Mechanism of the slow induction of apolipoprotein A-I synthesis by retinoids in cynomolgus hepatocytes: involvement of retinoic acid and retinoid X receptors

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Abstract We showed previously that retinoids stimulate apolipoprotein A-I (apoA-I) synthesis in cultured cynomolgus hepatocytes only after a 24-h lag phase. Here we report on the biochemical background of the slow response, the requirement for high retinoic acid concentrations, and the involvement of different retinoid receptors. The time course of the effect of 10 μM all-*trans* retinoic acid (at-RA) on apoA-I mRNA levels and protein secretion were comparable, i.e., minor increases were observed after a 24-h incubation and mRNA levels were increased 2.2- and 3.5-fold after 48 h and 72 h, respectively. In contrast, apoA-I gene transcription was already increased (2.6-fold) after a 4-h incubation with 10 μM at-RA. At-RA disappeared rapidly from the cultures: after 2 h of incubation 40% of the added amount was left and after 24 h only 2%. RARβ mRNA and gene expression were increased after incubation with 10 μM at-RA, whereas RARα and RXRα mRNA levels and expression remained unchanged. No transcriptional activity and mRNA for other retinoid receptors were detectable. Both RAR-selective (TTNPB) and RXR-selective (3-methyl-TTNEB) agonists induced apoA-I synthesis at 1 and 10 μM. These results show that *i*) the slow increase in apoA-I secretion is caused by a slow increase of its mRNA level; *ii*) the apoA-I gene transcription in cynomolgus hepatocytes is induced rapidly by retinoids; *iii*) the added at-RA disappeared rapidly from the cultures, explaining the necessity for high initial concentrations; *iv*) RARα and/or RARβ and RXRα are involved in the activation of apoA-I expression by retinoids.—**Kaptein, A., D. M. Neele, J. Twisk, H. F. J. Hendriks, T. Kooistra, and H. M. G. Princen.** Mechanism of the slow induction of apolipoprotein A-I synthesis by retinoids in cynomolgus hepatocytes: involvement of retinoic acid and retinoid X receptors. *J. Lipid Res.* *1997.* **38:** 2273–2280.

Supplementary key words apolipoprotein A-I • retinoids • receptor-selective ligands • RAR • RXR • transcriptional regulation • mRNA • cynomolgus hepatocytes

Decreased plasma levels of high density lipoprotein (HDL) cholesterol are associated with an increased risk of the development of coronary heart disease (1, 2). Apolipoprotein A-I (apoA-I), the major protein constituent of HDL, has been reported to be even more predictive than HDL cholesterol (3, 4). ApoA-I synthesis is reported to be associated with the HDL-cholesterol level in the circulation in vivo (5, 6). Furthermore, transgenic mice overexpressing human apoA-I have high plasma HDL and apoA-I levels and are more resistant to atherogenic diets (7). These data indicate that up-regulation of the apoA-I synthesis may be useful in increasing the HDL level, which may subsequently decrease the risk of development of atherosclerosis.

In a previous report (8) we showed that physiologically important modulators such as retinoids, including retinol (vitamin A), all-*trans* retinoic acid (at-RA) and its naturally occurring isomers, stimulate apoA-I synthesis in primary hepatocyte cultures from cynomolgus monkey. Similar data were obtained by others in HepG2 cells and rat hepatocytes (9–11). The stimulatory effect was observed only after prolonged incubation with retinoids and at relatively high concentrations (8). In the present study we further investigated the regulation of apoA-I secretion in cynomolgus monkey hepatocytes, and studied the biochemical background for the slow stimulation of apoA-I secretion and for the high initial retinoid concentrations needed.

It should be noted, however, that the effects of retinoids in isolated cultured cells may differ from their

Abbreviations: apo, apolipoprotein; RAR, retinoic acid receptor; RXR, retinoid X receptor; at-RA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid.

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effects in the more complex in vivo system in which modulation may be influenced by more factors. In contrast to the effect in vitro, retinoids showed a decreasing effect on apoA-I gene expression in vivo in rat (11), and decreased or unchanged HDL and apoA-I levels were reported in human studies with isotretinoin, the 13-*cis* isomer of at-RA (12, 13).

It is well known that retinoids can exert their biological action by binding to members of two families of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each family consisting of three receptor types; α, β, and γ (for reviews: 14–16). The effects of at-RA are mediated by the RARs, whereas the effects of 9-*cis* retinoic acid (9-*cis*-RA) are mediated by both RARs and RXRs (17, 18). The retinoid receptors control the transcription of genes by interacting with retinoic acid receptor response elements (RAREs) in the promoter region of these particular genes. One of the genes with an upstream RARE is the gene encoding apoA-I (19). The RARE in the human apoA-I promoter has been shown to bind both RXR-RXR homodimers and RAR-RXR heterodimers, and to be essential for apoA-I promoter activity in transactivation assays, in which the respective receptors are overexpressed (19, 20). However, which retinoid receptors are actively involved in the induction of apoA-I gene transcription in a physiological context is unknown. As a first step to elucidate this, we studied the transcriptional activity and the mRNA levels of the genes coding for the different retinoid receptors. In addition, we applied the RAR-specific ligand TTNPB (21) and the RXR-specific ligand 3-methyl-TTNEB (22) to investigate the involvement of RARs and RXRs in the induction of apoA-I gene transcription.

We found that transcription of the apoA-I gene is rapidly activated by at-RA, but that this is followed by a slow, time-dependent increase of its mRNA level. Our data suggest that activation of apoA-I gene expression requires both RARα and/or RARβ and RXRα.

METHODS

Materials

All-*trans*-retinoic acid (at-RA) was purchased from Sigma Chemical Co. (St. Louis, MO). 9-*Cis*-retinoic acid (9-*cis*-RA) was a generous gift from Mr. P. Weber and Dr. F. Schneider, Hoffmann-La Roche Ltd. (Basel, Switzerland). 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl] benzoic acid (3-methyl-TTNEB) and (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) were kindly provided by Dr. S. Karathanasis, American Cyanamid Company (Pearl River, NY) and by Dr. M. Issandou, Laboratories Glaxo, Centre de Recherches (Les Ulis, France). Stock solutions of 10 mM were prepared in 100% dimethylsulfoxide (DMSO) (freshly prepared just before each experiment) and stored at −20°C for no longer than 5 days. Immediately before use the retinoids were diluted in culture medium, in such a way that the final DMSO concentration did not exceed 0.1% (v/v). As the compounds are light-sensitive, all experiments were performed in subdued light. Materials used for the isolation and culturing of simian hepatocytes were obtained from sources described previously (8). [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (400 Ci/mmol) were obtained from Amersham International (Amersham, Buckinghamshire, U.K.).

Simian hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (*Macaca fascicularis*, 1.5–3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The monkeys were fed Primate Diet G.O. (Hope Farms B.V., Woerden, The Netherlands) ad libitum with one or two pieces of additional fruit per day and were overnight fasted before being killed. The isolation procedure was exactly as described (8). For each independent culture experiment hepatocytes from a single monkey were used. Total cell yields varied from 0.74 to 2.3 × 10^9 viable cells. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%), was 66–96%. The cells were seeded on culture dishes at a density of 2 × 10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated (30 min at 56°C) fetal bovine serum (FBS), 2 mM L-glutamine, 20 mU/ml insulin (135 nM), 50 nM dexamethasone, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml kanamycin at 37°C in a 5% CO_2/95% air atmosphere. After 14–16 h, the non-adherent cells were washed from the plates, using the same culture medium as above. After 24 h from seeding, the incubations with the retinoids were started with culture medium with a lower insulin concentration, 10 nM instead of 135 nM. Because the retinoids were added to the culture medium as a stock solution in DMSO, all incubations were performed with medium containing 0.1% (v/v) DMSO. The medium was renewed every 24 h.

Retinoid analysis

Retinoids were extracted from both cells and conditioned medium using the method described by Barua,

Batres, and Olson (23). Retinoids were analyzed by reverse phase high performance liquid chromatography (HPLC) using a Spherisorb (Phase Separations) S5ODS2 C_{18} analytical column (25 × 0.46 cm), developed by a non-linear gradient modified from Eckhoff and Nau (24) using two eluents, i.e., 60 mM ammonium acetate, pH 5.75 (A) and methanol (B). The gradient program with a flow rate of 1 ml/min was as follows: 5 min isocratic at 75% B, followed by a linear gradient to 85% B in 5 min and a further linear gradient to 99% B in 20 min. Absorbance was monitored at 340 nm using a 759A Absorbance Detector (Separations).

RNA hybridization

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski and Sacchi (25). Equal amounts of total RNA (3 and 6 μg, giving linear relation between the specific apoA-I and actin mRNA signal and the amount of RNA applied) from different incubations were slot-blotted to Hybond N+ (Amersham) in accordance with the manufacturer's instructions. RNA blots were hybridized and quantitated as described previously (8). Analysis of the mRNA level of the different retinoid receptors was performed by Northern blot hybridization of total RNA, as described (8). Twenty micrograms of total RNA was fractionated on a 0.8% (wt/vol) agarose gel containing 1 mol/L formaldehyde. The following fragments were used as probes in the hybridization experiments: 1.3 kb EcoRI-BgIII fragment of a human RARα cDNA, a 1.4 kb BamH1-XbaI fragment of human RARβ cDNA, a 1.4 kb EcoRI-XbaI fragment of a mouse RARγ1 cDNA, in which a few bases have been changed in the A and F regions by site-directed mutagenesis so that it encodes the human amino acid sequence, a 1.4 kb EcoRI-BgIII fragment of a human RXRα cDNA, and a 1.4 kb BamH1-EcoRI fragment of human RXRγ. The RARα and RXRα were cloned in the laboratory of Dr. J. Grippo, Hoffmann-La Roche, Nutley, NJ, and RARβ, RARγ, and RXRγ were cloned in the laboratory of Dr. P. LeMotte, Hoffmann-La Roche, Basle, Switzerland. A 2.2 kb EcoRI fragment of the mouse RXRβ (H-2RIIBP) cDNA was kindly provided by Dr. K. Ozato (26). Other probes were as described previously (8).

Nuclear run-off studies

Nuclear run-off studies were conducted as described (8, 27). The [^{32}P]UTP-labeled RNAs from the nuclear run-off studies were hybridized with target DNA, which was 5 μg of plasmid material containing DNA sequences of human apoA-I, different retinoid receptors, hamster actin, or the empty PUC vector. After hybridization the filters were exposed to Hyperfilm MP (Amersham) for 2–5 days. Corrections for nonspecific hybridization were made by subtracting the PUC signal. Quantitation of relative amounts of mRNA was conducted using the actin mRNA signal as an internal standard.

Statistical analysis

Statistical significance of differences was calculated by Student's *t*-test for paired data with the level of significance selected as $P < 0.05$. Values are expressed as mean ± SD.

RESULTS

Effect of retinoids on apoA-I mRNA levels and gene transcription

Previously we reported that retinoids increase the secretion of apoA-I time- and dose-dependently in primary cynomolgus hepatocytes. Incubation for 72 h with 1 and 10 μM at-RA resulted in a significant increase of apoA-I secretion (1.7- and 2.7-fold, respectively), whereas 0.1 μM at-RA had no effect. The effect of at-RA was observed only after a lag phase of 24 h (8). In the present paper, concomitant with the secretion of apoA-I, the time course of the effect of at-RA and 9-*cis*-RA on the apoA-I mRNA level was investigated. Only minor changes were observed in the apoA-I mRNA level after 24 h of incubation with 10 μM at-RA or 10 μM 9-*cis*-RA (**Fig. 1** and **Table 1**). In the second 24-h incubation period, the apoA-I mRNA level was 2.2-fold increased

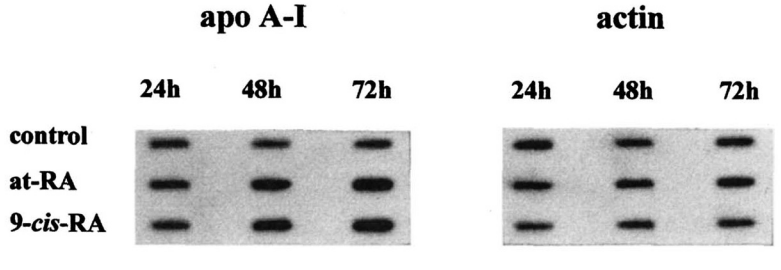

Fig. 1. Time course of the effect of at-RA and 9-*cis*-RA on apoA-I mRNA level in cynomolgus hepatocytes. Autoradiographs of slot-blot hybridization with [^{32}P]-labeled probes for apoA-I and actin, with 6 μg total RNA per slot. After a 24-h attachment period, total RNA was isolated from hepatocytes that had been incubated for 24, 48, or 72 h on standard medium without (control) or with 10 μM at-RA or 10 μM 9-*cis*-RA. Left and right autoradiographs show the results of the hybridization of apoA-I mRNA and actin mRNA, respectively. Actin mRNA was not affected by the retinoids. The experiment shown is representative of three independent experiments from which the data are shown in Table 1.

TABLE 1. Time course of the effect of at-RA and 9-*cis*-RA on apoA-I mRNA levels in cynomolgus hepatocytes

	A. Incubation Time ApoA-I mRNA (% of control)			B. Incubation Period ApoA-I Secretion (% of control)		
	24 h	48 h	72 h	0–24 h	24–48 h	48–72 h
Standard medium	100	83 ± 12	126 ± 19			
at-RA, 10 μM	146 ± 23	215 ± 18[a]	354 ± 33[a]	123 ± 17	188 ± 27[a]	334 ± 33[a]
9-*cis*-RA, 10 μM	142 ± 15[a]	226 ± 13[a]	379 ± 25[a]	128 ± 10[a]	219 ± 30[a]	304 ± 30[a]

After a 24-h attachment period, cynomolgus hepatocytes were cultured for 24, 48, or 72 h on control medium with at-RA (10 μM), 9-*cis*-RA (10 μM) or without retinoids (control). A: Effect of the retinoids on apoA-I mRNA levels. ApoA-I and actin mRNA levels at the indicated time points were assessed by densitometric scanning of the intensity of the bands on the autoradiographs (as illustrated in a representative autoradiograph in Fig. 1). Actin mRNA level, which was not affected by retinoids, was used as an internal standard to correct for differences in the amount of total RNA applied to the slot-blot. The results are expressed as percentages of the 24-h incubation in medium without retinoids, with analysis of 3 and 6 μg total RNA and are means ± SD of three independent hepatocyte isolations. B: Effect of retinoids on the apoA-I secretion during the 24-h culture periods in the experiments described above. ApoA-I secretion was measured by ELISA (8), normalized for cell protein, and is expressed as a percentage of control incubations without retinoids. ApoA-I production in control medium during the three consecutive 24-h culture periods was 780 ± 384, 698 ± 412, and 732 ± 368 ng/24 h per mg cell protein, respectively. These values are means ± SD of duplicate incubations from three independent hepatocyte isolations.

[a]Significant difference between control and treated cells, $P < 0.05$.

using at-RA and 2.3-fold with 9-*cis*-RA, followed by a further stimulation with at-RA (3.5-fold) and 9-*cis*-RA (3.8-fold) in the third 24-h period. As shown in Table 1B the time course of the effect of these retinoids on the apoA-I secretion was comparable to that on the apoA-I mRNA level. at-RA and its 9-*cis*-isomer gave similar stimulatory effects.

This slow increase in the apoA-I mRNA level may result from a retarded transcriptional activation of the apoA-I gene. Therefore, we investigated the transcription rate of the apoA-I gene, as assessed by a run-off assay, in hepatocytes incubated for different time periods in the presence or absence of at-RA (10 μM). As shown in **Fig. 2,** the transcriptional activity of the apoA-I gene was already markedly increased (2.6-fold) after a 4-h incubation with at-RA. After 10 h of incubation a comparable enhancement of the gene expression was observed. However, the transcription rate decreased after prolonged incubation: after 24 h of incubation stimulation was only 1.4-fold. To investigate whether the hepatocytes are equally able to respond to at-RA after prolonged culture, hepatocytes were incubated for 72 h in the presence of 10 μM at-RA, with renewal of the medium 8 h before isolation of the nuclei. This resulted in a similar extent of activation of apoA-I gene transcription (2.5-fold) as found after a 4- or 10-h incubation period (Fig. 2).

Disappearance of added at-RA from the cultures

An explanation for the fall in apoA-I gene expression after a 24-h incubation with at-RA may be active metabolism by the hepatocytes of the ligand involved in the retinoid receptor-mediated increase in the apoA-I gene expression. Therefore, we measured the turnover of at-RA in the presence and absence of hepatocytes. As shown in **Table 2A,** less than 2% of the added amount

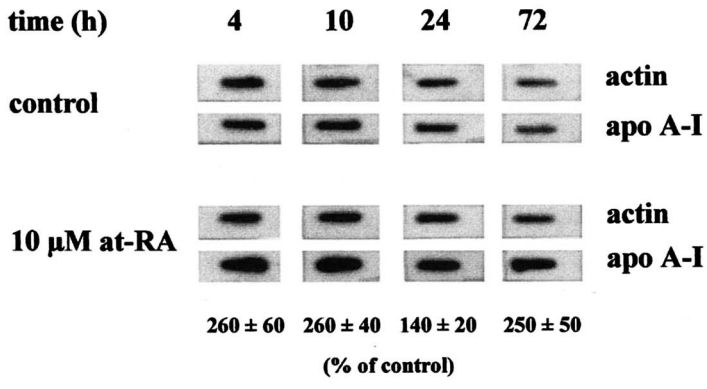

Fig. 2. Transcriptional activation of the apoA-I gene in response to at-RA. After a 24 h attachment period cynomolgus hepatocytes were treated for 4, 10, 24, or 72 h without (control) or with 10 μM at-RA. For the 72-h incubation period, the culture medium with or without at-RA was changed every 24 h and 8 h before isolation of the nuclei. At the bottom of the autoradiographs the transcriptional activity of the apoA-I gene in the presence of at-RA is listed, expressed as a percentage of the control incubation at the same time point. The density of the bands in the autoradiographs was assessed by densitometric scanning. The transcriptional activity of the apoA-I gene was normalized for the transcriptional activity of the actin gene, which was not affected by retinoids. Data shown are means ± SD for three independent hepatocyte isolations. All values differed significantly ($P < 0.05$) from the control incubation without retinoic acid.

TABLE 2. Disappearance of at-RA from the cultures

Incubation		Retinoid (nmol) at-RA	9-*cis*-RA	13-*cis*-RA	Total
A. With hepatocytes					
2 h	Cells	0.69 ± 0.04	0.01 ± 0.00	0.11 ± 0.02	4.15 ± 0.30
	Medium	2.74 ± 0.18	0.02 ± 0.00	0.58 ± 0.06	(41.5 ± 3.0%)
4 h	Cells	0.38 ± 0.20	0.01 ± 0.01	0.07 ± 0.01	3.31 ± 0.41
	Medium	2.30 ± 0.15	0.04 ± 0.02	0.51 ± 0.06	(33.1 ± 4.1%)
24 h	Cells	0.06 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.18 ± 0.09
	Medium	0.07 ± 0.05	0.00 ± 0.00	0.03 ± 0.03	(1.8 ± 0.9%)
B. Without hepatocytes					
2 h	Medium	9.01 ± 0.02	0.02 ± 0.00	0.91 ± 0.03	9.91 ± 0.05 (99.1 ± 0.5%)
4 h	Medium	6.72 ± 0.06	0.06 ± 0.06	0.76 ± 0.05	7.54 ± 0.17 (75.4 ± 1.7%)
24 h	Medium	4.23 ± 1.74	0.37 ± 0.19	1.20 ± 0.58	5.80 ± 2.21 (58.0 ± 22.1%)

Hepatocytes were allowed to attach for 24 h. One ml medium containing 10 nmol at-RA was added to culture dishes (A) with hepatocytes (0.7–0.9 mg cell protein per dish) or (B) without hepatocytes. Media and cells were harvested after a 2-, 4-, or 24-h incubation at 37°C. The amount of at-RA together with 9-*cis*-RA and 13-*cis*-retinoic acid (13-*cis*-RA) was measured as described in Methods. The amount of the retinoids, expressed as a percentage of the starting amount of at-RA, is indicated in parentheses. For the incubation with hepatocytes, the total amount of the retinoids is the total of cells and medium. Data shown are means ± SD for three independent isolations.

of at-RA could be recovered from cells and medium after a 24-h incubation. After a 2-h incubation, nearly 60% of the added at-RA was already metabolized by the hepatocytes. When medium with at-RA without hepatocytes was incubated at 37°C, we also observed the disappearance of at-RA (Table 2B), but at a much slower rate.

Effect of retinoids on mRNA levels and transcriptional activity of retinoid receptors

To investigate which retinoid receptors are involved in the transcriptional activation of the apoA-I gene, we studied the mRNA level and the transcriptional activity of the genes coding for the retinoid receptors in cynomolgus hepatocytes treated with at-RA or 9-*cis*-RA. Two RARα mRNAs (~3.6 and ~2.8 kb) were present in both control and treated hepatocytes (**Fig. 3**). Treatment with at-RA or 9-*cis*-RA did not affect the amount of the two RARα mRNA isoforms. In contrast, the RARβ mRNA (~3.4 kb) level, which was low in control hepatocytes, was induced about 6- to 8-fold by treatment with 10 μM at-RA and 4- to 5-fold by 10 μM 9-*cis*-RA relative to actin mRNA. No effect of retinoids on the RXRα mRNA (~5 kb) level was observed. In accordance with the mRNA levels, nuclear run-off assays showed clear gene expression of RARα and low expression of the RXRα gene; neither gene was induced by at-RA (**Fig. 4**). Gene expression of RARβ was very low in control cells, but was stimulated 1.8-fold by at-RA. No transcriptional activity and mRNA of the genes coding for RXRβ, RXRγ, and RARγ were detectable in cynomolgus hepatocytes during culture.

Effect of retinoid receptor-selective ligands on apoA-I synthesis

To assess which retinoid receptors are involved in the induction of apoA-I synthesis, hepatocytes were incubated with selective RAR-(TTNPB) and RXR-(3-methyl-TTNEB) agonists. **Figure 5** shows that both agonists induced apoA-I synthesis in a dose-dependent way and were equally active. Significant induction of apoA-I secretion was observed at 1 and 10 μM. In parallel experiments performed with the hepatocytes from the same isolations, at-RA showed a similar induction (166 ± 25% of control at 1 μM (n = 6) and 232 ± 51% of the control at 10 μM (n = 6)) as the agonists.

DISCUSSION

In this study we have shown that unlike a rapid induction of apoA-I gene transcription by retinoids, apoA-I mRNA levels in cynomolgus hepatocytes are increased slowly and time-dependently, explaining the slow increase of apoA-I synthesis. at-RA disappeared very quickly from the cultures, explaining the necessity for high initial concentrations. Active transcription of RARα, RARβ, and RXRα genes occurred and their mRNAs were present in cynomolgus hepatocytes, and

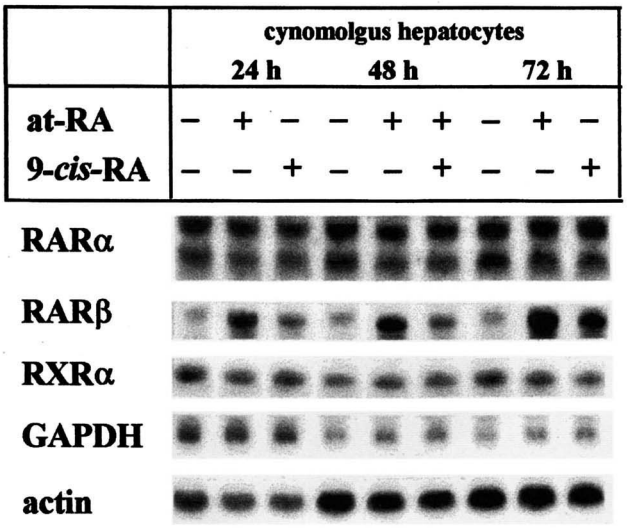

Fig. 3. Effect of at-RA and 9-*cis*-RA on RARα, RARβ, and RXRα mRNA levels in cynomolgus hepatocytes. Autoradiographs of a Northern blot hybridized with [^{32}P]-labeled probes for RARα, RARβ, RXRα, GAPDH, and actin mRNA. Total RNA from cynomolgus monkey hepatocytes was isolated after incubation for 24, 48, or 72 h on standard medium without (−) or with (+) 10 μM at-RA or 10 μM 9-*cis*-RA after a 24-h attachment period. Twenty μg of RNA was applied on to the gel. Medium was renewed every 24 h. Incubation conditions are indicated at the top of the autoradiographs. The experiment shown is representative of three independent experiments. The density of the bands in the autoradiographs was assessed by densiometric scanning. After correcting for the actin mRNA level, used to assess differences in the amount of total RNA applied to the gels, RARα and RXRα mRNA levels did not change with respect to control upon incubation with either at-RA or 9-*cis*-RA. RARβ mRNA values as % of control at the same time point were 806 ± 181 and 416 ± 90 (24 h); 636 ± 135 and 398 ± 83 (48 h); 617 ± 149 and 460 ± 66 (72 h) after incubation with 10 μM at-RA and 10μM 9-*cis*-RA, respectively. All data are means ± SD for three independent hepatocyte isolations. All data for RARβ mRNA differed significantly ($P < 0.05$) from control incubations without retinoic acid.

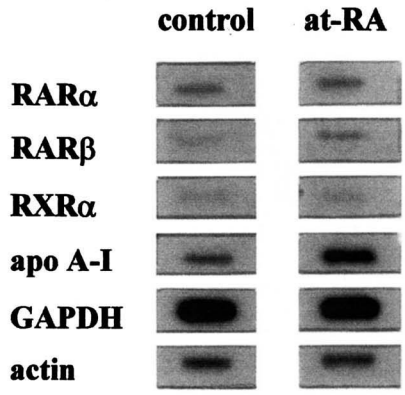

Fig. 4. Effect of at-RA on transcriptional activity of RARα, RARβ, and RXRα genes in cynomolgus hepatocytes. Autoradiographs of the run-off transcripts for RARα, RARβ, RXRα, apoA-I, actin, and GAPDH of nuclei from simian hepatocytes treated without (control) or with 10 μM at-RA (at-RA) for 24 h. Four hours before harvesting the cells, new medium with or without at-RA (10 μM) was added. The experiment shown is representative of three independent experiments. The density of the bands on the autoradiographs was assessed by densiometric scanning. Transcriptional activity of the actin gene was used as an internal standard. RARα and RXRα gene expression did not change with respect to control upon incubation with at-RA. RARβ gene expression was increased being 180 ± 50% of the control value (n = 3, $P < 0.05$ with respect to control) upon incubation with 10 μM at-RA. All data are expressed as % of control and means ± SD for three independent hepatocyte isolations.

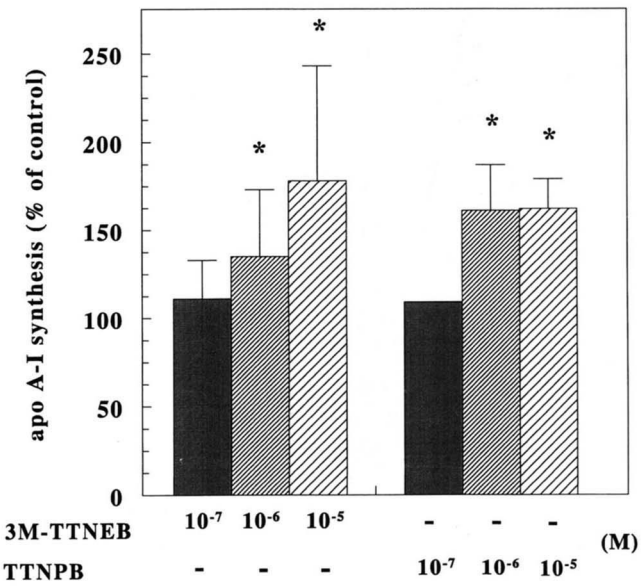

Fig. 5. Effect of retinoid selective agonists 3-methyl-TTNEB and TTNPB on apoA-I synthesis in cynomolgus hepatocytes. After a 24-h attachment period, hepatocytes were incubated for three consecutive 24-h periods with increasing concentrations of 3-methyl-TTNEB (RXR-agonist) and TTNPB (RAR-agonist). Apolipoprotein A-I synthesis was determined in the last 24-h period as described in Materials and Methods and expressed as a percentage of control. The values are normalized for the amount of cell protein in the culture dishes, and are means ± SD of duplicate incubations from 3–6 independent hepatocyte isolations. *Indicates a significant difference ($P < 0.05$) between control and treated cells.

RARβ mRNA levels and gene expression were induced by retinoids. Together with experiments using retinoid receptor-selective ligands, these findings suggest the involvement of RARα and/or RARβ and RXRα in the stimulation of apoA-I synthesis.

The reason for the discrepancy between apoA-I gene transcription and mRNA level is not fully clear. The transcription rate of the apoA-I gene increased 2- to 3-fold shortly after addition of at-RA to cynomolgus hepatocytes, but decreased after prolonged incubation in the same medium. We have shown that in the presence of cynomolgus hepatocytes, at-RA disappeared rapidly from the medium. This suggests that at longer incubation times without renewing of the medium, as was the case after a continuous 24-h incubation period, retinoids may not be present at high enough levels for maximal induction of apoA-I gene expression. We suggest that apoA-I mRNA levels are increased in a pulsatory

way and that the induction of RARβ may play a part in this process, possibly by extending the period of increased gene transcription. On the other hand, the possibility that the induction of RARβ is coincidental cannot be excluded. Nonetheless, the presence of a cellular pool of apoA-I mRNA, which is relatively stable and large with respect to the suggested small pulsatory increases, may explain the slow and time-dependent increase in apoA-I mRNA and secretion.

An increase in RARβ mRNA levels and transcriptional activity by at-RA and 9-*cis*-RA, as found in cynomolgus monkey hepatocytes, has also been reported in various other cell-culture systems such as F9 teratocarcinoma cells and primary rat hepatocytes after treatment with retinoids (9, 28–30). This induction is consistent with the presence of a retinoic acid responsive element (RARE) in the promoter region of the RARβ gene (31).

Widom, Rhee, and Karathanasis (32) and Zhang et al. (20) reported that the retinoid receptor RXRα is important in the regulation of the apoA-I gene expression. This was concluded from experiments using cells transfected with RXRα. From our experiments with an RXRα-selective agonist, we can conclude that the RXRα receptor also appears to be involved in the regulation of the transcriptional activation of apoA-I by retinoids in primary cultures of cynomolgus monkey hepatocytes; no expression of RXRβ and RXRγ was detectable. However, the presence of the mRNAs coding for RARα and RARβ and the finding that an RAR-selective agonist induced the apoA-I secretion indicate that these two receptors may also be involved in regulation. In agreement with the latter contention, it has been shown recently that both partners of the RAR/RXR heterodimer can activate transcription of RA-responsive genes (33–35). Whether retinoids act through activation of RXRα homodimers and/or via heterodimers of the RXRα receptor with the RARα or RARβ receptor remains to be elucidated.

In conclusion, we have shown that the slow increase in apoA-I secretion by retinoids in simian hepatocytes is not caused by a retarded transcriptional activation, but results from a slow time-dependent elevation of the apoA-I mRNA level. Transcriptional activation of the apoA-I gene by retinoids may occur via RXRα, possibly together with RARα or RARβ.

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