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REGULATION OF METABOLISM BY RETINOIC ACID AND ITS NUCLEAR RECEPTORS

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ABSTRACT

It is now well established that the pleiotropic effects of vitamin A—with the exception of the vision process—are mediated by its acid derivatives. Although all-*trans* retinoic acid has been known for some time to be an essential regulator for many important biological processes, critical roles for other acid derivatives have more recently emerged. The acid isoforms affect a large diversity of biological systems, including embryonal cells, lymphoid cells, and nerve and muscle cells, as well as essential developmental programs. Retinoic acid signals are mediated by specific nuclear receptors, the RARs and RXRs, which are part of a complex signaling network, allowing for receptor-receptor and receptor-DNA interaction, as well as for receptor interactions with other regulatory proteins. Dissection of the molecular mechanisms has been significantly advanced by the discovery of selective retinoids that in contrast to most natural retinoids activate only defined portions of the complex retinoid response. Some of these novel types of retinoids are also very promising candidates for the development of new therapeutics. Thus, the molecular analysis of the vitamin A derivative retinoic acid has opened new perspectives that form a connection between nutritional signals and the development of new therapeutic agents.

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INTRODUCTION

It is now well established that retinoic acid (RA), a product of oxidative metabolism of retinol (vitamin A), is the physiologically most important active vitamin A derivative. RA was first obtained synthetically in 1941 in the form of the all-*trans*-isomer, and its biological activity was analyzed in animal studies (3). All-*trans*-RA (tRA), originally called vitamin A acid, added to a diet lacking retinol supported growth in rats, but it was less efficient than retinol (3). Animals fed such a diet showed impaired vision and reproduction (26). These observations led to two conclusions: (*a*) tRA cannot be reduced in vivo to retinal, which is the chromophore of the visual pigment rhodopsin (34); and (*b*) tRA was not sufficient for the reproduction process (138). Both conclusions, however, have had to be modified to a certain extent. In the eye, involvement of tRA is indicated because RA binding sites were detected in the retina (130). In addition, formation and release of tRA after administration of all-*trans*-retinol was reported (37), as was the presence of the cellular RA-binding protein (CRABP) in amacrine and Müller cells of the retina (98), indicating that tRA at least has important accessory functions in the eye.

The discovery of high levels of CRABP in the testes (107) and the existence of a distinct androgen-regulated RA-binding protein in the epididymis also suggest that RA is involved in sperm formation and maturation (103, 110). The inability of dietary RA to support sperm formation may therefore be due to its inability to penetrate the blood-testes barrier when present in low concentrations. This is consistent with the observation that high doses of RA injected intraperitoneally supports spermatogenesis (142). Under physiologic

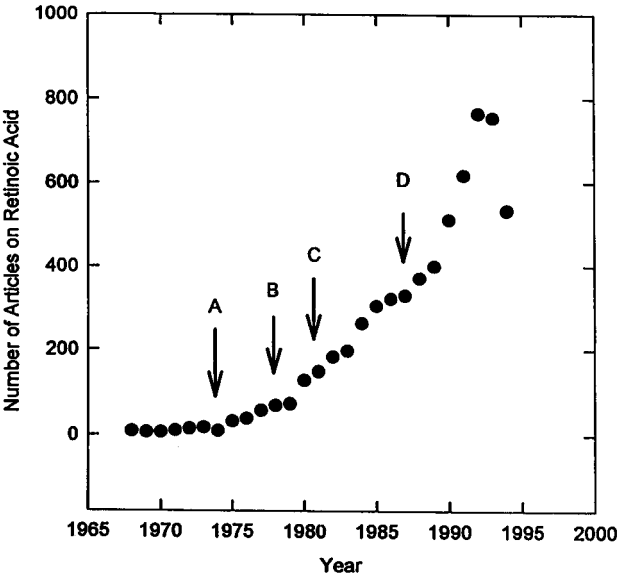


Figure 1 Number of articles on retinoic acid published per year.

conditions, RA is therefore likely to be supplied from within the testicular cell. In addition, dietary RA appears to be taken up by the testicular interstitial Leydig cells, as it supports testosterone production (2). Thus, although the tissue-specific roles (25) of RA at the molecular level in the eye and in the testis remain to be further elucidated, it is clear that vitamin A signals are mediated to a large extent by tRA. However, roles for additional active isomers in these organs cannot be excluded.

The research on RA attracted little attention for quite a number of years until the past two decades, when experimental work on the function of RA intensified amazingly because of important discoveries on the multiple effects of RA on cell cultures as well as on the whole animal. In fact, this precipitated the synthesis of thousands of novel RA analogues and derivatives—the retinoids. Figure 1 shows the yearly increase in scientific publications on RA, as compiled from the Medline data base. Until 1972, the number of publications per year on RA was very small. The slope of the curve changed dramatically in subsequent years, and several phases can be distinguished (Figure 1). The first phase (*A*) precedes the discovery of the first cellular retinoid-binding protein (5). The next phase (*B*) represents the time period following the report that tRA, when added to culture medium of teratocarcinomas of testicular origin, causes differentiation (129). The third phase (*C*) marks the time of the

observations that human leukemia cells (HL-60) in culture will differentiate in response to a very small amount of RA (8) and that retinol and RA can influence pattern formation in the regenerating limb (86). The last phase (*D*) is the discovery of the existence of specific nuclear RA receptors (7, 13, 32, 41, 90). This discovery also coincided with extensive work that provided evidence that RA is involved in animal development, where it appears to directly signal events connected to morphogenesis (54). The growth of interest in RA was reminiscent of the rise in research activity triggered by the discovery of another important signal molecule: cyclic AMP (60). If the 1994 drop in the number of publications on RA is significant, then besides shrinking research funds, one explanation may be that the field is awaiting the next series of important discoveries.

NATURAL ISOMERS OF RETINOIC ACID

Progress in the introduction of and improvement in methods of high-performance liquid chromatography and mass spectroscopy set the stage for the discovery of the existence of further novel RA derivatives in blood and tissues. Figure 2 presents the structural formulas of these compounds. Earlier, it was found that in tissue extracts and in animals, tRA is—at least to some extent—isomerized to 13-*cis*-RA (157). Conversely, when 13-*cis*-RA was given to rats, tRA was recovered (96). 9-*cis*-RA was identified as the principal ligand for one class of the nuclear retinoid receptors, the RXRs (89, 90), and most recently it was identified as a major circulating retinoid in bovine plasma (56). The presence of 9-*cis*-RA in the epididymis has also been reported (110), indicating again that RA is involved in male reproduction. The β-ionine ring of RA undergoes in vivo oxidation. The resulting derivatives are also depicted in Figure 2. Although some of these were previously available only as synthetic molecules and were thought to represent degradation products of RA, it is now clear that several of these molecules have high biological activities and are therefore likely to fulfill specific signaling roles (118). All-*trans*-4-oxo-RA was recently shown to possess high activity in modulating positional specification of early *Xenopus laevis* embryos (118). All-*trans*-4-oxo-RA is also biologically active and indeed has been detected after administration of 13-*cis*-RA (36). All-*trans*-hydroxy-RA and all-*trans*-4-oxo-RA were found to be formed in vitro by hamster trachea and liver (40). Isolation and identification of the 5,6-epoxy derivatives of RA has also been reported (97). All-*trans*-3,4-didehydro-RA has been isolated from chicken embryonic extract and has been shown to be another morphogenic signal like tRA (135). Another RA isomer, all-*trans*-18-hydroxy-RA, has been recently identified as a product of microsomal oxidation of tRA (39). Thus, the existence of many RA isomers may help to explain the multiplicity of RA action.

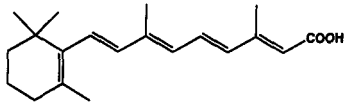
The addition of new chain isomers of RA brings up the question of the nature of the isomerization process. This problem has recently been approached experimentally (65, 140, 141). Although the isomerization of retinol in the visual process is an enzyme-catalyzed reaction that was found to occur at the level of retinyl esters (9, 18), no retinoyl esters have been detected in vivo thus far. The isomerization of tRA to 9-*cis*-RA is a direct process, as it could be demonstrated that bovine liver microsomes can isomerize tRA to 9-*cis*-RA and 13-*cis*-RA. However, the isomerization process is not stereospecific, as two products instead of only one were found. On the other hand, this process may be a physiologically relevant mechanism, allowing the formation of 9-*cis*-RA from tRA (140, 141).

The free carboxyl group of RA is important for its activity. The only known natural derivative of RA where the carboxyl group is blocked is retinoyl-β-glucuronide (4), which unlike other RA derivatives is water soluble. Although most glucuronides are considered a product of detoxification of lipid-soluble compounds like steroid hormones, retinoyl-β-glucuronide is biologically active. The glucuronide was originally isolated from the bile and was subsequently also detected in the blood and tissues (4). Liver microsomes are responsible for formation of all-*trans*-β-retinoyl-glucuronide as well as for 9-*cis*-β-retinoyl-glucuronide, which has been detected after oral administration of tRA to rats and mice (123). However, the tRA glucuronide could not be shown to bind to nuclear RA receptors. This suggests that the biological activation of the glucuronide involves the hydrolysis to free acid. This is in contrast to retinoyl-taurine, which was also isolated from bile (126). It, too, has a blocked carboxyl group but does not show biological activity. Apparently this compound represents an excretory form of RA and is not hydrolyzed in vivo.

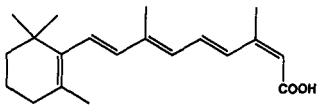
In summary, the number of isomers of RA is now approaching that of steroid hormone isomers, which show species differences in their occurrence in the blood (17). Whether or not isomers of RA have specific roles in various species remains to be shown.

SOURCES OF RETINOIC ACID

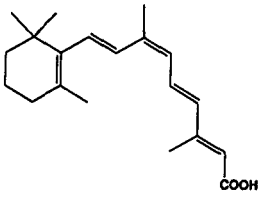
The major source of RA in the diet is its precursor retinol and retinyl ester. Animal diet does not contain substantial amounts of RA that could contribute to RA homeostasis. On the other hand, the findings that rat intestinal cytosol can produce RA from β-carotene (100) pointed to a new system responsible for the generation of RA in vivo. The phenomenon of RA formation from β-carotene has been corroborated by experiments using homogenates from human intestinal mucosa (145), and in addition, it was shown that RA can originate from eccentric cleavage of β-carotene via a series of β-apocareotenols



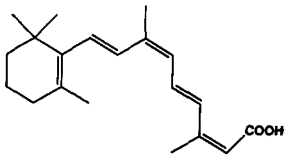
all-trans-retinoic acid



13-cis-retinoic acid

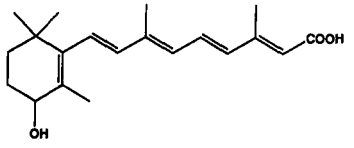


9-cis-retinoic acid

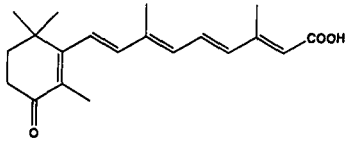


9,13-dicis-retinoic acid

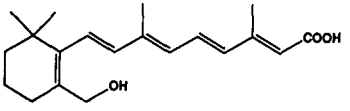
a.



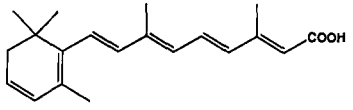
4-hydroxyretinoic acid



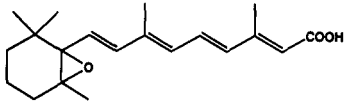
4-oxoretinoic acid



18-hydroxyretinoic acid



3,4-didehydroretinoic acid

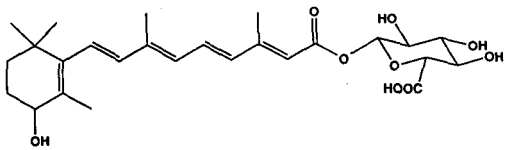


5,6-epoxyretinoic acid

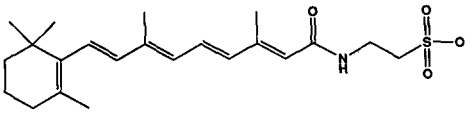
b.

as intermediates (144). Interestingly, orally administered RA is absorbed less efficiently than is retinol. Nevertheless, its action on protein synthesis in the testes (46) or on the regulation of expression of specific genes in the lung can be observed 1 or 2 h after oral administration (47, 48). Thus, the effects of RA are rapid.

Several isomers of RA are found in the blood. In addition to tRA, normal plasma contains 13-*cis*-RA (144), all-*trans*-RA-β-glucuronide (4), 13-*cis*-RA-β-glucuronide (144), and 9-*cis*-RA-β-glucuronide (123). After dosing with

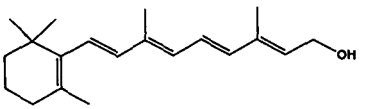


retinoyl-β-glucuronide

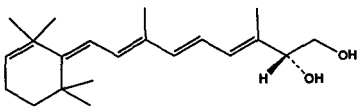


retinotaurine

c.



all-trans-retinol



14-hydroxy-4,14-retro-retinol

d.

Figure 2 Retinoic acid (RA) isomers. (*a*) Naturally occurring isomers of RA (chain modified); (*b*) naturally occurring derivatives of RA (ring modified); (*c*) naturally occurring derivatives of RA (carboxyl group blocked); (*d*) all-*trans*-retinol and 14-hydroxy-4,14-retro-retinol.

13-*cis*-RA, an additional form, 13-*cis*-4-oxo-RA, has been detected (143). From published reports it can be calculated that tRA and 13-*cis*-RA circulate in similar concentrations—approximately 4.3 x 10^{-9} M. The steady-state concentrations of all-*trans*-RA-β-glucuronide and 13-*cis*-glucuronide are below those of RA (approximately 2.9 x 10^{-9} M). Together, then, a total concentration of RA isomers of approximately 1.4 x 10^{-8} M exists in the blood. Although these concentrations of RA isomers are high enough to induce, for instance, differentiation of HL-60 cells in culture (14), many of the physiologic functions of plasma RA remain to be characterized. In addition, the exact origins of RAs in the blood is not known either and may include diverse processes, including cellular apoptosis.

The various RA isoforms are taken up by the cell. Although orally and

intraperitoneally administered RA are biologically effective, the molecular mechanisms of RA uptake into the cell are not clear but appear in most situations to be independent of specific cell surface receptors. For therapy, both tRA and 13-*cis*-RA are used at considerably higher doses than those found normally in the blood, which suggests that uptake from the blood is against a concentration gradient in the cells. Indeed, tissue levels of tRA can be much higher than those found in the blood (98). The levels of tRA determined in various organs range in ascending order from about 35 pmol/g in the rat epididymis, lung, and liver, to as high as 130 pmol/g in the kidney. Thus, the concentration gradient is organ specific and is 2.5 times higher in epididymis and as much as 10 times higher in the kidneys than in blood. The underlying mechanisms of the tissue-specific uptake of RA have not yet been elucidated. One possible explanation for the diverse RA content of various tissues assumes intracellular tissue-specific metabolism of RA (101).

Perhaps more important is the question of how RA is synthesized in various tissues. This subject has been reviewed recently (100). Isolation and characterization of the enzymatic systems responsible for RA synthesis is of prime importance for understanding the involvement of RA in animal development, organogenesis, or organ maturation. Clarification of RA synthesis and its regulation may shed light on the diverse functions of RA in cellular metabolism and development.

One supposition is that the putative enzymes catalyzing oxidation of retinal must have a very low K_m. Several approaches have been taken to study the oxidation of retinol to RA, including the use of cytosolic fractions from different organs (10). Participation of the cellular retinol–binding protein (CRBP) in the synthesis of RA via CRBP-retinal as well as the CRBP-retinol complex has been proposed (98). In addition, some microsomes were shown to catalyze RA synthesis from retinal via RA-bound CRBP. Furthermore, formation of RA from retinol in various cell lines has been described (98). Other experiments suggest the involvment of cytochrome P-450 isozymes (119). These observations point to the complexity of the systems, as P-450 isoforms also metabolize RA, retinol, and retinal to multiple products (119). Although the physiologic systems responsible for the formation of RA remain to be fully characterized, an important step toward this has been the recent report at a conference of the cloning of specific RA-dehydrogenases.

As mentioned above, RA is further metabolized, and this metabolism can be self-regulated. Induction of total hepatic microsomal cytochrome P-450 was observed after feeding rats a diet containing an excess of retinyl acetate, which resulted also in an increase of 4-hydroxy- and 4-oxo-RA (76). One member of the cytochrome P-450-3A family appears to be involved in the RA 4-hydroxylation (36). In addition to these products, 18-hydroxy-RA has been identified when testis microsomes were incubated with RA and CRABP type I

(CRABPI). Thus, it appears that CRABPI is necessary for the oxidation of RA. However, the physiologic importance of this was recently challenged by the report that CRABP type I (CRABPI)-deficient mice are normal (43). Deciphering the regulation of synthesis and degradation of RA still represents a major and important challenge.

BIOLOGICAL SYSTEMS AFFECTED BY RETINOIC ACID

Compelling evidence that RA is involved in many biological systems has accumulated. One very important feature of RA action resides in its involvement in cell differentiation. A large volume of data suggests now an essential role for RA in the molecular mechanism(s) of cell determination, the elucidation of which represents one of the most important issues of developmental biology. Some of the most-studied model systems in which RA plays a pivotal role—as shown by direct or indirect experimental evidence—are summarized below.

Embryonal Carcinoma Cells

RA can induce differentiation of embryonal carcinoma cells (129). Here the concentration of RA is of prime importance. These cells normally develop into muscle cells, whereas at relatively high concentrations of RA (above 5×10^{-7} M) in the medium, aggregates of these cells develop into neuronal and glial tissue (95). These and other experiments have shown that RA is a very potent differentiation agent, which can be active at very low concentrations. However, differential sensitivity and the concentration dependency of RA action also suggest that RA-sensitive cells may possess a mechanism that acts as a sensor of RA. The family of nuclear RA receptors, some of which are inducible by RA (48, 53, 71, 74, 99), is likely to be involved in this process. RA not only causes the embryonal carcinoma cells to differentiate, but under the influence of RA these cells also lose their malignant character (129).

Human Leukemic Cells

Human leukemic cells (HL-60) in culture also differentiate when exposed to small concentrations of RA (14). This discovery eventually led to the successful use of tRA as a differentiation-inducing therapeutic agent in the treatment of human promyelocytic leukemias (19, 21), as discussed below.

Morphogenesis

Another most interesting role of RA was discovered when it was observed that RA could induce morphogenesis, i.e. act as a factor that can carry positional information leading to a change in morphology (54, 84). Although the question

of whether or not RA is a direct morphogen has been disputed, compelling evidence has been presented that RA can alter profoundly the development of the chicken limb (132). In addition, it has been shown that RA forms a concentration gradient in the developing limb, which suggests that RA may be responsible for triggering developmental changes (54). The exact molecular mechanisms responsible for the developmental processes remain to be elucidated. However, it has been domonstrated that expression of HOX genes that specify positional information is influenced by RA (62a, 92, 93, 105). That RA via its receptors directly regulates these genes is supported by the observation that several HOX genes, including HOXB1, have RA response elements (RARE) in the 5′ or 3′ region (106). Whether or not some of these elements show differential RA responses remains to be elucidated.

Central Nervous System

The development of the central nervous system is also influenced by RA. First evidence for this came from the observation that RA induces neuronal or mesodermal differentiation in the pluripotent embryonal carcinoma cell line P19 (95). Gradients of RA are believed to provide information in several processes during vertebrate development, including positional information necessary for the proper formation of the nervous system (35, 86, 87). Evidence for participation of RA in develoment and homeostasis of the central nervous system was further provided by the observation that nuclear RA receptors (RARs) (7, 99) and CRABP are expressed in the brain (87). Finally, null mutations in the retinoid receptors were subsequently shown to lead to abnormal neural development (88). At the molecular level, RA can alter HOX gene expression patterns, altering hindbrain HOX codes by inducing transformation of rhombomers (62a, 105). Regulation of the HOX genes by RA can be direct because, as mentioned above, specific RAREs have been demonstrated in HOX gene promoter regions (93, 106).

Muscle and Cartilage

RA has a modifying effect on the determination of myogenic and cartilage cells in early chicken embryo (20). This enlarges the repertoire of the RA diversified effects on additional systems. In addition, it has been established that targeted loss-of-function mutation in the knock-out RXRα gene in the mouse germ line leads to death of the homozygous embryos. The major defect responsible for mice lacking the expression of this gene has been localized in the hypoplastic development of the ventricular chambers of the heart (131). Thus, RA appears to be a signal for cardiac muscle morphogenesis. Null mutations in RARs (78, 88), especially when combined with RXR mutations, lead to various defects often blocking development.

Pulmonary System

Development of the pulmonary system appears to also be influenced by RA (24). For instance, expression of the nuclear RA receptors in the lung tissue can be influenced by exogenous all-*trans*-RA (48), as well as by the presence of the CRABP, and its regulation during lung development and maturation indicates such involvement (24, 107). The branching of the tracheoalveolar system can be induced by exogenous RA. Several HOX genes influenced by RA have been identified in the lungs of newborns, and the effects of the gestational age on their expression have been described (11).

Immunity

Considerable experimental evidence shows that RA is involved in cell-mediated as well as humoral immunity (23, 33, 120). More recently, it was reported that RA, when added to the retinol-deficient diet, can normalize production of natural killer cells, a process depressed in retinol-deficient rats (156).

In contrast, proliferation of B cells is elevated in the presence of retinol, whereas RA is not active in this system (15). Subsequently, the active retinoid in this system was characterized as 14-hydroxy-4,14-retro-retinol, the structure of which is shown in Figure 3. Thus far, this is a rather unique case, as in most biological systems RA is more active than retinol.

THERAPEUTIC USE

tRA, 13-*cis*-RA, and synthetic retinoids are used clinically for the treatment of several dermatoses (for review see 111). Topical application of tRA is used successfully in the therapy of acne vulgaris (111). When taken internally, 13-*cis*-RA is the most effective agent against this skin disease. Topical application of tRA can also considerably improve photodamaged skin. Adapalene (CD271), a synthetic retinoid that activates selectively RARβ and -γ (8, 32), is the first of a new series of synthetic retinoids approved in several countries for the topical treatment of acne. This retinoid causes fewer side effects than the natural retinoids do.

Natural and synthetic retinoids have also shown promise for the prevention and treatment of cancers. A striking success has been achieved more recently in the treatment of human promyelocytic leukemias with tRA. Here, high oral doses of this compound lead to a remission of the disease (146) and when combined with chemotherapy lead to an apparent cure. One of the most promising synthetic retinoids for the treatment and prevention of various cancers is N-(4-hydroxyphenyl)retinamide (4HPR or fenretinide). Because of its very low toxicity, this compound is currently being evaluated in at least a dozen clinical trials (62).

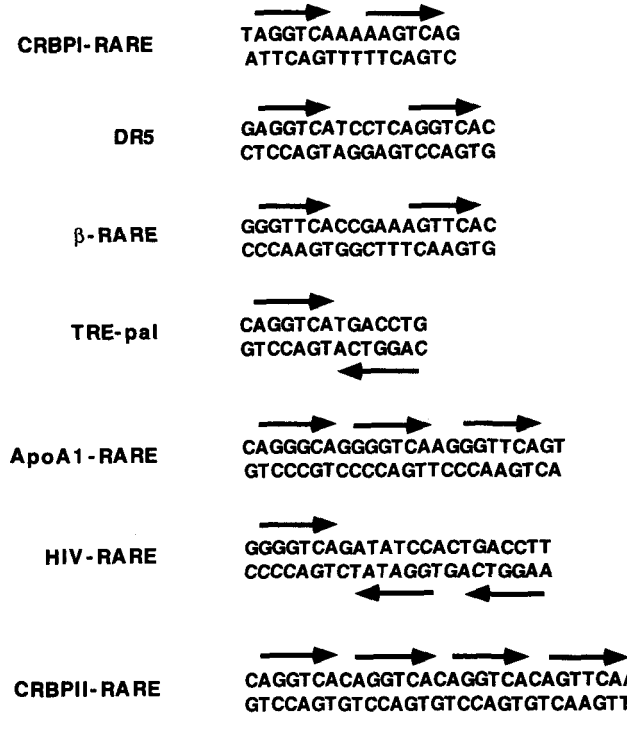


Figure 3 Response elements. Retinoic acid (RA) response elements contain a minimum of two half-sites (*over-* or *underscored by arrows*) that can be separated by spacers of different length and that can be oriented in different directions. The structurally distinct RAREs allow for diversification of the RA response (67).

PHYLOGENESIS

Little is known about which stage of phylogenesis RA first becomes functional as an essential biological signal molecule. Although insects (*Drosophila*) contain a gene that encodes a product with high structural similarities to the human retinoid X receptor (108), the ligand for this receptor is not known. However, this gene, ultraspiracle, is required for pattern formation. In addition, the *Drosophila* ecdysone receptor can heterodimerize with ultraspiracle or RXR (136). Thus, an RA-like molecule could signal morphogenesis in invertebrates. When in evolution RA or its precursor retinal becomes essential for normal development still remains to be elucidated.

In vertebrates, RA functions as a differentiation-inducing agent in many biological systems. This property also underlies some of its therapeutic usage.

In spite of the diversified action of RA, however, the molecular action of RA has a common target: the cell nucleus.

RETINOIC ACID SIGNALING MECHANISMS

The Nuclear Receptors

It is now well established that RA signals are mediated by specific receptors—the retinoid receptors, which are usually located in the cell nucleus. These receptors, the three RA receptors (RARα, -β, -γ) (7, 13, 41, 42, 66) and the retinoid X receptors (RXRα, -β, -γ) (89, 90), are part of a large family of nuclear receptors that also includes the steroid and thyroid hormone receptors. The relationship of RARs and RXRs to the other receptors, as well as their structural domains, has been reviewed elsewhere in detail (117). Briefly, the receptors contain a highly conserved DNA binding domain that allows specific DNA interaction, as well as protein-protein interaction. The second general feature of the receptors is the ligand binding domain (LBD), comprising the carboxy terminal half of the receptors. The LBD is less well conserved among the receptors and besides specific ligand binding also contains a transcriptional activation function, as well as a strong dimerization domain. In addition to the six major receptor subtypes, isoforms for each of the receptors have been identified that differ in their aminoterminal regions, a region that also can contain a transactivation function. The various receptor isoforms are generated by differential promoter usage and alternative splicing (71, 99).

Although the RARs and RXRs are the only receptors that directly interact with or bind retinoids, an increasing number of other receptors are being identified that interact with the retinoid receptors either by heterodimer formation or by competing for the same specific DNA binding sites—the RAREs. These receptors include the thyroid hormone receptors (TRs), vitamin D_3 receptor (VDR), peroxisome proliferator activator receptor (PPAR), and a number of "orphan receptors," receptors for which specific ligands have not yet been identified. Thus, the retinoid hormone receptors are part of a subfamily of receptors that interact through a complex network, allowing for cross talk between various hormones and vitamin derivatives and for modulation of the RA response.

Transcriptional Regulation Via Response Elements

Like the steroid hormone receptors, retinoid receptors regulate gene expression by binding as dimeric complexes to specific DNA sites, the RAREs, usually located 5′ of the promoter region in susceptible genes. As observed for the steroid hormone responsive elements, these RAREs are made up of a minimum of two identical or similar half-sites (usually PuGGTCA or a closely related

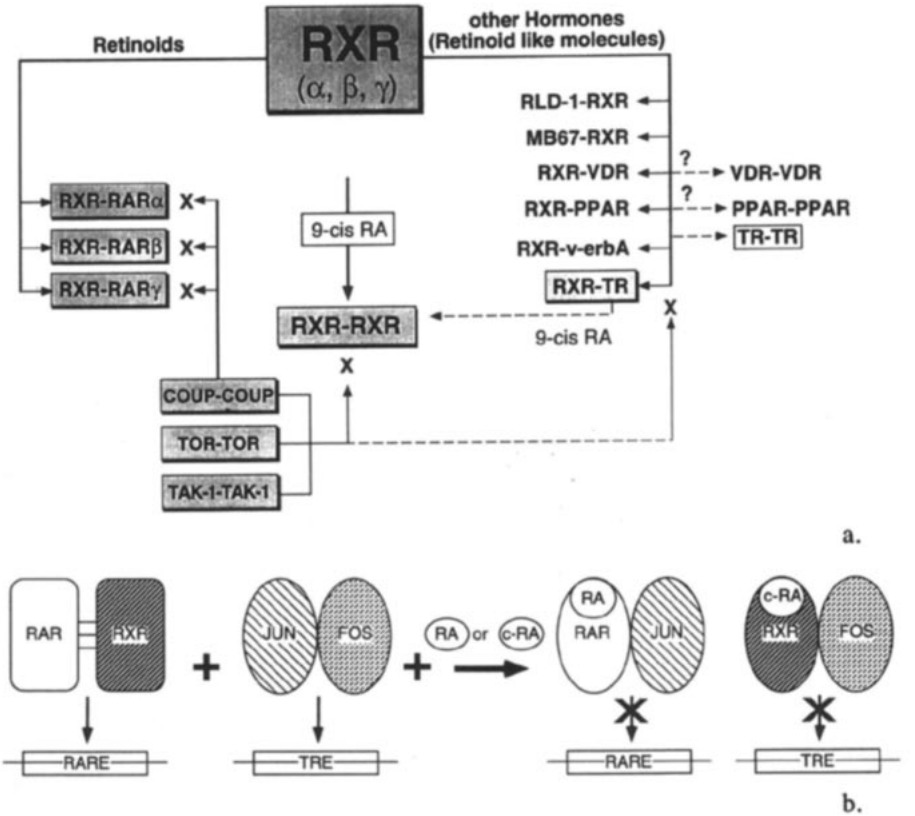


Figure 4 Retinoic acid (RA) signal transduction. (*a*) Interactions among the retinoid-thyroid hormone receptor subfamily. RXTs form heterodimers with RARs that are activated by retinoids. RXRs also form heterodimers with thyroid hormone receptors (TRs) (including v-*erbA*), the vitamin D_3 receptor (VDR), the peroxisome proliferator activator receptor (PPAR), and probably other receptors yet to be determined. In the presence of RXR-specific retinoids like 9-*cis*-RA, RXR homodimers that recognize a subset of RAREs are formed. The presence of ligands that induce the formation of RXR homodimers can inhibit the formation of certain heterodimers and, for instance, lead to a reduction of T3 responsive genes, whereas RAR containing heterodimers do not appear to be negatively affected by RXR-specific ligands. COUP receptors form homodimers that bind with high affinity to several RAREs and that can repress RAR/RXR heterodimer, as well as RXR homodimer, activity. Thus, COUP receptors can restrict retinoid responses to a subset of retinoid-responsive genes. COUPs may also antagonize other receptors. VDR homodimers bind to a subset of vitamin D response elements. TR homodimers only bind in the absence of T3 and can function as ligand-responsive repressors. Other homodimers (RAR, PPAR) may also form on specific response elements. (For a more detailed discussion of these mechanisms see 117.) (*b*) Nuclear receptor/AP-1 interaction. A model is proposed for transrepression. Retinoid receptors and the transcription factor AP-1 (Jun/Fos) can inhibit each others' activities. RAR and RXRs form heterodimers in solution that bind with high affinity to RAREs. Similarly, the components of AP-1, Jun and Fos, form heterodimers that bind with high affinity to TREs. In the presence of RA or 9-*cis*-RA (c-RA), RARs or RXRs undergo a conformational change that allows them to form complexes with Jun and/or Fos. These receptor/Jun or receptor/Fos complexes do not bind to DNA. Thus, when the retinoid receptors are in excess, AP-1 activity is inhibited whereas an excess of Jun or Fos can lead to the inhibition of the retinoid receptors. (For further details, see 114.)

sequence). However, although steroid hormone response elements are palindromes with a 3-bp spacer (6), RAREs comprise a variety of differently arranged half-sites, including direct repeats with 1-, 2-, 4-, 5-, or 8-bp spacers, as a palindrome with no or with a 9-bp spacer, and as inverted palindromes with an 8-bp spacer (reviewed in 116). Examples for RAREs are shown in Figure 3.

Although the steroid hormone receptors bind as homodimers, RARs interact with their response elements as heterodimers with RXR (16, 63, 75, 91, 151, 152). RARs or RXRs alone bind poorly to DNA. Since effective DNA binding is required for receptor function, it can be assumed that RARs and RXRs mostly function as heterodimers. In the presence of RA or 9-*cis*-RA, the heterodimers act as activators of transcription (Figure 4*a*). However, DNA binding of the heterodimers is independent of the ligand, which allows the receptors to have dual functions such that they can operate as gene repressors in the absence of ligand and as gene activators in the presence of ligand (45). Interestingly, RXRs not only enhance RAR DNA binding but are also required for efficient DNA binding of TRs, VDR, PPAR (61, 64), and the v-*erb*A oncogene—a mutated form of TR (49), as well as of several orphan receptors (1, 112, 147). The various RXR containing heterodimers bind to distinct response elements that can have overlapping specificities (116). Thus, one class of retinoid receptors, the RXRs, has a very central role and function as coregulators of several other nuclear receptors, which are activated by structurally unrelated hormones and signal molecules (see Figure 4). This role is maintained through evolution because the RXR homologue ultraspiracle in *Drosophila* was found to heterodimerize with the ecdysone receptor (136, 150).

Besides their central role as coreceptors, RXRs can also function as homodimers. In the presence of 9-*cis*-RA, a natural ligand of RXR, RXR homodimers are induced in solution (153) (Figure 4*a*). RXR homodimers mediate a distinct retinoid signaling pathway by interacting selectively with a subset of RAREs, including those present in the promoter regions of the CRBP type II (CRBPII) and the apolipoprotein AI genes (72, 153).

The unique capability of RXRs to form heterodimers with a large number of receptors that bind structurally unrelated ligands, in combination with its ability to form homodimers in response to 9-*cis*-RA, allows RXR to mediate cross talk among retinoids and other hormones, thereby setting the stage for a central physiologic role of vitamin A derivatives. One mechanism for the cross talk is the competition of various receptors for RXR. In addition, the induction of RXR homodimers by specific retinoids like 9-*cis*-RA can shift the equilibrium from heterodimers to RXR homodimers, resulting in the inhibition of heterodimer-mediated signals. When we investigated, for instance, the effect of 9-*cis*-RA on TR/RXR activity, we indeed observed that thyroid hormone (triiodothyronine; T3) induction of a T3-responsive gene was strongly

repressed by 9-*cis*-RA and by synthetic retinoids that allow RXR homodimer formation. A detailed analysis of the phenomena suggested a mechanism where 9-*cis*-RA induces the sequestering of RXR molecules from TR-RXR heterodimers into RXR homodimers, thereby leading to a repression of the T3 response (73) (see Figure 4*a*). The vitamin D_3 response, it appears, can also be inhibited by 9-*cis*-RA via this mechanism (83). Thus, certain retinoids can control the availability of RXR molecules for heterodimerization with other receptors and thereby allow cross talk between retinoids and other hormones and vitamin derivatives.

One other possible mechanism by which retinoids can control the availability of RXR for other receptors is the induction of RARs by tRA and 9-*cis*-RA. The RARs can be classified as so-called master regulators because they can control their own synthesis. RARα, -β, and -γ isoforms have been shown to be up-regulated in the presence of RA through RAREs in their promoter regions (115). In particular, RARβ was found to be rapidly increased in lung and other tissues when vitamin A–starved rats were fed tRA (48). Such an RA-induced increase in RAR molecules is likely to affect the equilibrium of heterodimers in the cell under conditions where RXR molecules are limited and therefore could affect signal responses of other hormone and vitamin receptors that require RXR for DNA binding. Thus, a complex network of receptor interactions has been unraveled that allows retinoids to influence a network of receptors and therefore other hormone and vitamin signals.

Specifying the RA Response and the Role of Orphan Receptors

Although the various heterodimers and ligand-induced RXR homodimers allow for a diversity and specificity of the retinoid responses, an important question is how certain RA responses can be limited to certain cell types. It now appears that the receptors themselves, as well as some members of the extended retinoid receptor subfamily for which ligands have not been identified, i.e. orphan receptors, play an important role in determining cell type–specific RA-sensitive gene regulation. RARγ1, for instance, could be shown to repress certain RAREs, in particular the βRARE (53, 58). Thus, in cells that produce RARγ1, RA-responsive genes that contain βRARE-like response elements may show a reduced or no response to RA. In an extension of this, it was shown that different RA-responsive promoters can be activated in the presence of individual RAR subtypes to varying degrees. Recently, we carried out a detailed study to investigate how the RA responsiveness of a particular RARE is dependent on specific heterodimers. We observed that different response elements (such as those shown in Figure 3) show different receptor responsiveness. For instance, the RARβ/RXRα heterodimer is a strong activator of the CRBPI RARE whereas the RARα/RXRα heterodimer is a poor

activator of this response element (67). Thus, the expression pattern of the RARs and RXRs determines at least to some degree the RA responsiveness of a gene. The RA isoform present further modulates this response. 9-*cis*-RA is in most cases the optimal activator, whereas tRA often leads to only a partial activation of a heterodimer/RARE complex (67). Further specification of cell type–specific restriction of RA responses is achieved through the tissue or cell type–specific expression of orphan receptors. The orphan receptor COUP and its closely related homologue, ARP-1 (also called COUPα and -β), are the most highly conserved members of the nuclear receptor family. Interestingly, COUPα and -β were found to bind with high affinity to several RAREs but did not function as activators of these RAREs (69, 139). However, COUPs were able to inhibit activation of RAREs by RAR/RXR heterodimers and RXR homodimers. Inhibition of retinoid receptors was only observed on some RAREs, in particular those to which these orphan receptors were found to bind with high affinity. Response elements not recognized by COUP were also not repressed (28, 69, 139, 148). Various regions of the developing brain are major expression sites for COUP (82). Thus, COUP orphan receptors may have particular roles in regulating RA responses in the brain. Are there then other orphan receptors that could play similar roles in other tissues? Recently, we characterized two new orphan receptors that both bind strongly as homodimers to certain RAREs and that can repress RA induction of genes containing such elements. One, TAKI, is expressed in a broad collection of tissues, including testis. TAKI can repress the tRA response on βRARE-type elements, as well as 9-*cis*-RA–induced RXR homodimer activity on the CRABPII RXRE (52). Another orphan receptor that represses RAREs is TOR. This receptor is expressed almost exclusively in the thymus and mature T cells. TOR represses selectively a subset of DR-4 and DR-5 elements (109).

Thus, one subgroup of orphan receptors appears to exist that is expressed in selected tissues and can repress subsets of RAREs. Thus, the tissue-specific expression of these orphan receptors (repressors) allows for a mechanism to restrict the RA response in a tissue-specific manner.

Receptor Interaction with Other Transcription Factors

An important alternative mechanism by which retinoid receptors can mediate signals is by interacting directly with other transcription factors. The best-studied example for this is the transcription factor AP-1 (for a recent review see 114). The products of the two proto-oncogenes c-Fos and c-Jun and several related proteins form a complex in the nucleus, termed activator protein 1 (AP-1), that binds to a DNA sequence motif not recognized by the retinoid receptors and that is referred to as AP-1 binding site, or TPA-responsive element (TRE). By binding to this sequence, AP-1 mediates signals from

growth factors, inflammatory peptides, oncogenes, and tumor promoters, usually resulting in cell proliferation. In the presence of their ligands, RARs as well as RXRs can interact with AP-1 and inhibit its activity (122, 124, 149). The retinoid receptors can thus directly interfere with many cell proliferation signals when blocking AP-1 activity. However, vice versa, AP-1 can also inhibit RAR activity via the same mechanism. Thus, depending on the concentrations of the AP-1 components and that of the nuclear receptors, AP-1 or the receptors can be inhibited, thus allowing for a major switch between proliferation and differentiation. In Figure 4*b*, a model for the interaction between the retinoid receptors and AP-1 (c-Jun/c-Fos) is presented that has been reviewed in detail elsewhere (114).

SYNTHETIC DERIVATIVES AND ANALOGUES OF RA

The antiproliferative activities of RA have raised much interest for the usage of RA in the treatment of proliferative diseases. As mentioned above, retinoids used today are the most effective drugs for the treatment of a number of skin diseases. In addition, tRA treatment leads to complete remission in promyelocytic leukemia, and 13-*cis*-RA has shown promise in the treatment and prevention of many other cancers (reviewed in 12, 127). However, the broad clinical usage of RAs has been hindered by their undesirable side effects, which can range from mild skin rashes to severe nausea, bone fractures, and malformations in fetuses. It has therefore been a long-time goal of chemists to separate the beneficial effects of the retinoids from the undesirable side effects. Progress in this met with limited success for a long time because of a lack of suitable test systems and because of the absence of an understanding of the molecular mechanisms by which retinoids function. The cloning of multiple nuclear receptors and the deciphering of their mechanism of action set the stage for using simple test systems to separate diverse retinoid activities on the basis of receptor specificities.

RAR Subtype–Selective Compounds

Although tRA activates only RARs and not RXRs, little difference in the activation capacities of naturally occurring RA isomers for the RAR subtypes α, β, and γ has been observed. Close examination of the RARs from several different species revealed that amino acid sequence differences among the RARs were well conserved among species, i.e. human RARα was essentially identical in its ligand-binding domain to mouse RARx, whereas both were clearly distinct in their LBDs from mouse and human RARβ and RARγ. This suggested that the evolutionarily conserved differences in the receptor LBDs might serve to allow for differential ligand responses. Lehmann et al (70) and

Graupner et al (44) were able to demonstrate that small differences between the receptors in ligand sensitivities could be largely enhanced by conformationally restricted synthetic retinoids. For instance, certain retinoids showed striking differences in their activation capacity of individual RARs, in that they were efficient activators of RARβ and RARγ but poor activators or nonactivators of the RARα (44, 70). The RARβ/γ selectivity of these retinoids may be related to the higher degree of sequence similarity between the ligand-binding domain of RARβ and RARγ compared to the ligand-binding domain of RARα (7, 41, 42, 66, 90). Similar results were obtained when in vitro binding assays were used (32). In contrast, two retinobenzoic acid derivatives, Am80 and Am580, representatives of a distinct class of all-*trans*-RA analogues (44), showed completely different receptor subtype selectivity in that they preferentially bind to RARα and also induce transcriptional activation of RARα (29, 32, 44).

Bernard et al (8) were able to show that retinoids that distinguish between RARβ and RARγ can also be designed. Surprisingly, such retinoids were derived from common parental compounds, indicating that a simple chemical modification can be sufficient to differentiate between the ligand binding pockets of these two RAR subtypes. Thus, retinoids with unique profiles of RAR subtype selectivity can be defined. These retinoids could serve as regulators of specific cellular programs and thus might be useful for therapeutic applications. Recent results from our laboratory indicate that one of the RARα/β-selective retinoids is indeed much more effective at inhibiting a prostate cancer cell line than tRA is (81), whereas a RARγ-selective retinoid was reported to inhibit cell proliferation and induce apoptosis in breast cancer cells and melanoma cells (125).

RXR–Selective Retinoids

The central role of RXR in regulating distinct hormonal response pathways has been addressed before (see Figure 4*a*). RXR homodimers were shown to activate RAREs different from RAR/RXR heterodimers. However, the only naturally available ligand for RXRs, 9-*cis*-RA, also activates RARs. Lehmann et al (72) were able to demonstrate that conformationally restricted 9-*cis*-RA analogues can be designed to separate RXR from RAR activities. Several retinoids were identified that selectively activate RXR homodimers but not RAR/RXR heterodimers (72). Selectivity of these retinoids for RXR homodimers was shown by using cotransfection assays with several different reporter constructs. Similar to 9-*cis*-RA, the RXR-selective retinoids were strong activators of the CRBPII-RARE (for response elements, see Figure 3), which is activated only by RXR homodimers. However, in contrast to 9-*cis*-RA, the RXR-selective retinoids did not induce the rat CRBPI-RARE, which is activated only by the RAR/RXR heterodimers (57, 72).

These novel types of RXR-selective retinoids are now being evaluated in various biological systems. As expected, they show a much more restricted biological activity spectrum and, for instance, do not induce differentiation in F9 terato carcinoma cells, whereas they can induce apoptosis in HL-60 cells in the presence of tRA.

Anti-AP-1 Selective Retinoids

Considering that retinoid receptors can function via at least two major mechanisms, by binding to response elements and/or by interacting with the transcription factor AP-1, we investigated more recently whether or not retinoids can be designed that function primarily in one of these mechanisms, in particular the anti-AP-1 pathway. We found that retinoids can indeed be defined (38) that only inhibit AP-1 activity but do not induce transcriptional activation. These retinoids turned out to inhibit growth of certain cancer cell lines, including lung cancer and breast cancer cells. However, these retinoids do not induce differentiation in F9 cells, which is known to require the activation of several genes via RAREs. Anti–AP-1 selective retinoids thus only effect some of the cellular retinoid response pathways and, in contrast to the natural retinoids tRA, 9-*cis*-RA, and 13-*cis*-RA, which induce a very broad spectrum of biological responses, are likely to have fewer side effects. This new class of retinoids is therefore a good candidate for a new generation of retinoid therapeutics.

PERSPECTIVES

The molecular analysis of the retinoid response has revealed a complex network of signaling pathways that allow retinoids to play a central role in hormonal and nutritional responses. Pro–vitamin A is usually taken up in the form of β-carotenes and related compounds, which are then converted into the active vitamin A–derivatives tRA and 9-*cis*-RA, which essentially function like hormones, interacting with specific nuclear receptors. One class of these receptors, the RXRs, also plays an important role in mediating other vitamin and hormone responses, in particular vitamin D_3 (derived from cholesterol) and thyroid hormone, which requires iodine for its synthesis. Moreover, it is well known that RA is necessary for erythrocyte differentiation, thereby controlling usage of iron. The nuclear receptors themselves require zinc atoms for their DNA binding domains. Thus, vitamins and micronutrients can directly affect the complex retinoid response network.

It is now clear that several retinoids and their precursors can inhibit the carcinogenic process, and interestingly, many of them are natural products present in various foods. Vitamin A has a preventive effect on the induction of experimentally induced precancerous conditions, like benign epithelial tu-

mors and both metaplasia and carcinoma in animals (22, 30, 121a, 121b, 133). The central role of vitamin A and its derivatives in the growth and differentiation of normal epithelial tissue and its preventive effects further underlined the natural role of this vitamin. But the serious side effects, known as hypervitaminosis A syndrome (128), led to the search for synthetic, less-toxic derivatives. With molecular biology–based analysis systems, it was possible to show that synthetic RA derivatives with receptor- or pathway-selective activities can be designed. These retinoids induce only limited spectra of the RA response and are thus likely to serve as valuable tools for further deciphering the roles of specific receptor subtypes. In addition, these retinoids may serve as more potent therapeutic agents that also induce fewer side effects. Using more complex analysis systems, we have now also observed that the various RAREs can influence the activity of selective retinoids, which suggests that gene-selective retinoids can be obtained that may even be able to interfere more specifically with certain diseases (67). In addition, retinoid antagonists have been obtained, one of which is able to inhibit RA induction of an RARE present in the HIV-1 promoter (69). HIV and some other viruses have been shown to contain RAREs that allow enhancement of viral transcription in the presence of RA. Therefore, RA antagonists could serve in some cases as antiviral agents. Overall retinoids with selective activity, therefore, show promise as new therapeutics for a large variety of diseases. Applications of such compounds in precancerous stages have by now been extended to various cancers, including bladder, mammary gland, and skin cancer. However, it is clear that the most interesting synthetic retinoids, in particular those that induce apoptosis of cancer cells, have yet to be analyzed in the clinic; they have the potential to induce a new area for the highly specific treatment of proliferative diseases. Whether or not natural ligands for RAR and/or RXR exist that have comparable selectivity and potency remains to be seen.

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