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Nuclear Signaling Pathways for 1,25-Dihydroxyvitamin D_3 Are Controlled by the Vitamin A Metabolite, 9-*cis*-Retinoic Acid

*The vitamin A metabolite, 9-*cis*-retinoic acid, may be the effector molecule that controls which genes respond to the 1,25-dihydroxyvitamin D_3 receptor.*

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Ever since E.V. McCollum[1] first resolved cod liver oil into antirachitic "fat-soluble D" and growth-promoting "fat-soluble A" fractions, knowledge concerning the mechanism of action of these two nutrients has grown in parallel. We now know that both compounds are extensively metabolized to form one or more hormonelike compounds and, furthermore, that some of these metabolites regulate gene expression by interacting with specific nuclear receptors. A recent report by Carlberg and coworkers[2] indicates an even more subtle relationship: the

vitamin A metabolite, 9-*cis*-retinoic acid, can alter the specificity of the 1,25-dihydroxyvitamin D_3 receptor for regulatory DNA sequences in target genes.

Receptors for 1,25-dihydroxyvitamin D_3, all-*trans*-retinoic acid, and 9-*cis*-retinoic acid are members of the nuclear hormone receptor superfamily, a gene family having at least 30 members, including receptors for the classic steroid hormones (estrogen, progesterone, glucocorticoids, androgens, mineralocorticoids), thyroid hormone, and several others for which the activating ligand is unknown (also known as "orphan receptors").[3] All family members exhibit sequence homology at protein and DNA levels and have a characteristic structure consisting of distinct hormone and DNA-binding domains and a transcriptional modulation domain. Another feature common to this class of molecules is that they control gene expression by interacting with specific DNA sequences or regulatory elements in control regions of target genes. The interaction of the hormone:receptor complex with its response element either alone or in complex with other factors alters the overall DNA structure of the gene promoter so as either to stimulate or to inhibit transcription.

Although all superfamily members share these characteristics, receptors for 1,25-dihydroxyvitamin D_3 (VDR), retinoic acid (RAR), and thyroid hormone (TR) also have some unique features not found in classic steroid receptors. Thus, steroid receptors bind DNA as homodimers while the VDR, RAR, and TR can form heterodimers with an accessory nuclear factor. This factor was recently identified as the receptor for 9-*cis*-retinoic acid, also called the retinoid X-receptor or RXR.[4] RXR-containing heterodimers interact with regulatory DNA sequences having an inherently different structure from those recognized by classic steroid receptors. Although steroid receptors bind to palindromic DNA sequences (e.g., the estrogen response element, 5'-AGGTCANNNTGACCT 3'), VDR, RAR, and TR heterodimers bind to directly repeating structures related to the estrogen response element half-site (i.e., the hexameric sequence, AGGTCA). The number of spacer bases between this hexameric repeat is a major determinant of receptor binding specificity; a three-base pair (bp) spacer designates VDR:RAR binding (also called DR3 for direct repeat with a three bp spacer), four bp (DR4) for TR:RXR, and five bp (DR5) for RAR:RXR. This relationship is known as the 3-4-5 rule.[5] In addition to existing as RXR heterodimers, the VDR, RAR, TR, and RXR may, like classic steroid receptors, also form homodimers. These homodimers appear to bind different DNA sequences. For example, RXR homodimers bind to hexameric direct repeats with a one-bp spacer (DR1), whereas VDR homodimers may bind hexameric core sequences in a variety of orientations other than DR3 (see ref. 6 for review).

These possible differences in DNA-binding behavior between VDR:VDR homodimers and VDR:RXR heterodimers form the basis for the recent report by Carlberg et al.,[2] in which two different types of 1,25-dihydroxyvitamin D_3 response elements (VDREs) were compared in terms of their ability to be stimulated by 1,25-dihydroxyvitamin D_3 in the presence or absence of the RXR and 9-*cis*-retinoic acid. The two VDREs chosen for comparison were from the 5'-regulatory regions of the genes for mouse osteopontin and human osteocalcin. Both genes encode bone-related proteins whose synthesis is stimulated by 1,25-dihydroxyvitamin D_3. The osteopontin VDRE contains the sequence 5'-GGTTCANNNGGTTCA-3', which is a DR3 structure and, therefore, a binding site for the VDR:RXR heterodimer. In contrast, the osteocalcin VDRE contains the sequence 5'-GGGTGANNNNNNGGGTGA-3', a hexamer direct repeat with a six-bp spacer (i.e., a DR6 sequence).

Experiments used *Drosophila* SL-3 cells, which have no endogenous VDR or RXR. Cells were transfected with expression plasmids capable of directing the synthesis of VDR or RXR and a reporter plasmid containing the desired VDRE upstream to the chloramphenicol acetyltransferase (CAT) gene, which was under the control of the thymidine kinase (tk) promoter. By measuring CAT enzyme activity, the authors[2] could determine if a particular VDRE was able to act as an enhancer of the tk promoter. When cells contained reporter plasmid with the osteopontin VDRE or related DR3 sequences, 1,25-dihydroxyvitamin D_3 or 9-*cis*-retinoic acid conferred a twofold CAT induction when the appropriate VDR or RXR expression plasmid was cotransfected. If cells were transfected with both receptor expression plasmids and both hormones, a clear synergistic response was obtained. In contrast, when cells were transfected with CAT reporter plasmid containing the osteocalcin VDRE (a DR6 sequence), a large 1,25-dihydroxyvitamin D_3 induction of CAT was seen as long as cells were cotransfected with the VDR expression vector. Simultaneous transfection with RXR and treatment with 9-*cis*-retinoic acid did not further enhance the vitamin D response. Similar results were obtained if the hexameric sequence of the osteocalcin VDRE was arranged as a palindrome without spacing or as an inverted palindrome with a 12-bp spacer. Similar overall results were obtained when osteopontin or osteocalcin VDRE reporter plasmids were transfected into the human MCF-7 cell line, which con-

tains endogenous VDR and RXR. These results show that the osteocalcin VDRE is maximally stimulated by the 1,25-dihydroxyvitamin D_3:VDR complex in the absence of RXR, while the osteopontin VDRE requires both 1,25-dihydroxyvitamin D_3:VDR and 9-*cis*-retinoic acid:RXR complexes.

Further evidence for the selectivity of these two VDREs was obtained when receptor:DNA binding was examined by band shift assays. VDR and RXR prepared by in vitro translation were mixed with VDREs from the osteopontin and osteocalcin genes, and the formation of receptor:DNA complexes was measured by looking for reduced mobility of labeled VDRE DNA after gel electrophoresis. When binding to the osteopontin VDRE was examined, a prominent shifted species was only observed in the presence of both VDR and RXR. In contrast, the osteocalcin VDRE produced a shifted species when incubated with either VDR or RXR. No further enhancement of binding was observed when both receptors were present. In contrast to the results obtained with transfection experiments, where CAT expression was only stimulated in the presence of 1,25-dihydroxyvitamin D_3 and 9-*cis*-retinoic acid, RXR and VDR induced band shifting in the absence of ligand.

It should be pointed out that these studies are somewhat artificial because responses to 1,25-dihydroxyvitamin D_3, 9-*cis*-retinoic acid, and their receptors were measured using an artificial gene containing the relevant VDREs. One wonders whether intact osteopontin and osteocalcin genes would exhibit the same response as the CAT reporter genes used in these experiments. If this proves to be the case, these studies show that the VDR can regulate at least two separate types of genes: 1) those containing VDREs regulated by VDR:VDR homodimers and 2) those responding to VDR:RXR heterodimers. Of even greater significance is the possibility that responses to retinoic acid and thyroid hormone are regulated by a similar mechanism.

Because VDRs and RXRs are found in most cells, the presence or absence of 9-*cis*-retinoic acid likely determines which set of genes is regulated by 1,25-dihydroxyvitamin D_3. The isomerization of all-*trans*-retinoic to the 9-*cis* isomer occurs in several tissues, but details of its regulation are not known.[7] One interesting possibility would be the demonstration that 9-*cis* isomerase activity changes during cell differentiation or at critical periods during tissue morphogenesis, thereby altering the overall responsiveness of cells to hormonal stimuli. In this way, 9-*cis*-retinoic acid and RXRs could double the regulatory potential of 1,25-dihydroxyvitamin D_3 and related hormones.

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Hypercholesterolemia of Copper Deficiency Is Linked to Glutathione Metabolism and Regulation of Hepatic HMG-CoA Reductase

Copper deficiency in rats causes hypercholesterolemia and increases in hepatic glutathione concentration and HMG-CoA reductase activity. Prevention of the increase in hepatic glutathione abolished the other changes.

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The occurrence of hypercholesterolemia as a consequence of copper deficiency in the rat was first reported by Klevay[1] 20 years ago. Subsequent attempts to determine the biochemical basis of this phenomenon have demonstrated that copper deficiency does not impair cholesterol catabolism and excretion;[2,3] rather, it increases the activity of the rate-controlling enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR).[4,5] Because physiologic concentrations of copper do not inhibit this enzyme, its increase in