Opposite In Vitro and In Vivo Regulation of Hepatic Apolipoprotein A-I Gene Expression by Retinoic Acid

Absence of Effects on Apolipoprotein A-II Gene Expression

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Abstract We studied the pharmacological potential of retinoids to modulate apolipoprotein (apo) A-I and apoA-II gene expression and production in vitro in the rat and in vivo in the rat. In HepG2 cells, addition of all-trans retinoic acid (RA) doubled apoA-I mRNA within 24 hours and protein secreted in the culture medium after 48 hours. The induction of apoA-I mRNA by RA was completely blocked by actinomycin D, suggesting that RA acts at the transcriptional level in HepG2 cells. In primary cultures of rat hepatocytes, addition of RA increased apoA-I mRNA in a dose- and time-dependent manner as well as the secretion of apoA-I protein. Similar changes in apoA-I mRNA were observed with 9-cis RA. However, in vivo, hepatic apoA-I mRNA levels decreased after a single administration of RA at 10 mg/kg and remained low after prolonged treatment or at a higher dose, and serum apoA-I concentrations did not change. Furthermore, RA treatment did not substantially affect apoA-II mRNA levels or protein secretion either in vitro or in vivo. As a control, RA receptor-β mRNA levels increased after RA both in vitro and in vivo. In conclusion, RA treatment selectively induces apoA-I and not apoA-II expression in vitro but not in vivo. These results therefore show additional regulatory effects of RA on apoA-I gene expression in vivo and raise questions about the usefulness of RA in the treatment of atherosclerosis. (Arterioscler Thromb. 1994;14:1657-1664.)

Key Words • atherosclerosis • gene regulation • apolipoproteins • retinoic acid • steroid hormone receptors

Decreased plasma concentrations of high-density lipoprotein (HDL) cholesterol are associated with an increased risk for the development of coronary heart disease.1-3 The major protein constituents of HDL are apolipoprotein (apo) A-I and apoA-II. Recent epidemiological studies have suggested that the protective effect of HDL on atherosclerosis development may be correlated to specific particles within HDL containing apoA-I and not apoA-II (lipoprotein A-I/A-II), as opposed to particles containing both apoA-I and apoA-II (lipoprotein A-I/A-II/IIa).4 In addition, overexpression of human apoA-I in transgenic mice confers resistance to early atherogenesis,5 whereas less protection is observed in mice overexpressing human apoA-I and apoA-II simultaneously.6 The atherosclerosis susceptibility in mice also has recently been linked to the apoA-II gene locus,7 and transgenic mice that overexpress mouse apoA-II exhibit increased atherosclerotic lesion development.8 Taken together, these observations suggest that agents capable of upregulating apoA-I gene expression and production, without affecting or even decreasing apoA-II synthesis, may be of use in the prevention and treatment of atherosclerosis.

The cloning and characterization of the apoA-I and apoA-II gene promoters have provided the opportunity to study the regulation of these genes at the molecular level (for review, see Reference 9). Furthermore, several factors, such as various hypolipidemic drugs and hormones, have been shown to modulate the expression of the apoA-I and apoA-II genes, essentially in vivo.10-18 Several of these factors are ligands for receptors belonging to the steroid/thyroid hormone receptor superfamily. These receptors are trans-acting factors, some of which have been shown to interact with and transactivate the apoA-I and apoA-II gene promoters in cotransfection assays.19-22 It has been shown that transcription from the apoA-I gene promoter is induced after cotransfection with the retinoid receptor RXRα in the presence of all-trans retinoic acid (RA).23 These observations have raised the possibility that activation of apoA-I gene transcription by vitamin A and its derivatives may provide a physiological mechanism for atherosclerosis prevention. However, data on the physiological importance of RA and its isomers on apolipoprotein gene expression are still lacking.

We therefore decided to evaluate the physiological role of retinoids in the regulation of apoA-I gene expression and production using different in vitro and in vivo model systems. In addition, this article addresses the question of whether RA regulates apoA-I and apoA-II genes coordinately. First, we investigated the effects of RA on apoA-I and apoA-II mRNA and protein in vitro on the human hepatoma cell line HepG2.
and then on primary cultures of adult rat hepatocytes. Finally, because both human and rat cells responded in a qualitatively similar fashion, we studied the regulation of apoA-I and apoA-II gene expression in vivo using the rat as a model. The results from these studies demonstrate that RA selectively induces hepatic apoA-I but not apoA-II gene expression in both in vitro models tested but decreases liver apoA-I mRNA levels in vivo.

Methods

Materials

All-trans RA was purchased from Sigma Chemical Co, and 9-cis RA was synthesized at Rhône-Poulenc Rorer Ltd as previously described.24 Stock solutions (10 mmol/L) in dimethyl sulfoxide or dimethylformamide were prepared under reduced light conditions and stored at -20°C in the dark.

Culture of HepG2 Cells

The human hepatoblastoma cell line HepG2 was obtained from the European Collection of Animal Cell Cultures. Only cells from early passages (<20) were used. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) without sodium pyruvate supplemented with 10% (vol/vol) heat-inactivated calf serum (FCS), 2 mmol/L glucose, and antibiotics for 3 days. At 60% confluence, cells were changed for medium without FCS supplemented with 1% (wt/vol) bovine serum albumin (BSA, Sigma) with or without RA. At the RA concentrations used, no cell cytotoxicity could be detected in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay.25 At the end of the experiments, medium was collected and stored at -20°C until measurement of apoA-I and apoA-II concentrations. Cells were washed cold PBS and harvested by lysis in 4 mol/L guanidinium isothiocyanate homogenization buffer.26 Cellular protein concentrations were determined using the Coomassie protein assay reagent (Pierce Chemical Co).

Isolation and Culture of Rat Hepatocytes

Hepatocytes were isolated by collagenase perfusion of livers from male Sprague-Dawley rats (body weight, 150 to 250 g). Cell viability, as determined by trypan blue exclusion, was higher than 85%. The hepatocytes were cultured in monolayers (1.5 x 10^6 cells per centimeter squared) in Leibovitz-15 (L-15) medium (GIBCO/BRL) supplemented with 10% (vol/vol) FCS, 0.2% (wt/vol) fatty acid-free BSA, 26 mmol/L NaHCO_3, 2 mmol/L glucose, and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. Treatments were started immediately after the cells were seeded and continued for the indicated time periods. No morphological differences in cell adhesion or cell toxicity as determined by the MTT test (IC_50 > 10^-4 mol/L) were observed between control and treated cells at the doses used. At the end of the experiments, cells were harvested and medium was collected as described above.

Animals and Treatments

Male Sprague-Dawley rats weighing approximately 200 g were treated daily by oral gavage with an RA suspension in a 1% (vol/vol) Tween solution at the indicated doses. Sham-treated animals received vehicle only. At the end of each experiment, animals were fasted overnight and killed by exsanguination under ether anesthesia. Blood was collected and serum stored at -20°C for determination of cholesterol, triglyceride, and apoA-I concentrations. Livers were removed immediately, rinsed with 0.9% NaCl, and frozen in liquid nitrogen.

RNA Analysis

Total cellular RNA was isolated from cultured cells and tissues by the acid guanidinium thiocyanate–phenol–chloroform method.26 Tissue RNA was further purified by an additional precipitation step with 1 vol of 8 mol/L LiCl for 3 hours at -26°C. Northern and RNA dot blot hybridizations were performed exactly as described.10,10 Human and rat apoA-I, apoA-II, and apoE cDNA probes were used for HepG2 and rat RNA hybridizations, respectively. As a positive control for gene regulation in response to RA, a mouse RA receptor-β (RAR/β) cDNA probe was used.30 All probes were radiolabeled by random primed labeling (Boehringer Mannheim). Filters were hybridized to 1.5 x 10^6 cpm/mL of each probe as described.10 They were washed in 500 mL of 0.5 x SSC and 0.1% sodium dodecyl sulfate for 10 minutes at room temperature and twice for 30 minutes at 65°C and subsequently revealed by autoradiography (X-OMAT-AR, Eastman Kodak Co). Autoradiograms were analyzed by quantitative scanning densitometry (Hoefer GS-300 scanning densitometer) as described.16

ELISA of ApoA-I and ApoA-II

HepG2-secreted apoA-I and apoA-II concentrations were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) adapted from Fruchtart et al.29 For measurement of apoA-I in rat plasma and medium from rat hepatocyte cultures, a specific rat apoA-I sandwich ELISA was developed. Briefly, a specific rabbit antiserum was raised against a long synthetic polypeptide corresponding to the C-terminal part of rat apoA-I (Tartar et al, personal communication, 1994). The IgG fraction was subsequently purified by affinity chromatography using diethylaminoethyl Affi-gel Blue (Bio-Rad Laboratories) and used as primary antibody in a sandwich ELISA. Peroxidase-conjugated IgG was prepared as described29 and used as secondary antibody. Intra-assay and interassay coefficients of variation were 4% and 5%, respectively.

Statistical Methods

All experiments were reproduced at least twice. ANOVA was used to evaluate the results of the in vivo experiments. If
Results

RA Induces ApoA-I but Not ApoA-II Expression in HepG2 Cells

Since HepG2 cells retain many of the normal biochemical functions of human liver parenchymal cells, including the ability to synthesize and secrete all major human plasma apolipoproteins, we decided to investigate the influence of RA on apoA-I and apoA-II gene expression and secretion. Addition of RA (10 μmol/L) to HepG2 cells slightly increased apoA-I mRNA levels after 6 hours, and a further induction (twofold) was observed after 24 hours (Fig 1, left). In contrast, apoA-II mRNA remained constant during the treatment period (Fig 1, left). The induction of apoA-I gene expression was followed after 48 hours of RA by a similar increase in apoA-I protein secreted in the culture medium, whereas apoA-II secretion was similar in RA-treated and control cells (Fig 1, right). Quantitatively similar results were obtained in five independent experiments.

Because previous in vitro studies using transient transfection assays have shown that RA is able to activate gene transcription from the apoA-I promoter, we determined whether the increase of apoA-I mRNA steady-state levels in HepG2 cells after RA was at the transcriptional level. When gene transcription was blocked by addition of actinomycin D (5 μg/mL) to the culture medium 90 minutes before treatment with RA, the induction of apoA-I mRNA levels was abolished, whereas treatment with RA alone again resulted in a twofold increase in apoA-I mRNA (Fig 2, left). Again, apoA-II mRNA levels did not change after RA treatment (Fig 2, left). The alterations in apoA-I and apoA-II mRNA levels as measured by dot blot hybridization were confirmed by Northern blot analysis (Fig 2, right).

RA Selectively Induces ApoA-I Expression in Primary Rat Hepatocyte Cultures

Because HepG2 is a transformed cell line showing some characteristics of fetal hepatocytes, we decided to investigate the effects of RA on primary cultures of adult rat hepatocytes. Similar to HepG2 cells, addition of RA (10 μmol/L) increased apoA-I mRNA levels slightly after 6 hours, and a further rise to 182% and 204% of control levels was observed after 12 and 24 hours, respectively (Fig 3, left). When primary hepatocytes were treated for 12 hours with different RA doses, apoA-I mRNA increased slightly at 1 μmol/L RA, and a maximal effect was observed at 10 μmol/L RA (Fig 4). In contrast, hybridization of the same blots to apoA-II revealed no change in apoA-II mRNA levels at any time or dose tested (Figs 3 and 4). In addition, the increase in apoA-I mRNA after RA was accompanied by a significant increase in apoA-I secretion in the culture medium as measured by ELISA. After a 24-hour treatment, apoA-I concentrations reached 951±60 and 1259±99 ng/mg per 24 hours in the absence and presence of RA (10 μmol/L), respectively. These results were confirmed in two independent experiments.

RA and 9-cis RA Induce ApoA-I Gene Expression to a Similar Extent

Because the effects of RA on the apoA-I gene promoter appear to be mediated by the RXRα recep-
Influence of Retinoic Acid on Body and Liver Weights in Rats

<table>
<thead>
<tr>
<th>Days and Treatment</th>
<th>Body Weight, g</th>
<th>Liver Weight, mg/g body wt</th>
</tr>
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<tr>
<td>Control</td>
<td>236 ± 9</td>
<td>27.9 ± 0.8</td>
</tr>
<tr>
<td>RA</td>
<td>237 ± 8</td>
<td>31.3 ± 1.0</td>
</tr>
<tr>
<td>Control</td>
<td>247 ± 8</td>
<td>28.2 ± 2.5</td>
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<td>RA</td>
<td>249 ± 5</td>
<td>31.2 ± 3.0</td>
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<tr>
<td>Control</td>
<td>267 ± 6</td>
<td>27.2 ± 1.7</td>
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<tr>
<td>RA</td>
<td>268 ± 1</td>
<td>29.1 ± 2.2</td>
</tr>
<tr>
<td>Control</td>
<td>282 ± 12</td>
<td>26.1 ± 1.2</td>
</tr>
<tr>
<td>RA</td>
<td>284 ± 21</td>
<td>29.0 ± 1.1</td>
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RA indicates retinoic acid. Adult male rats were treated with 10 mg/kg per day RA or vehicle (Control) during indicated number of days. Each group consisted of four animals. Values are mean ± SD.

shown in Fig 6, RA treatment did not affect apoE mRNA levels, indicating that the decrease in apoA-I gene expression was not associated with a nonspecific effect of RA on liver gene expression.

Because the induction of apoA-I mRNA in primary cultures of rat hepatocytes occurred within a few hours, we investigated whether RA could induce apoA-I mRNA after shorter treatment periods. To avoid secondary effects via changes in food intake, observed at the highest RA dose tested and which may affect hepatic apoA-I gene expression, we chose to treat rats with 10 mg/kg per day RA.

No differences in body weight were observed between RA-treated and control animals throughout the treatment period, but liver weights were slightly higher in RA-treated versus control rats (Table).

Liver apoA-I mRNA levels decreased slightly after 1 day of RA and remained lower throughout the treatment period (Fig 7A). In contrast, liver apoA-II mRNA levels remained constant at all time points tested (Fig 7B). Serum apoA-I concentrations were similar in control and RA-treated animals (Fig 7D). Furthermore, as shown in Fig 7C, RA treatment did not change apoE mRNA levels.

![Graph showing dose-dependent effects of retinoic acid (RA) on apoA-I and apoA-II mRNA levels in primary rat hepatocytes.](image)

Fig 5. Line graphs show dose-dependent effects of all-trans retinoic acid (RA) and 9-cis RA on apolipoprotein (apo) A-I mRNA levels in HepG2 cells (left) and primary cultures of adult rat hepatocytes (right). Cells were treated for 24 hours (HepG2 cells) or 12 hours (primary cultures) with all-trans RA or 9-cis RA at indicated concentrations. RNA was extracted, and apoA-I mRNA levels were measured by dot blot analysis.10 Values from one representative experiment are expressed as described in Fig 1.
FIG 6. Bar graph shows influence of retinoic acid (RA) on liver apolipoprotein (apo) mRNA levels from adult rats. Rats were treated intragastrically with 10 (light hatched bars) or 60 (dark hatched bars) mg/kg per day RA for 7 days. Control animals received vehicle only (open bars). Tissue RNA was extracted, and apoA-I, apoA-II, and apoE mRNA levels were measured as described in Methods. Each value represents mean±SD of four animals. *P<.05 compared with controls, ANOVA.

RA Treatment Increases Hepatic RARβ mRNA Levels Both In Vitro and In Vivo

In view of the opposite effects of RA on apoA-I gene regulation in vitro and in vivo, we performed control experiments to verify whether RA treatment at the doses used efficiently induced rat liver gene expression. RA has been shown to increase RARβ mRNA levels in rat liver31 as well as in various cell culture models,39,40 such as HepG2 cells, so we analyzed the effects of RA on RARβ gene expression.

Treatment of primary rat hepatocytes with RA (10 μmol/L) increased RARβ mRNA levels after 12 hours of treatment (Fig 8, left). Furthermore, after a 12-hour treatment RARβ mRNA levels increased in a dose-dependent fashion (Fig 8, right). Compared with the

induction of apoA-I mRNA, the increase in RARβ mRNA was maximal at RA concentrations in the same concentration range (Fig 8, right). As also shown in Fig 8 (right), 9-cis RA and all-trans RA induced RARβ mRNA levels to the same extent.

In good agreement with the in vitro data, RA administration to rats resulted in an upregulation of RARβ gene expression in liver. Rat liver RARβ mRNA levels increased after 3 days of treatment and remained higher thereafter (Fig 9, left). Furthermore, the increase in RARβ mRNA levels was observed at both RA doses tested (10 and 60 mg/kg per day), at which liver apoA-I gene expression is specifically downregulated (Fig 9, right). These results show that RA has qualitatively similar effects on RARβ gene expression in vivo and in vitro.

Discussion

In this article we show that RA plays a physiological role in regulating the expression of the apoA-I gene. However, opposing responses are observed in vivo and in vitro.

In vitro, RA treatment results in an induction of apoA-I gene expression at the RNA level, in both human HepG2 cells and primary cultures of adult rat hepatocytes. A report that appeared during the preparation of this article demonstrated a similar induction on stimulation of isolated primary hepatocytes from cynomolgus monkeys.41 These results extend previous reports showing that RA is able to transactivate the apoA-I gene promoter in transient transfection assays.35 In all in vitro models tested, rather high RA doses are required to increase apoA-I mRNA levels, whereas lower, more physiological levels are not active. The induction of apoA-I gene transcription by RA has been shown to occur through interaction of the receptor RXRa, with a specific RA-responsive element in the
apoA-I promoter. The natural ligand for this receptor is believed to be the RA isomer 9-cis RA. However, no major differences in activity between RA and 9-cis RA were observed. These results suggest that both RA and 9-cis RA may be metabolized to other as yet unidentified RA isomers that are responsible for the observed effects. Alternatively, addition of RA or 9-cis RA in the culture medium may be equally effective in promoting the formation of RAR-RXR heterodimers, which may be more efficient than their respective homodimers in activating the apoA-I gene promoter. On the other hand, it is possible that the activation of the apoA-I gene requires a prolonged presence of RA in the culture medium, which is only obtained using rather high starting doses, because of the high rate of metabolic conversion of retinoids by the liver. Nevertheless, the effects of RA are qualitatively similar in human and rat liver cells, indicating that primary cultures of rat hepatocytes can be useful in the study of the regulation of apoA-I gene expression by retinoids.

In contrast to the in vitro situation, treatment of adult rats with RA decreases apoA-I mRNA levels in liver. We chose doses used in the in vivo experiments according to multiple studies addressing the pharmacokinetics and toxicology of RA in vivo. In fact, administration of similar doses was found to result in RA concentrations in the micromolar range in plasma and even higher in target organs, such as the liver. Second, in contrast to the apoA-I gene, RA treatment increased RARβ mRNA levels both in vivo and in vitro. Therefore, in vivo RA treatment does not result in an aspecific decrease in liver gene expression. These data confirm previous reports describing the autoregulation of RARβ gene expression by RA in vitro as well as in vivo.

Several mechanisms may explain the observed discordance between the results obtained in vivo and in vitro. First, the observed effects of RA on apoA-I gene expression in vivo may be indirect via changes in lipoprotein metabolism, as exemplified by the RA-induced hypertriglycerideremia. Alternatively, in vivo, RA could induce factors downregulating apoA-I synthesis. Such inhibitory factors have been recently found to be produced by nonparenchymal cells present in rabbit liver. Second, retinoids are morphogens, whose functions are most pronounced during development and cellular differentiation. It is conceivable that their stimulatory effects on apoA-I gene expression are restricted to a particular period during fetal or early neonatal development. Indeed, RA induces apoA-I gene expression in HepG2 cells, which exhibit some characteristics of fetal hepatocytes. Studies in hepatoma cell lines exhibiting different characteristics of cellular differentiation are presently under way to test this hypothesis. In addition, isolation and cultivation of hepatocytes are known to be associated with early alterations in cellular functions, which might at least partially contribute to the stimulatory effect of RA. Serum apoA-I concentrations remain fairly constant. The discordance between serum apoA-I concentrations on the one hand and liver apoA-I mRNA levels on the other suggests that additional regulatory mechanisms are operative in the in vivo regulation of apoA-I gene expression by RA in rats. Since both human and rat liver cells responded in a similar fashion in vitro, these effects are unlikely to be a consequence of species differences. Furthermore, serum triglyceride concentrations increase, without any significant change in plasma cholesterol concentrations (data not shown). In humans, treatment with vitamin A derivatives was shown to be associated with a similar increase in serum triglycerides, whereas plasma apoA-I did not change substantially. In contrast to the situation observed in humans and rats, plasma apoA-I concentrations have been reported to increase in rabbits on treatment with RA.

Finally, it is remarkable that the expression of the apoA-I and apoA-II genes is differently regulated by
RA. A similar differential regulation has been reported previously after treatment with estrogens, thyroid hormones, and corticosteroids. Together with RA, these hormones are ligands for receptors belonging to the thyroid/steroid hormone superfamily. Since both apolipoproteins may perform opposite functions in the process of reverse cholesterol transport, this differential regulation by vitamin A derivatives may have important implications for the atherothrombotic properties of the HDL particles.

In conclusion, the results from the present study show that RA regulates apoA-I gene expression in the opposite direction in vitro and in vivo. This stresses the fact that great caution should be taken in extrapolating results from the in vitro to the in vivo situation. To be useful as a pharmacological agent in the treatment and prevention of atherosclerosis, RA derivatives should be designed that activate apoA-I gene expression directly, without displaying secondary effects on plasma lipid concentrations, which may be responsible for the observed decrease in apoA-I mRNA after RA in vivo.

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