, TTA, in combination with dexamethasone as this combination had the strongest stimulatory effect on both the peroxisomal β-oxidation enzymes and PPARα shown in earlier studies (34–38). Already after 4 h a marked regulation was observed, with a 3.3-fold induction of the RXRα mRNA steady-state level (**Fig. 5**). A maximum stimulation was obtained after 12 h with a 6.0-fold induction of the RXRα

mRNA level. After 12 h the mRNA steady-state level kept more or less constant throughout the experimental period up to 7 days (Fig. 5).

DISCUSSION

Our results demonstrate that in both rat hepatocytes and hepatoma cells, RXRα gene expression is regu-

Fig. 4. A: Time-curve effects of the RXRα mRNA levels after treatment with linolenic acid alone or in combination with dexamethasone in cultured hepatocytes. The mRNA steady-state levels of RXRα were measured after semiquantitative Northern blot analysis where the effects of 1 mm linolenic acid (Lin), 0.25 μm dexamethasone (Dex), and the combination of the two (Lin + Dex) were determined in cultured hepatocytes. The treatment lasted for the indicated time periods, from 2 h to 3 days. The values are presented relative to the control (Ctrl) and given as mean ± SEM. B: Time-course effects of the RXR protein levels after treatment with linolenic acid alone or in combination with dexamethasone in cultured hepatocytes. The protein steady-state levels of RXRα were measured after semiquantitative Western blot analysis where the effects of 1 mm linolenic acid (Lin), 0.25 μm dexamethasone (Dex), and the combination of the two (Lin + Dex) were determined in cultured hepatocytes. The treatment lasted for the indicated time periods, from 8 h to 3 days. The values are presented relative to the control (Ctrl) and given as mean ± SEM. –■–, Lin; --▲--, Dex;— -●— -, Lin + Dex.

lated by hormones, including insulin and glucocorticoids, and by different fatty acids. The glucocorticoid analogue, dexamethasone, alone and especially together fatty acids, has a pronounced inductive effect on RXRα steady-state mRNA and protein levels, while insulin shows a general inhibitory action. These findings suggest that RXRα expression in liver might be subject to hormonal and fatty acid regulation. It is particularly interesting that the heterodimeric partners, PPARα and RLD-1 are also controlled by glucocorticoids, insulin and fatty acids (H. H. Steineger, S. Alberti, W. Eskild, D. Feltkamp, K. M. Gautvik, Ø. Spydevold, J-Å. Gustafsson, and H. N. Sørensen, unpublished results). We have also shown similar regulation of several PPARα target genes (34–38). These results indicate that the elevated mRNA levels observed were a combined result of an induction of RXRα gene expression and a prolonged half-life of the mRNA. However, the stimulation of RXRα mRNA levels by dexamethasone, alone or in combination with fatty acids, was higher than for the protein levels (Tables 1 and 3). This is in contrast to previous studies of the three β-oxidation enzymes (36, 37) and preliminary reports of PPARα (H. H. Steineger, S. Alberti, W. Eskild, D. Feltkamp, K. M. Gautvik, Ø. Spydevold, J-Å. Gustafsson, and H. N. Sørensen, unpublished results) where the mRNA and protein levels were essentially the same. The lower protein levels compared to the mRNA levels that were observed could indicate a regulation on the post-transcriptional level.
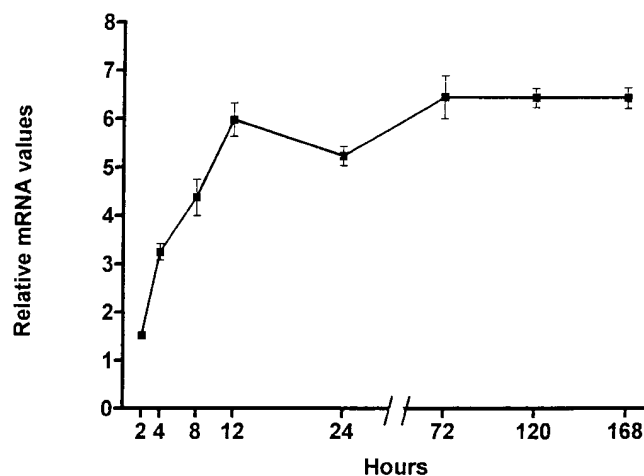
Fig. 5. Time-curve effects of TTA in combination with dexamethasone in 7800C1 Morris hepatoma cells. The mRNA steady-state levels of RXRα were measured after semiquantitative Northern blot analysis where the effects of 50 μm TTA combined with 0.25 μm dexamethasone were determined in 7800C1 Morris hepatoma cells. The treatment lasted for the indicated time periods, from 2 h to 7 days. The values are presented relative to the control (Ctrl) and given as mean $\pm$ SEM; ■, TTA + Dex.

This study clearly shows that the RXRα gene is regulated by fatty acids or its metabolites as well as being under hormonal control, demonstrating a link between the hormonal/retinoid and the intermediate metabolic pathways. The effects observed with dexamethasone treatment confirm studies done by Wan, Wang, and Wu (50), where three hepatoma cell lines were treated with different hormones and they showed that dexamethasone was the only hormone tested with effects on the mRNA levels of RXRα in these cell lines. We observed a quantitative difference in the inductive effects of dexamethasone when we compare hepatoma cells and the cultured hepatocytes, where we found a stronger stimulation by dexamethasone in hepatocytes. The magnitude of stimulation by dexamethasone in the 7800C1 hepatoma cells was comparable to the results in the three hepatoma cell lines tested by Wan et al. (50). The stronger effects we observed in hepatocytes with treatment of dexamethasone alone, or in combination with fatty acids, are in concordance with previous results on acyl-CoA oxidase and PPARα mRNA regulation (37, 38). This may be due to the fact that hepatoma cells are clonal strains of hepatic tumors, and that in these experiments, cultured hepatocytes represent a more physiological system.

In contrast to the other fatty acids used in this study, treatment with myristic acid resulted in an inhibition of the induced RXRα mRNA level by dexamethasone in cultured hepatocytes after 72 h (Table 1). Similar effects by myristic acid were also observed earlier of the PPARα mRNA level (38), but not for the peroxisomal β-oxidation enzymes (34–37). The mechanisms underlying this fatty acid effect are obscure and our previous observation that myristic acid had a stimulatory effect in hepatoma cells may indicate that the effect may be rather complex. To our knowledge, myristoylation of transcription factors has not been described but may be involved, as myristic acid has been shown to be active in several signal transduction studies (reviewed in ref. 51). We used 1 mm initial concentration of the different fatty acids in the culture medium to ensure a constant and sufficient concentration of the fatty acids. Although this concentration is superphysiological, it was well tolerated by the cells.

All-*trans* retinoic acid showed a stimulatory effect on the RXRα mRNA expression in hepatocytes after 24 h, giving a 6.5-fold induction (Table 2). The combined treatment of retinoic acid with dexamethasone did not further induce the RXRα mRNA expression. The combination with retinoic acid and arachidonic acid or EPA reduced the mRNA level (Table 2). The effect of retinoic acid on RXRα mRNA expression could partly explain the induction of peroxisomal β-oxidation enzymes by retinoic acid in cultured rat hepatocytes observed by

Hertz and Bar-Tana (52) as the heterodimer that regulates the gene transcription of these genes is PPARα/RXRα.

It has been speculated that all RXR isoforms, as well as all RAR isoforms, are mostly, if not fully, functionally redundant for the transcriptional control of all retinoic acid target genes (30, 32, 53, 54). The only requirement would be to reach a certain threshold of RXR and RAR in a given cell at a given time. This could be achieved through any combination of RXR and RAR isoforms and by regulating the levels of the individual receptors by increased transcriptional rate. In this respect, both the hormonal regulation by increased or decreased levels of glucocorticoids due to stress or metabolic demands, or to the dietary effects of natural fatty acids present at the time, would affect the steady state level of RXRα, as shown earlier for PPARα (40). In addition, RXR homodimers have been shown to activate RXRE in vivo, but it was shown that certain cells that exogenously express RXRα and RARα (even at low levels) do not yield any response with DR-1-containing reporter genes unless RXRs are overexpressed in these cells (55).

The present results show that RXRα gene regulation and the steady-state level of the dietary fatty acids in the cells are under hormonal control. Because changing the levels of nuclear receptor protein can drastically affect the signaling of the cognate ligand, the study of the regulation of nuclear receptor genes is very important for understanding the signal transduction of these ligands.

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