The influence of different retinoids on apolipoprotein A-I (apoA-I) synthesis and secretion was investigated in primary monolayer cultures of hepatocytes from cynomolgus monkeys. Addition of retinol (vitamin A) and retinoic acid to the culture medium resulted in a time- and dose-dependent increase in the secretion of apoA-I. No effect was observed during the first 24-hour incubation period; however, apoA-I secretion was enhanced 1.5-fold in the following 24-hour period in the presence of 10 μmol/L retinoic acid. Maximal stimulation (2.7-fold) was obtained at 10 μmol/L retinoic acid during a third 24-hour incubation. In these experiments apoB-100 secretion was unaffected. When [35S]methionine incorporation studies were performed de novo synthesis of apoA-I was increased, whereas total protein synthesis remained constant. These observations indicated that the induction of apoA-I synthesis is not part of a general effect of retinoic acid on hepatic protein synthesis. Among different natural and synthetic retinoids, retinoic acid and its 9-cis and 13-cis isomers were equally active and were the most potent inducers of apoA-I synthesis, whereas the maximal stimulation induced by retinol was lower (1.6-fold).

Apolipoprotein A-I mRNA abundance was increased threefold in hepatocytes exposed for 72 hours to 10 μmol/L retinoic acid, which was associated with a twofold increase in the transcriptional rate of the apoA-I gene. In contrast, no changes were found in the apoB-100 mRNA level and transcriptional activity of the apoB-100 gene. We conclude that retinoids enhance apoA-I synthesis in simian hepatocytes by transcriptional regulation.

KEY WORDS • apoA-I • apoB-100 • retinoids • vitamin A • transcriptional regulation • hepatocytes • cynomolgus monkey
of lipid and lipoprotein metabolism and the development of atherosclerosis. The primary hepatocyte model has a potential advantage over hepatoma cell lines in that the cells in primary culture might reflect more accurately what occurs in the liver.

In this article we report that retinoids enhance apoA-I synthesis in hepatocyte cultures from cynomolgus monkey by induction of apoA-I gene transcription. Among various natural and synthetic analogues, retinoic acid and its natural isomers were the most potent stimulators. In contrast to apoA-I, synthesis of apoB-100 was found to be unchanged by retinoids.

Methods

Materials

Retinol, retinoic acid, and 13-cis-retinoic acid were purchased from Sigma Chemical Co, St Louis, Mo. 9-cis-Retinoic acid, RO 13-4306, RO 13-7410, and RO 13-6298 were generous gifts from Mr P. Weber and Dr F. Schneider, Hoffmann-La Roche Ltd, Basel, Switzerland. Fenretinide was a generous gift from Dr M. Rosenthal, the RW Johnson Pharmaceutical Research Institute, Raritan, NJ. Pelretin was a generous gift from Dr B. Janssen, BASF, Ludwigshafen, FRG. The structures of the various retinoids are shown in Fig 1. All retinoids were stored at −20°C in sealed containers. Stock solutions of 10 mmol/L were freshly prepared just before each culture experiment in 100% dimethyl sulfoxide (DMSO) and preserved at −20°C for no longer than 5 days. Immediately before use the retinoids were diluted in culture medium, such that the DMSO concentration did not exceed 0.1% (vol/vol). Since 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, except for fetal bovine serum, which was purchased from BioWhittaker, Walkersville, MD, USA.

Simian Hepatocyte Isolation and Culture

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (Macaca fascicularis; 1.5 to 3 years old) that 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 isolation procedure was essentially as described for human hepatocytes with a few modifications. The simian liver was perfused with 1.5 L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 10 mmol/L HEPES, 132 mmol/L NaCl, 6.7 mmol/L KCl, and 20 mmol/L glucose at a rate of 100 mL/min. After the perfusion, the liver was washed successively, first with 500 mL of another HEPES buffer, pH 7.6, containing 100 mmol/L HEPES, 67 mmol/L NaCl, 6.7 mmol/L KCl, and 5 mmol/L CaCl2 without circulation, and second with 200 mL of the same buffer containing 0.05% collagenase with recirculation for 30 minutes. Liver tissue was dissociated in Hanks’ buffer containing 2% bovine serum albumin, and the cells were filtered through a 250-μm filter, centrifuged at 60g for 5 minutes, and washed three times in cold culture medium to remove damaged and nonparenchymal cells. Total cell yields varied from 0.74 to 2.3 × 107 viable cells. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%), was 66% to 96%. The cells were seeded on culture dishes at a density of 2 × 103 viable cells/cm2 and were maintained in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum (30 minutes at 56°C), 2 mmol/L L-glutamine, 20 mU insulin/mL (135 nmol/L), 50 nmol/L dexamethasone, 100 U penicillin/mL, 100 μg streptomycin/mL, and 100 μg kanamycin/mL at 37°C in a 5% CO2–95% air atmosphere. After 14 to 16 hours the nonadherent cells were washed from the plates with the same culture medium as above. Twenty-four hours after seeding, the incubations with the various retinoids were started in the same culture medium but with a lower insulin concentration (10 nmol/L rather than 135 nmol/L), using two or three separate wells per culture condition. Since the retinoids were added to the culture medium as a stock solution in DMSO, all incubations, control and with retinoids at various concentrations, were performed with medium containing 0.1% (vol/vol) DMSO. The medium was renewed every 24 hours thereafter. At the end of an incubation period, medium was collected and centrifuged for 20 seconds in an Eppendorf centrifuge (type 5414) to remove detached cells and debris. The supernatant was frozen immediately in dry ice and stored at −20°C until measurement of apoA-I and apoB-100 concentrations. After the last incubation, cells were washed three times with cold phosphate-buffered saline (sodium/potassium phosphate buffer, 11 mmol/L, pH 7.5, containing 150 mmol/L NaCl). The cellular protein was determined as described by Lowry et al.

Enzyme-Linked Immunosorbent Assay of ApoA-I and ApoB-100

ApoA-I and apoB-100 concentrations in culture medium were measured in triplicate using a sandwich enzyme-linked immunosorbent assay (ELISA) procedure with polyclonal antibodies to human apoA-I or human apoB-100, respectively, both as catching and detecting antibodies as described. The standard curves for apoA-I and apoB-100 in human and cynomolgus monkey sera and in culture medium of the cynomolgus hepatocytes were parallel, indicating that similar epitopes on apoA-I and apoB-100 of the two species are recognized.

Protein Synthesis

Overall secretion of newly synthesized proteins was determined by measuring the incorporation of [35S]methionine into the 10% (wt/vol) trichloroacetic-acid-precipitable fraction of radiolabeled culture medium. The metabolically radiolabeled proteins secreted in the medium were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by the method of Laemmli, with resolving gels containing a gradient of 4% to 20% (wt/vol) acrylamide and stacking gels of 3.5% (wt/vol) acrylamide. Protein molecular-mass standards (BioRad, Richmond, Calif) were used for calibration of the gel. For autoradiography, the gel was treated with an...
Autoradiography enhancer (ENhance; New England Nuclear Du Pont, Boston, Mass) in accordance with the manufacturer's instructions, dried, placed on x-ray film (Kodak X-Omat AR films; Eastman-Kodak Co, Rochester, NY), and stored at -80°C for the appropriate time.

**Immunoblotting**

After SDS-PAGE as described above, the gel was electroblotted on nitrocellulose in a blot buffer containing 192 mmol/L glycine, 25 mmol/L tris(hydroxymethyl)aminomethane (Tris)-HCl, and 20% (vol/vol) methanol, pH 8.3. The further processing of the blot was as described.41 ApoA-I and apoB-100 on the blot were identified using affinity-purified goat anti-apoA-I and rabbit anti-apoB-100, followed by incubations with goat anti-rabbit immunoglobulin (Ig) G conjugated to peroxidase and rabbit anti-goat IgG conjugated to peroxidase, respectively.

**RNA Hybridization**

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski and Sacchi.42 After washing the RNA pellets with 70% (vol/vol) ethanol, RNA samples were dissolved in water. The RNA concentration in each sample was determined spectrophotometrically, with the assumption that one A260 unit corresponds to 40 µg RNA/mL.

Equal amounts of total RNA from different incubations were fractionated by electrophoresis in an 8% (wt/vol) agarose gel containing 1 mol/L formaldehyde and were transferred to Hybond-N+ (Amersham) in accordance with the manufacturer's instructions. RNA blots were hybridized with different probes at 65°C in a sodium phosphate buffer (0.5 mol/L, pH 7.5) containing 7% (wt/vol) SDS and 1 mmol/L EDTA. DNA fragments used as probes were isolated from low-melting-point agarose.43 One blot was hybridized with a 25-ng probe, which was labeled by the random-primer method (Multiprime, Amersham) to approximately 2x10^6 to 10^8 cpm/µg DNA.

After hybridization, the blots were washed twice with 2x saline–sodium citrate (SSC)/0.1% SDS (30 minutes at 65°C; 1x SSC = 0.15 mol/L NaCl/0.015 mol/L sodium citrate, pH 7.0); twice with 1x SSC/0.1% SDS (30 minutes at 65°C); and twice with 0.1x SSC/0.1% SDS (30 minutes at 65°C), successively. The blots were exposed to Hyperfilm-MP (Amersham) and an intensifying screen (Eastman-Kodak Co) for 15 to 96 hours at -80°C. For quantitation of the relative amounts of mRNA, the autoradiographs were scanned with a Shimadzu CS 910 chromatograph scanner, and areas under the peaks were quantified of the relative amounts of mRNA using the random-primer method (Mulprime, Amersham) to approximately 2x10^6 to 10^8 cpm/µg DNA.

**Isolation of nuclei.** Cells were washed twice and scraped in a 0.9% (wt/vol) saline solution by using a rubber policeman and then collected by centrifugation at 500g for 4 minutes. The cells were resuspended in NP40 lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L MgCl2, 1 mmol/L NaCl, 3 mmol/L MgCl2, 0.5% NP40, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 1 mmol/L diithiothreitol [DTT]); after being left on ice for 5 minutes, they were homogenized in a Potter Elvehjem tube with pestle B for 25 strokes at 4°C. This homogenate was left on ice for 5 minutes and was again homogenized for 25 strokes at 4°C. Resulting nuclei were centrifuged at 500g and resuspended in NP40 lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. Nuclei were then taken up in a glycerol storage buffer (50 mol/L Tris-HCl, pH 8.3, 40% glycerol, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L PMSF, and 5 mmol/L DTT), counted, and divided into aliquots at approximately 4x10^6 nuclei/mL before being frozen at -80°C.

**RNA labeling and isolation.** An aliquot of frozen nuclei (2x10^6) was added to 200 µL transcription buffer (10 mmol/L Tris-HCl, pH 7.9, 140 mmol/L KCl, 2.5 mmol/L MgCl2, 0.5 mmol/L MnCl2, 1 mmol/L each of dGTP, dATP, and dCTP, 0.1 mmol/L S-adenosyl-L-

**Nuclear Run-on Studies**

Nuclear run-on studies were conducted essentially as described by Groudie et al47 with minor modifications.48

**Isolation of nuclei.** Cells were washed twice and scraped in a 0.9% (wt/vol) saline solution by using a rubber policeman and then collected by centrifugation at 500g for 4 minutes. The cells were resuspended in NP40 lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L MgCl2, 1 mmol/L NaCl, 3 mmol/L MgCl2, 0.5% NP40, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 1 mmol/L diithiothreitol [DTT]); after being left on ice for 5 minutes, they were homogenized in a Potter Elvehjem tube with pestle B for 25 strokes at 4°C. This homogenate was left on ice for 5 minutes and was again homogenized for 25 strokes at 4°C. Resulting nuclei were centrifuged at 500g and resuspended in NP40 lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. Nuclei were then taken up in a glycerol storage buffer (50 mol/L Tris-HCl, pH 8.3, 40% glycerol, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L PMSF, and 5 mmol/L DTT), counted, and divided into aliquots at approximately 4x10^6 nuclei/mL before being frozen at -80°C.

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**Hybridization.** Target DNA, being 5 µg of plasmid material containing DNA sequences of human apoA-I, human apoB-100, hamster actin, or empty plasmid DNA as negative control (for details see "RNA Hybridization"), was slot blotted on strips of Hybond-N+ filter (Amersham) and cross-linked with 0.4 mol/L NaOH for

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H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂O\text{H}

(all-E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol, Retinol (vitamin A)

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

(all-E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, Retinoic acid (vitamin A acid; Tretinoin)

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

13-cis-retinoic acid (Isotretinoin; Accutane)

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

9-cis-retinoic acid

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

RO 13.4306 (all-E)-3-Methyl-7-(1,1,3,3-tetramethyl-5-indanyl)-2,4,6-octatetraenoic acid

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

RO 13.7410 \text{p}-(\text{E})-2-\{5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthyl\}-1-propenyl]benzoic acid

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

RO 13.6298 Ethyl \text{p}-(\text{E})-2-\{5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl\}-1-propenyl]benzoate

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

N-[4-hydroxyphenyl]retinamide, Fenretinide

H₂C\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\[\begin{array}{c}
\text{H}_3 \text{C} \\
\text{H}_2 \text{C}
\end{array}\]CH₂CH₂\text{COOH}

(all-E)-1-(4-carboxyphenyl)-4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1,3,5-hexatriene, Pelretin
FIG 1. Chemical structures and chemical names or code numbers of retinoids.

30 minutes. The filters were preincubated for 30 minutes at 65°C in a sodium phosphate buffer as described before and hybridized with the labeled RNA for 36 hours in the same buffer. The various filters were washed once for 5 minutes and twice for 30 minutes in 2×SSC/1% SDS at 65°C and exposed to Hyperfilm-MP (Amersham) for 2 to 5 days. Quantification of relative amounts of mRNA was conducted using 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<.05. Values are expressed as mean±SD.

Results

Time Course and Dose Dependency of the Effect of Retinol and Retinoic Acid on ApoA-I and ApoB-100 Secretion

Fig 2 shows the time course of the effect of retinol and retinoic acid on apoA-I and apoB-100 secretion by cynomolgus hepatocytes. Effects of retinol and retinoic acid are expressed as percentages of control during the same incubation period. Both the secretion of apoA-I and apoB-100 remained constant or almost constant during the three consecutive 24-hour incubation periods in all four independent hepatocyte cultures.

With 10 μmol/L retinoic acid, a significantly accelerated accumulation of apoA-I (1.5-fold) was observed during a 24-hour incubation after a 24-hour preincubation with the same concentration of retinoic acid. A further significant increase in the accumulation of apoA-I in the culture medium was observed in the third 24-hour incubation in the presence of 10 μmol/L retinol (1.6-fold) or 10 μmol/L retinoic acid (2.7-fold). The increase of the apoA-I secretion caused by retinoic acid was significantly higher than with retinol. Both with retinol and retinoic acid the secretion of apoB-100 was not affected. In subsequent experiments the effect of retinol, retinoic acid, or other retinoids was studied over a 24-hour period after two 24-hour preincubation periods in the presence of the same retinoid concentration.

Fig 2 shows that the addition of increasing amounts of retinol or retinoic acid resulted in an increase in the secretion of apoA-I without affecting the apoB-100 secretion. The effect of retinoic acid on the apoA-I secretion is more pronounced than that of retinol. A significant increase of the apoA-I secretion was observed with 1 and 10 μmol/L retinoic acid and 10 μmol/L retinol.

Structural Specificity Studies

Because of the difference in the induction of the apoA-I secretion caused by retinoic acid and retinol, we studied the effect of other natural and synthetic retinoids. Fig 1 shows the structure of the various retinoids; the Table lists the effects of these retinoids (all tested at a concentration of 10 μmol/L) on the secretion of apoA-I and apoB-100 by the hepatocytes from the...
Retinoic acid proteins secreted into the culture medium, the proteins that retinoic acid may alter the pattern of the experiment were separated by SDS-PAGE and autoradiographed (Fig 3A). ApoA-I and apoB-100 were identified by immunoblotting of the culture medium with or without retinoic acid. RA and C indicate incubation with or without retinoic acid, respectively. None of the retinoids affected the secretion of apoB-100.

Specificity of the Stimulation of ApoA-I Secretion by Retinoic Acid

Our finding that the various retinoids do not affect the apoB-100 secretion by cynomolgus hepatocytes indicated that the increase of apoA-I is not part of a general effect of retinoids on the hepatocytes. In addition, the effect of retinoic acid on the total protein secretion by the hepatocytes was studied by using \[^{35}S\]methionine incorporation in newly synthesized and secreted proteins. Retinoic acid had no effect on the total amount of \[^{35}S\]methionine-labeled proteins secreted by the simian hepatocytes. To explore the possibility that retinoidic acid may alter the pattern of the proteins secreted into the culture medium, the proteins in the medium of the \[^{35}S\]methionine incorporation experiment were separated by SDS-PAGE and autoradiographed (Fig 3A). ApoA-I and apoB-100 were identified by immunoblotting of the culture medium with or without retinoic acid (Fig 3B). The autoradiograph showed that retinoic acid increases the secretion of newly synthesized apoA-I. The effect on apoA-I appeared to be rather specific, although there are a limited number of proteins that are also affected by retinoic acid. Both increases and decreases of some of the secreted labeled proteins were observed, indicating that both positive and negative regulation of synthesis of proteins by retinoic acid may occur.

Primary hepatocytes from the cynomolgus monkey were incubated with different retinoids (10 \(\mu\)mol/L) for 24 hours after two 24-hour preincubation periods with the same retinoid at the same concentration. The concentrations of apolipoprotein (apo) A-I and apoB-100 in the medium were measured as described in "Methods" and were normalized for the amount of cell protein on the culture dishes. Results are expressed as mean±SD for three to five independent experiments. ApoA-I and apoB-100 secretion in control medium was 709±334 and 599±167 ng/24 h per milligram cell protein, respectively. ApoA-I but not apoB-100 secretion in incubations with all retinoids was significantly different \((P<.05)\) from control. *\(P<.05\) between retinol and other retinoids.

### Table: Structural Specificity of Retinoids

<table>
<thead>
<tr>
<th>Compound Added to the Medium</th>
<th>ApoA-I Secretion, % of Control</th>
<th>ApoB-100 Secretion, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>163±38</td>
<td>101±13</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>272±49*</td>
<td>104±28</td>
</tr>
<tr>
<td>13-cis-Retinoic acid</td>
<td>238±37</td>
<td>117±7</td>
</tr>
<tr>
<td>9-cis-Retinoic acid</td>
<td>247±14*</td>
<td>124±17</td>
</tr>
<tr>
<td>RO-13-4306</td>
<td>233±41</td>
<td>99±14</td>
</tr>
<tr>
<td>RO-13-7410</td>
<td>262±13*</td>
<td>122±24</td>
</tr>
<tr>
<td>RO-13-8298</td>
<td>267±28*</td>
<td>110±23</td>
</tr>
<tr>
<td>Fenretinide</td>
<td>191±32</td>
<td>125±12</td>
</tr>
<tr>
<td>Pelretin</td>
<td>185±23</td>
<td>107±5</td>
</tr>
</tbody>
</table>

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**FIG 3.** A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of metabolically radiolabeled proteins in the medium of cynomolgus monkey hepatocytes. Hepatocytes were incubated for 24 hours in standard medium with or without 10 \(\mu\)mol/L retinoic acid containing 20 \(\mu\)Ci \[^{35}S\]methionine/mL after 24-hour preincubation periods in the same medium without labeled methionine. B, Apolipoprotein (apo) A-I and apoB-100 immunoblot of the metabolically radiolabeled medium described above after separation by SDS-PAGE. Molecular-mass standards are indicated on the left of the autoradiograph. At the right side of the autoradiograph the positions of apoA-I and apoB-100 are depicted. RA and C indicate incubation with or without retinoic acid, respectively.

**Effect of Retinoic Acid on ApoA-I and ApoB-100 mRNA Levels and Transcriptional Activity**

To investigate the mechanism of induction of apoA-I secretion, the effect of retinoic acid on the mRNA levels of apoA-I and apoB-100 was assessed by Northern blot hybridization (Fig 4). The apoA-I and apoB-100 mRNA levels were compared with the actin mRNA level, which was not affected by retinoic acid. A threefold increase in the apoA-I mRNA level was observed when cynomolgus monkey hepatocytes were treated with 10 \(\mu\)mol/L retinoic acid for 72 hours. In contrast to the apoA-I mRNA level, the apoB-100 mRNA level was not changed by retinoic acid.

To further investigate the mechanism of regulation of apoA-I mRNA induction, nuclear run-on studies were performed. Nuclear run-on transcripts were analyzed after a 72-hour incubation of the simian hepatocytes with or without 10 \(\mu\)mol/L retinoic acid (Fig 5). The transcriptional activity of the actin gene was used as an internal standard. The empty vector pUC 18 showed
that there was no aspecific hybridization of the gene transcripts. A twofold increase in the transcriptional activity of the apoA-I gene was observed without changes in the expression of the apoB-100 gene, indicating that the elevated apoA-I mRNA level resulted from an increased transcriptional activity.

**Discussion**

In this study we showed that retinoids increase the secretion of apoA-I in primary hepatocyte cultures from cynomolgus monkey in a time- and dose-dependent way without affecting apoB-100 secretion. $[^{35}S]$Methionine incorporation experiments demonstrated that retinoic acid stimulates the secretion of newly synthesized apoA-I without changing total protein synthesis. Although there were changes in a few other proteins synthesized by the hepatocytes, these findings indicated that the effect of retinoids on apoA-I synthesis is not part of a general increase in protein synthesis by simian hepatocytes. The enhanced apoA-I synthesis was accompanied by a comparable increase of the apoA-I mRNA level and of the transcriptional activity of the apoA-I gene in the retinoic acid–treated hepatocytes. The latter data demonstrated that retinoids regulate apoA-I synthesis in simian hepatocytes by induction of gene expression. In contrast with apoA-I, retinoic acid did not change apoB-100 mRNA level and transcriptional activity.

We found, to our knowledge for the first time, that retinoids stimulated apoA-I synthesis and secretion in a physiological system. This may not seem surprising, as the presence of a retinoic acid–responsive element in the 5'-flanking region of the A-I gene has recently been reported that responds preferentially to the retinoic acid–responsive receptor RXRα. However, the addition of retinoic acid to the human hepatoma cell line HepG2 did not lead to the induction of the apoA-I gene in these cells or to the secretion of apoA-I in the culture medium (A. Kaptein, PhD, and H.M.G. Prin- cen, PhD, unpublished data, May 1990), even after prolonged exposure to the retinoid. In contrast to retinoids, butyrate stimulates apoA-I synthesis in HepG2 cells, indicating that regulation of apoA-I is possible. The lack of responsiveness could only be overcome by transfection of a high amount of RXRα, leading to overexpression of this receptor in HepG2 and subsequent transcriptional activation in the presence of retinoic acid. These data may indicate that HepG2 cells do not contain and do not have the capability of inducing significant amounts of RXRα to stimulate apoA-I gene expression and secretion on addition of retinoids. In contrast, as we demonstrated, primary simian hepatocytes are responsive to retinoids. We suggest that this difference in responsiveness between hepatoma cells and primary hepatocytes results from the well-known phenomenon of dedifferentiation in hepatoma cells, which is accompanied by loss of certain functions, eg, the loss of RXRα. Therefore, hepatocytes in primary culture may give a more accurate reflection of the actual physiology of the liver.

Retinoic acid is known to be an active metabolite of retinol, which exhibits a variety of potent effects on cell growth and differentiation, such as suppression of carcinogenesis in vivo and regulation of pattern formation.
in developing and regenerating limbs. Retinoic acid, in turn, can be converted into physiologically active compounds such as its 9-cis and 13-cis isomers. We compared the potency of these naturally occurring retinoids to induce apoA-I synthesis with several synthetic compounds. Our results suggested that a terminal carboxylic acid moiety in the retinoids is of importance in the modulation of apoA-I synthesis in the hepatocytes. Replacement of this group by a hydroxy group (as in retinol) or amidation of the carboxylic function (as in fenretinide) diminished the activity of the compounds compared with the carboxyl group-containing analogue retinoic acid. On the other hand, esterification of the carboxylic acid moiety (RO 13-6298) did not lead to a decrease in activity compared with the free carboxylic acid-containing analogue RO 13-7410, suggesting that this compound is active as such or that hydrolysis of the ester proceeds rapidly in the hepatocytes, in contrast with hydrolysis of the amide bond in fenretinide. Structural modification of the side chain such that a free carboxylic acid moiety is retained, as in RO-13-4306, RO-13-7410, and in the 9-cis and 13-cis isomers of retinoic acid, resulted in a comparable effectivity in stimulation of the apoA-I synthesis as that found with retinoic acid itself. Pelretin, which also contains a free carboxylic acid, appeared to be less effective than retinoic acid, although the difference between retinoic acid and pelretin was not statistically significant. The importance of a terminal carboxylic acid group in retinoids has also been shown by other investigators using different types of cultured cells. This may point to a common mechanism responsible for all these different effects. However, the actual molecular mechanism of regulation of gene expression by retinoids may be more complicated, since two different families of nuclear retinoid receptors have now been characterized (RARs and RXRs), each consisting of three receptor subtypes. Our finding that induction of apoA-I secretion is regulated by transcriptional activation in primary hepatocytes is in line with two recent reports showing that apoA-I promoter constructs can be regulated by incubation of cells transfected with retinoid receptors and with retinoic acid and its 9-cis isomer. However, the exact molecular mechanism of induction of apoA-I gene expression by retinoids is not fully understood. Whereas Widom et al report that formation of RXRa homodimers in the presence of retinoic acid may abolish the inhibition of transcription of the apoA-I gene by the nuclear factor ARP-I, Zhang et al show that the heterodimer RARa-RXRa is more efficient in activation of the apoA-I retinoic acid–responsive element, and it is most efficiently activated in the presence of 9-cis-retinoic acid. This intracellularly generated stereoisomeric metabolite of retinoic acid is believed to be the natural ligand for the retinoid receptor RXRa. We did not observe a significant difference in the magnitude of stimulation of apoA-I secretion in simian hepatocytes with either retinoid. This is the case not only for the 10-μmol/L concentration listed in the Table but also for lower concentrations, ie, 0.01, 0.1, and 1 μmol/L (data not shown). A possible explanation for the comparable potency of retinoic acid and its 9-cis isomer may be intracellular isomerization of the retinoid.
noids to each other in metabolically active hepatocytes. Our finding that the apoA-I synthesis is stimulated after a lag phase of at least 24 hours, ie, during the second and third day of culture, suggests that one or more of the receptors must be induced to sufficient levels to activate apoA-I gene expression.

The results in the present study showing that retinoids activate apoA-I secretion and gene expression raise the possibility that physiological signals relevant to vitamin A metabolism play an important role in the regulation of plasma apoA-I and HDL levels and consequently the atherosclerotic process. In this context it is interesting that ingestion of \( \beta \)-carotene, which is considered to be a precursor of retinol and retinoic acid, has been reported to increase HDL levels. Similarly, feeding of retinoid acid to rabbits appears to enhance plasma HDL cholesterol and apoA-I levels. On the other hand, it should be noted that the therapeutic use of retinoids, eg, isotretinoin and etretinate in dermatologic applications, has been associated with increased concentrations of serum triglycerides and cholesterol contained predominantly in very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), respectively. No change in apoA-I levels and no or a slight decrease in HDL cholesterol were found. The mechanism of these changes has recently been suggested as involving reduced VLDL uptake by the liver after isotretinoin treatment in rat, possibly as a consequence of a decreased lipoprotein lipase activity. The reduction of HDL cholesterol may be secondary to the increase of VLDL, ie, decreased production of surface components because of decreased catabolism of VLDL, or a reflection of the greater cholesterol ester- and triglyceride exchange between HDL and VLDL, intermediate-density lipoprotein, and LDL. Thus, it is still conceivable that retinoids in vivo enhance apoA-I synthesis, which is, however, not reflected in increased plasma apoA-I levels as a result of the concomitant elevation of the triglyceride level. In view of the reported stimulation of apoA-I synthesis by retinoids in hepatocytes, we suggest that development of retinoid analogues that do not cause hypertriglyceridemia may be suitable in regulating HDL levels.

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Retinoids stimulate ApoA-I synthesis by induction of gene transcription in primary hepatocyte cultures from cynomolgus monkey (Macaca fascicularis)
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