Retinoic acid affects the expression of nuclear retinoic acid receptors in tissues of retinol-deficient rats

(Rungs/liver/testes/α, β, γ receptors)

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ABSTRACT The multitude of biological effects of the vitamin A metabolite, retinol, are mediated by nuclear retinoic acid receptors (RARs), which are members of the steroid/thyroid hormone receptor superfamily. RAR-α, -β, and -γ are encoded by three genes from which multiple isoforms can be generated. Recent studies suggest that the expression of at least some RAR isoforms can be regulated by retinoic acid in certain cell lines. Here we examined regulation of RAR expression in the adult animal. RARs were analyzed by Northern blots from lung, liver, and testes of retinol-deficient rats. Retinol deficiency caused a 65-70% decrease in the mRNA levels of lung and liver RAR-β, whereas no change was observed in RAR-α and -γ mRNA levels in these organs. In the testes of retinol-deficient animals, two transcripts, RAR-α2 (3.7 kb) and RAR-α3 (2.8 kb), were detected as compared with one RAR-γ (3.7 kb) transcript in retinol-sufficient testes. When retinol-deficient rats were orally administered 1 dose of retinoid acid (100 μg per rat), lung RAR-β mRNA levels started to increase after 1 hr and reached a 16-fold higher level after 4 hr; after 4 hr these retinoid acid-fed rats also showed a 7-fold increase in liver RAR-β mRNA levels as compared with levels in the retinol-deficient rats. In contrast, liver, lung, and testes RAR-α transcripts remained either unchanged or showed only a slight increase in response to retinoic acid. RAR-γ was constitutively expressed in lung, and its mRNA levels were induced 2-fold by retinoic acid. These results show tissue diversity in the rapid induction of RAR-β and RAR-γ by retinoic acid in the adult animal and suggest distinct roles for the various receptor isoforms in the control of the retinoid response.

Retinol and its active metabolite, retinoic acid, have profound effects on cell proliferation and differentiation and appear to play a key role in vertebrate development. Retinol deficiencies or excess induce embryonic malformations in several mammalian species, including humans. Studies of the chick wing bud development suggest that retinoid acid acts like a morphogen and determines the digit pattern along the anteroposterior axis (3-9). In the adult animal, retinol or retinoic acid administration lead to a rapid change in the synthesis of many proteins (ref. 10; for a review, see ref. 11). Retinoids are also known as powerful therapeutic agents that have been used to treat a number of skin diseases. As anticancer agents, particular success was observed in the treatment of second primary carcinomas of the head and neck (12) and acute promyelocytic leukemia (13).

The molecular mechanisms underlying the many effects of retinoic acid are not well understood. Important insight has been gained through the discovery of cellular binding proteins and nuclear receptors for retinoids. The roles of the binding proteins are not yet clearly understood. Cellular binding proteins for retinol (CRBP) and retinoic acid (CRABP) have been implicated in the delivery of their respective ligands to the cell nucleus (14-16). In addition, retinoid-binding proteins appear to be involved in regulating the concentration of free retinoids (17), and CRABP has been reported to play a role in the generation of a concentration gradient of retinoid acid (18-20).

The nuclear retinoic acid receptors (RARs) belong to a large family of ligand-activated enhancer proteins that include the steroid and thyroid hormone receptors as well as the vitamin D3 receptor. The RARs bind to specific DNA sequences near the promoters of responsive genes and modulate gene transcription in the presence of retinoids (21, 22). Three different RAR types, RAR-α, -β, and -γ, have been described (23-28) that are encoded by different genes. From each gene, several isoforms can be derived by alternative splicing and alternative promoter usage (29-31). An additional class of more distantly related retinoid receptors has also been described (32). The highest amino acid sequence homology exists in the DNA-binding domains of different RARs, while the receptors differ more in their amino and carboxyl-terminal domains (23-28, 33, 34). The differences between the receptor subtypes have been conserved from mouse to human, suggesting important specific physiological roles for the individual receptors.

Distinct tissue distributions of the RARs have been described in adult mouse, rat, and human tissues. RAR-α is widely distributed in most tissues, while expression of RAR-β transcript is limited (26, 28, 35, 36). RAR-γ is expressed in mouse embryo, skin, and lung (28). Similar to adult tissues, the distribution of various RARs has a differential pattern of expression during mouse organogenesis (17). RAR-α transcripts are ubiquitously expressed, whereas distinct, mostly nonoverlapping distribution is observed for RAR-β and RAR-γ transcripts in various developing organs (17). The existence of multiple forms of RARs is intriguing in terms of their regulation. For example, addition of retinoic acid to embryonal carcinoma cells (F9 cells) induced mRNA levels for RAR-β (37, 38). In contrast, RAR-α and RAR-γ remained constant after addition of retinoic acid to F9 cells (38). RAR-β can regulate its own synthesis by activating a retinoic acid-responsive element in the RAR-β promoter (21, 39). We have recently found that the gene encoding RAR-γ also contains a retinoic acid-responsive promoter (J. Lehmann and M.P., unpublished data). This raises the question whether in the adult animal RAR expression in various tissues is regulated by retinoids. The purpose of the present study was to examine the expression of RARs as a function of the retinoid status and their response to retinoic acid in the adult animal.

MATERIALS AND METHODS

Animals. Rats (21 days old) were made retinol deficient as described (40). The serum retinol level of retinol-deficient rats was consistently lower than normal levels (15). All animals were maintained on a diet containing 50 mg retinol per kg food. For each experiment, the serum of 10-12 rats was analyzed for retinol and all-trans retinoic acid.

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rats ranged between 0.5 and 1.6 μg/100 ml and those of retinol-sufficient rats ranged between 23 and 33 μg/100 ml as determined by fluorometry (41). Retinol-deficient rats were fed 0.2 ml of cottonseed oil alone or with 100 μg of retinoic acid by intubation into the stomach. Retinol-sufficient rats, used as controls, also were fed 0.2 ml of cottonseed oil. Animals were killed by decapitation after 1 or 4 hr, and various tissues were excised for RNA preparation. For each experiment three individual animals were analyzed with similar results.

**RAR Probes.** RAR-specific probes were obtained from cDNA clones for RAR-α, -β, and -γ as described (26, 42, 43). All of the fragments used as probes spanned the ligand/carboxyl-terminal domain of RAR-α, -β, or -γ. The β-actin probe used to normalize RNA levels was a human β-actin cDNA (Clontech). cDNA probes were labeled with [32P]dCTP (3000 Ci/mmol, DuPont; 1 Ci = 37 GBq) by using a NEN random primer extension labeling system according to the manufacturer’s instructions.

**RNA Isolation and Northern Analysis.** Total RNA was isolated from 0.2-0.4 g of lungs, liver, or testes by the guanidinium thiocyanate/cesium chloride procedure as described (44). The RNAs (25 or 40 μg) were fractionated by size on a 1% formaldehyde/agarose gel and transferred to Nytran membranes (Schleicher & Schuell) in 10× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7). The membranes were then washed briefly in 5× SSC, dried in air, and baked in an oven for 2 hr at 80°C. Nytran membranes were prehybridized (4 hr) and hybridized (1 × 10⁶ cpm/ml) overnight at 42°C in 50% formamide containing 5× SSPE (1× SSPE = 0.15 M NaCl/0.01 M Na2HPO4/H2O/0.001 M EDTA, pH 7.4), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficol, 10% dextran sulfate, 1% SDS, and 200 μg of sheared salmon sperm DNA per ml. The membranes were washed four times in 2× SSC at room temperature, followed by two 30-min washes in 2× SSC/0.1% SDS at 65°C. The membranes were then exposed to Kodak XAR-5 film with intensifying screens at −70°C. After 24 or 48 hr of exposure, band intensities were quantitated by densitometric scanning. Sizes of RAR transcripts were estimated by comparison with 28S (4.7 kb) and 18S (1.8 kb) rRNA.

**RESULTS**

**Retinoid-Dependent Changes in RAR-α Gene Expression.**

Rapid effects of a single dose (100 μg per rat) of retinoic acid on RAR-α mRNA levels in retinoid-deficient rats were examined in liver, lung, and testes 1 hr or 4 hr after the feeding. Northern blot analyses detected two transcripts of ~3.7 kb (RAR-α1) and 2.8 kb (RAR-α2) in liver and lung (Fig. 1). There was no change due to retinoid deficiency in the mRNA levels of RAR-α in liver and lung of cottonseed-fed retinoid-deficient rats (lanes 4–6) and retinol-sufficient rats (lanes 7–9 in Upper and 7 and 8 in Lower). A small increase was observed in the mRNA levels of RAR-α in liver and lung after 4 hr of feeding retinoic acid to retinoid-deficient rats (lanes 10–12) in Upper and 9–11 in Lower compared with levels in retinoid-sufficient rats (lanes 7–9 in Upper and 7 and 8 in Lower).

In contrast to transcripts in liver and lung, RAR-α was the major transcript in the testis of retinoid-sufficient rats, whose RAR-α2 was expressed at very low levels (Fig. 2, lanes 7–9). However, RAR-α2 expression was 3-fold higher in testes of cottonseed-fed retinoid-deficient rats (Fig. 2, lanes 4–6) than of similarly fed retinoid-sufficient animals (Fig. 2, lanes 7–9). Moreover, retinoic acid feeding to retinoid-deficient rats caused a further 30% increase in the accumulation of this transcript (Fig. 2, lanes 10–12). Retinoic acid also increased the mRNA levels of RAR-α by 33% when compared with retinoid-deficient testes (Fig. 2, compare lanes 4–6 vs. 10–12).

**FIG. 1.** Effect of retinoic acid on RAR-α mRNA levels in liver (Upper) and lung (Lower). Each lane contained 40 μg of total RNA from an individual animal. (A) Liver RAR-α. Retinol-deficient rats were fed retinoic acid (100 μg per rat) and sacrificed after 1 hr (lanes 1–3) or 4 hr (lanes 10–12). Control retinoid-deficient rats were fed cottonseed oil and sacrificed after 4 hr (lanes 4–6); similarly, retinol-sufficient rats were fed cottonseed oil and sacrificed after 4 hr (lanes 7–9). (Lower) Lung RAR-α. Feeding conditions were as in Upper. Retinoid-deficient animals were fed retinoic acid and sacrificed after 4 hr (lanes 9–11) or 1 hr (lanes 1–3). Control retinoid-deficient rats (lanes 4–6) and retinol-sufficient rats (lanes 7 and 8) were fed cottonseed oil and sacrificed after 4 hr.

**FIG. 2.** Effect of retinoic acid on RAR-α mRNA levels in the testes. Each lane contained 40 μg of total RNA from an individual animal. Retinoid-deficient rats were fed retinoic acid (100 μg per rat) and sacrificed after 1 hr (lanes 1–3) or 4 hr (lanes 10–12). Control retinoid-deficient rats (lanes 4–6) and retinol-sufficient rats (lanes 7–9) were fed cottonseed oil and sacrificed after 4 hr.
and sacrificed after mRNA after 4 hr. Feeding fed retinoic acid and sacrificed were as in A. Retinol-deficient animals were fed retinoic acid and sacrificed after 1 hr (lanes 1–3) or 4 hr (lanes 9–11). Control retinol-deficient rats (lanes 4–6) and retinol-sufficient rats (lanes 7 and 8) were fed cottonseed oil and sacrificed after 4 hr.

the mRNA levels of lung RAR-β (Fig. 3 Upper, compare lanes 4–6 vs. 7–9). However, lung RAR-β mRNA levels started to rise 1 hr after retinoic acid feeding to retinol-deficient rats (Fig. 3 Upper, lanes 1–3) and showed a 16-fold increase within 4 hr (Fig. 3 Upper, compare lanes 4–6 vs. 10–12). Thus, in the lung, retinoic acid-induced RAR-β mRNA reaches ~5-fold the level in retinol-sufficient rats.

RAR-β message levels in livers of retinol-deficient rats were also decreased by 70% when compared with message levels in livers from retinol-sufficient rats (Fig. 3 Lower, compare lanes 4–6 vs. 7 and 8). Retinoic acid feeding to retinol-deficient rats, induced RAR-β mRNA levels 7- to 9-fold within 4 hours (Fig. 3 Lower, compare lanes 4–6 vs. 9–11). The induced levels of RAR-β were also much higher than the levels in retinol-sufficient liver. Thus, RAR-β mRNAs were strongly inducible in both lung and liver. Lung RAR-β mRNA levels increased more drastically than liver RAR-β transcripts.

**Retinoid-Dependent Changes in RAR-γ Gene Expression.**

Total RNA from liver, lung, and testes was probed by RAR-γ cDNA. A 3.4-kb RAR-γ transcript was detectable only in lung tissue (Fig. 4 Upper). These data show that all three cDNA probes detected their specific RAR transcripts under the stringent conditions used here, and cross-hybridization between RAR-α, RAR-β, and RAR-γ sequences was not observed.

In cottonseed oil-fed retinol-deficient animals, a decrease in RAR-γ mRNA levels in the lungs was observed as compared with similarly fed retinol-sufficient animals (Fig. 4 Lower, lanes 3 and 4 vs. 5–7). Retinoic acid feeding to retinol-deficient rats induced the mRNA levels of RAR-γ 2-fold after 4 hr (Fig. 4 Lower, lanes 8–10).

**DISCUSSION**

In this study, we used retinol-deficient rats to investigate the regulation of RAR-α, -β, and -γ expression in the adult animal. This model system has been used in the past to study the expression of the gene for the cellular retinol-binding protein and overall changes in mRNA synthesis (45–47). A single dose of retinoic acid was given to retinol-deficient rats to examine the short-term effects of retinoic acid on the expression of RARs in liver, lung, and testes.

RAR-α was detectable in all three tissues. Liver and lung RAR-α and RAR-α2 remained unchanged or showed a slight increase in response to retinoic acid. These data are comparable to those reported for F9 and murine melanoma cells (37, 48, 49). Retinol also has been reported to induce the expression of both transcripts in retinol-deficient rat testes (50). However, this study could not distinguish whether retinol has a direct effect or whether retinoic acid formed from retinol influences the expression of RAR-α transcripts in the testes. In contrast, our results showed that RAR-α1 and RAR-α2 were induced in retinol-deficient testes within 4 hr of retinoic acid treatment.

The expression of RAR-α2 in retinol-deficient testes appears to be cell specific because under these conditions this organ contains Sertoli and not germinal cells (51). Thus, Sertoli cells express RAR-α1 and RAR-α2 transcripts. This finding is in accordance with a recent study that investigated the expression of RAR-α in different testicular cells and found that Sertoli cells expressed two transcripts, whereas germinal cells had only the RAR-α1 transcripts (50). The functional significance of cell-specific expression of RAR-α1 and RAR-α2 in testes is presently not known. It is possible that two RAR-α transcripts might influence the expression of different genes within the same cell.
Two RAR-β gene transcripts were detected in liver and lung, and no RAR-β mRNA was detectable in testis. This absence of RAR-β gene expression in the testis has also been observed in mouse (28, 35), while other studies showed the presence of RAR-β in Sertoli cells (50). This discrepancy might be due to the use of total RNA from the mature testis, which is composed of many different cell types, including Sertoli cells. The rapid increase in RAR-β mRNA levels in lung and liver of whole animals suggests an important role for RAR-β in modulating the action of retinoic acid in these two organs. Similar induction of RAR-β transcripts has been characterized in several cell lines, such as F9, PLC/PRE/S, HEPG2, and murine melanoma (36–38, 48). We have not measured the rate of RAR-β transcription in nuclei, but it has been shown that the RAR-β gene contains a retinoic acid-responsive promoter and that RAR-β can regulate its own synthesis (21, 39). Our observation that RAR-β expression is down-regulated in lung and liver of retinol-deficient animals further indicates that RAR-β expression is directly controlled by retinoids and RARs in certain tissues of the adult animals. In this regard, it is also interesting to note that RAR-β protein has a 10-fold higher affinity for retinoic acid than RAR-α protein. Because of this difference in binding affinities, RAR-β gene might be autoregulating its own synthesis more effectively than RAR-α. In addition, our data suggest that RAR-β and RAR-α must have distinct roles in these tissues and that the expression of RAR-β requires tight control. In this context, it is of interest to note that RAR-β is expressed constitutively in several hepatoma cell lines (36) and appeared to be activated in a hepatocellular carcinoma by hepatitis B virus integration (42).

The tissue distribution of RAR-γ is limited to mouse skin and lung. RAR-γ is also constitutively expressed in F9 and S91-C2 cell lines (35, 48). In the present study, a single RAR-γ transcript present in lung is induced within 4 hr by retinoic acid. This transcript encodes most likely the RAR-γ2 (34). RAR-γ2 is transcribed from a special promoter within the RAR-γ gene (52) that is also regulated by RARs (J. Lehmann and M.P., unpublished data). The presence of all three RARs in lung might also explain why this organ responds to vitamin A more rapidly than testes and other tissues. For example, expression of cellular retinol-binding protein mRNA levels in the lungs is induced within 1 hr after retinoic acid feeding to retinol-deficient rats, whereas no changes occur in testes mRNA levels (46). The present study clearly demonstrates the reverse regulation of RARs by retinoic acid in the adult animal and suggests distinct roles for the various receptor isoforms.

The physiological roles for retinoic acid-induced increases in RAR-β and -γ expression are not known. At the molecular level, increased levels of RARs could lead to increased levels of expression of retinoic acid-responsive genes. A more striking effect can be expected through a novel mechanism recently described in which RARs can antagonize the activity of the cellular oncogenes c-jun/c-fos (X. K. Zhang and M.P., unpublished data). The inhibition of jun/fos activity by RARs is strongly dependent on the concentration of RARs and will be significantly affected by the cellular levels of RAR expression. Therefore, since c-jun/c-fos stimulate cell proliferation, the regulation of their activities through the induction of RAR levels by retinoids could represent an important molecular mechanism of growth control in these organs.

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